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The role of the microbiome in plant and soil health in a changing climate

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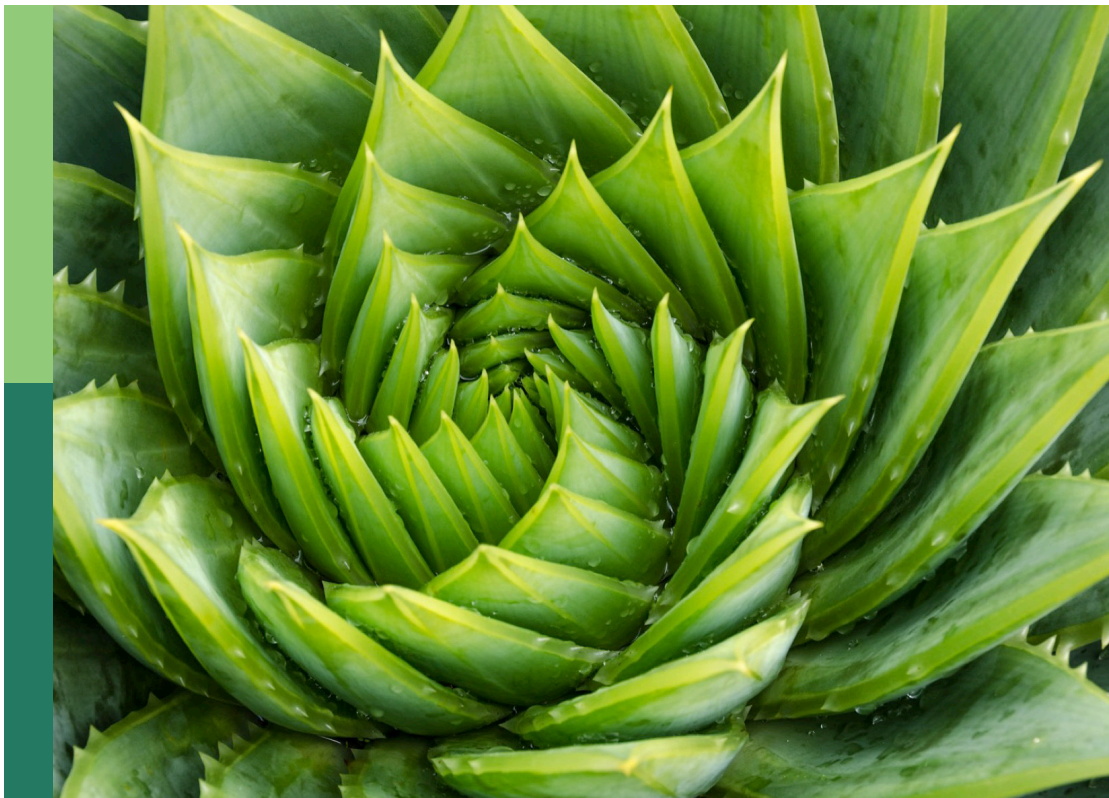
Published in
Frontiers in Plant Science



ISSN 1664-8714
ISBN 978-2-8325-5554-5
DOI 10.3389/978-2-8325-5554-5

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The role of the microbiome in plant and soil health in a changing climate

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Citation

Kaundal, A., Yadav, D., Srivastava, A. K., eds. (2024). *The role of the microbiome in plant and soil health in a changing climate*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-5554-5

Table of contents

frontiers | Frontiers in Plant Science

TYPE Editorial
PUBLISHED 02 October 2024
DOI 10.3389/fpls.2024.1491438

OPEN ACCESS

EDITED AND REVIEWED BY
Maurizio Chiurazzi,
National Research Council (CNR), Italy

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RECEIVED 04 September 2024
ACCEPTED 11 September 2024
PUBLISHED 02 October 2024

CITATION
Kaundal A, Srivastava AK and Yadav D (2024) Editorial: The role of the microbiome in plant and soil health in a changing climate.
Front. Plant Sci. 15:1491438.
doi: 10.3389/fpls.2024.1491438

Editorial: The role of the microbiome in plant and soil health in a changing climate

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KEYWORDS

microbiome, plant growth-promoting microbes, plant health, climate change, soil salinity, drought, heavy metal, elevated CO2

Editorial on the Research Topic
The role of the microbiome in plant and soil health in a changing climate

Industrialization during the mid-twentieth century drastically increased the earth's temperature over the past few decades due to increased concentration of greenhouse gases, primarily carbon dioxide, by burning fossil fuels (Houghton, 2001). This rise in global temperature has led to extreme weather events worldwide, such as intense summers or harsh winters, and altered precipitation patterns, leading to prolonged droughts or severe floods (Ripple et al., 2022). The resulting environmental stress, a consequence of climate change, affects all living beings, including humans and plants. These stresses, especially the extreme heat and water conditions, negatively affect crop production and threaten food security (Ahmad et al., 2023). Soil salinity is another cause of concern due to elevated sea levels and extreme droughts (Munns and Tester, 2008; Sandhu and Kaundal, 2018). In nature, plants often face various stresses sequentially or simultaneously (Zandalinas and Mittler, 2022). Several studies reported the negative effect of combined environmental biotic and abiotic stresses on crop production and yield (Mahalingam, 2015; Ramegowda and Senthil-Kumar, 2015). The constant increase in world population, which is expected to reach 9 billion by 2050, demands an increase in food production by 70-85% (FAO, 2009). On top of that, anthropogenic activities and overuse of chemical fertilizers deteriorate soil health (Pahalvi et al., 2021; Santorufo et al., 2021). The root microbiome is one of the most diverse communities on the earth. It is mainly composed of rhizosphere microbes colonizing the immediate soil surrounding the plant root and endosphere microbes colonizing the internal tissues of the roots (Pascale et al., 2020; Bai et al., 2022) These microbes in the rhizosphere exhibit various plant growth-promoting activities such as nitrogen fixation, phosphate solubilization, siderophore, catalase, and IAA production and help plants' growth and development (Mohanty et al., 2021; Ganesh et al., 2024). The plant growth-promoting bacteria significantly mitigates environmental abiotic and biotic stresses (Beneduzi et al., 2012; Kumar et al., 2019; Burlakoti et al., 2024).

The Research Topic, which has received diverse contributions, is a testament to the collaborative nature of scientific research. It is a collective effort to understand the role of the microbiome in plants and soil health in a changing climate, highlighting the role of

soil microbes in mitigating salinity stress, drought, heavy metal toxicity, flooding, and elevated CO_2. The first article suggested the potential of the cell-free supernatant in the novel *Devosia* sp. SL43 strain to sustain the soybean seed germination rate under salt stress (Monjezi et al.). Another study on soybean under elevated CO2 and flooding revealed higher bacterial and fungal diversity upon combined treatments compared to non-flooding control. The individual treatment of elevated CO2 and flooding revealed a significant abundance of *Chitinophaga, Clostridium*, and *Bacillus*. However, the combined treatments showed a considerable abundance of *Trichoderma* and *Gibberella*, offering hope for the future of plant and soil health in a changing climate (Coffman et al.). Another study focused is on phyllosphere epiphytic microbes' diversity of five medicinal plants in summer and winter. The phyllosphere microbiome plays a significant role in plant physiological metabolism. The study revealed the seasonal effect on the bacterial and fungal phyllosphere compared to host species. The summer phyllosphere is more heterogeneous for microbial diversity than winter. The network connections of the bacterial and fungal communities significantly increased during season transition compared to plant connections. This study shed light on the understanding of the plant microbial community's composition in small-scale agriculture and their ecological roles (He et al.). The article on the utilization of *Bacillus amyloliquefaciens* QST713-based product on potatoes revealed that this PGPB enhanced the potato yield and improved potato peel nutrient profile with a minor impact on the soil microbiome diversity (Adamo et al.). Another study on the PGPB revealed the biofertilizer and biocontrol properties of *Stenotrophomonas maltophilia* BCM. This PGPB significantly increased the wheat seed germination rate in the presence of two phytopathogens, *Rhizoctonia solani* and *Fusarium oxysporum*, as well as saline conditions. Genomic analysis of *S. maltophilia* revealed the presence of genes known for nutrient assimilation plant growth promoting traits such as plant growth and antifungal activities (Sharma et al.). The report on the impact of two PGPBs, *B. subtilis*, and *B. aryabhattai*, on mitigating salt stress in rice revealed the potential of these isolates for sustainable agriculture in the era of climate change. PGPB treatment in rice during salt stress improved the ionic and water balance, antioxidation defense, photosynthesis, nutrient uptake, and phytohormone production (Siddika et al.).

Drought and salinity, often in tandem, are an important climate conundrum affecting crop growth and development due to ominous auxin imbalance as a function of microbial diversity. However, functional microbial diversity is more impactful than mere numerical diversity, the former undergoing lesser reduction in water scarcity under organic production practices than conventional practices with assured irrigation (del-Canto et al.). The study on *Phaseolus vulgaris* recommends organic management rather than using agrochemicals to maintain enhanced rhizobia abundance, nodulation, and diversity (del-Canto et al.). Efforts must be made to develop sustainable and eco-friendly approaches for preserving and strengthening soil microbiota biodiversity.

Further, it has been recommended that many microbes as auxin-producing endophytes are reported to neutralize drought and salinity through auxin balance with coordinated auxin biosynthesis involving plant-indigenous auxin, microbes-associated auxins, and carriers of auxin transporters, apart from upregulation of stress-induced auxin-responsive microbial genes (Mal and Panchal). The intervention of omics-driven research in understanding the action mechanism and interaction of plants and associated plant rhizobacteria has been nicely reviewed (Verma et al.). The revelation of omics-based adaptive regulatory mechanisms underlying the plant adaptation under microbes-mediated abiotic stress reduction with improved plant nutrition as the second line of plant defense has been reported (Verma et al.). In another review, the potential of plant growth-promoting microorganisms for salinity tolerance in plants has been elucidated (Acharya et al.). Interestingly, rhizosphere microbes, as the second genome, put forth stressing plant defense through the elevated supply of growth-promoting hormones such as auxins, gibberellins, and cytokinins, coupled with a reduced level of stress causing ethylene, thereby striking a balance osmoprotectant secretion and further oxidative cellular damage (Acharya et al.). An interesting review highlighting the microbial intervention in the remediation of heavy metal toxicity, emphasizing the mechanism involved, ensures better rhizosphere health resilience (Tang et al.). Further, attempts have been made to enlist diverse approaches, including the recent nanotechnology, to improve the microbial remediation of heavy metal-polluted soils.

While developing a combative strategy against drought and salinity, photobomb-induced soil legacy effects (developing functional bridge accommodating pathogenic microbes, antagonists, and repeated recruitment of fresh microbial diversity, all collectively surviving through competitive coexistence) featuring rhizosphere secretions, non-preferential salinity-tolerant microbes coupled with the use of halophytes are highly pivotal (Ma et al.).

In conclusion, this research topic has a significant collection of articles shedding light on the role of plants' phyllosphere, rhizosphere, and endosphere microbiome in plant growth and development and soil health under critical environmental stresses.

Author contributions

AK: Writing – original draft, Writing – review & editing. AS: Writing – original draft, Writing – review & editing. DY: Writing – review & editing.

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

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TYPE Original Research
PUBLISHED 28 March 2023
DOI 10.3389/fpls.2023.1071346

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Plant Symbiotic Interactions, a section of the journal Frontiers in Plant Science

RECEIVED 16 October 2022
ACCEPTED 20 February 2023
PUBLISHED 28 March 2023

CITATION
Monjezi N, Yaghoubian I and Smith DL (2023) Cell-free supernatant of *Devosia* sp. (strain SL43) mitigates the adverse effects of salt stress on soybean (*Glycine max* L.) seed vigor index. *Front. Plant Sci.* 14:1071346. doi: 10.3389/fpls.2023.1071346

Cell-free supernatant of *Devosia* sp. (strain SL43) mitigates the adverse effects of salt stress on soybean (*Glycine max* L.) seed vigor index

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Soil salinity is a major constraint for soybean production worldwide, and the exploitation of plant growth-promoting bacteria (PGPB) and their bioactive metabolite(s) can improve plant salinity tolerance. With this objective, two experiments were performed, aiming to test 4 culture media (YEM(A), TYE(A), TS(A), and LB(A)) for growing a novel *Devosia* sp. (strain SL43), and then evaluating cell-free supernatants (CFS) from the *Devosia* sp. on germination of soybean (*Glycine max* L.) seeds under salinity stress. Soybean seeds were subjected to three salinity levels (0, 100, and 125 mM NaCl) and 6 levels of *Devosia* sp. CFS dilution (0, 1:1, 1:100, 1:250, 1:500, 1:1000). The results indicated that 125 mM NaCl concentration caused the greatest reduction in the total number of germinated seeds (15%), germination rate (43.6%), root length (55.2%), root weight (39.3%), and seed vigor (68%), and it also increased mean germination time by 71.9%. However, *Devosia*-CFS improved soybean germination, and the greatest effect was obtained at 1:1 dilution. Under the highest salinity level, application of CFS at 1:1 dilution increased final germination (17.6%), germination rate (18.6%), root length (162.2%), root weight (239.4%), seed vigor index (318.7%), and also shortening mean germination time by 19.2%. The results indicated that seed vigor index was positively correlated with other traits except for mean germination time. Our study suggested that the highest productivity of Devoisa sp. was obtained from the YEM medium. Results also suggested that CFS produced by the novel Devosia sp. (SL43 strain) can successfully alleviate salt stress effects on soybean seed germination and manipulating the chemical composition of the growth medium can influence the effectiveness of these bioactive metabolites.

KEYWORDS

soybean, salinity, *Devosia* sp., cell-free supernatants, seed germination, vigor index, culture medium optimization

1 Introduction

Soybean (*Glycine max* L.) is one of the world's more widely produced crops, due to its high nutritional value, which plays a vital role in global food security (Waqas et al., 2014; Chung et al., 2020). Previous studies have shown that soybean growth, production and quality are strongly influenced by abiotic stresses (Chung et al., 2020; Yaghoubian et al., 2021).

As our climate continues to be affected by global warming/climate change, the consequences of that warming grow more intense, and the frequency of extreme weather events increases. This shifting of weather patterns is the biggest challenge currently facing farmers and farm communities worldwide (Kheiri et al., 2021; Clarke et al., 2022). Rising demand for agricultural commodities persists despite continuous warming, which is driving the agricultural sector seek new ways of crop production (Chandio et al., 2020; Yadav et al., 2021). Further, climate-induced environmental stressors such as salinity, drought, and heat are among the principal factors reducing crop productivity worldwide, which diminishes global food security and environmental sustainability. The situation with regard to soil salinization is worsening substantially faster than researchers had predicted less than a decade ago, putting the world on alert for the potential spread of salinity issues into currently unaffected regions (Mukhopadhyay et al., 2021). Salinity impairs many plant functions, from the seed germination stage to final seed production; seedling emergence at the initiation phase of the lifecycle of plants is highly susceptible to salinity (Naamala et al., 2022; Yaghoubian et al., 2022a). However, once the plants are overcome the seedling stage, they are better able to cope with the adverse consequences of salinity stress (Gholizadeh et al., 2021; Shah et al., 2022).

Building climate resilience requires relying on sustainable farming methods and practices in the face of climate change; hopefully these technologies can relieve pressure on the environment, cut greenhouse gas emissions, and also aid in managing future risks (Dhankher and Foyer, 2018; Adegbeye et al., 2020; Yadav et al., 2021). One of the interesting methods for increasing the resilience of our food systems is linking farming communities and scientists together to work toward easing dependence on chemical products while increasing reliance on eco-friendly product options such as plant growth-promoting bacteria (PGPB), which are increasingly being employed as bio-stimulant formulations, promoting plant health, development, and sustainability (Naamala and Smith, 2020; Fiodor et al., 2021; Yaghoubian et al., 2022b). There are various mechanisms by which microbes effectively promote plant growth in saline soil. Several studies have shown that beneficial microbes can directly improve plant growth under salinity stress, perhaps by synthesizing specific growth-stimulating hormones such as auxins, cytokinin, and gibberellins or by downregulation ethylene formation. Indeed, this kind of hormonal coordination by PGPB will result in minimizing plant ethylene levels and therefore reducing the detrimental and inhibitory effects of this phytohormone on plant growth under salt stress. On the other hand, some beneficial microbes might either promote plant growth through bacterial auxin production or increase the production of endogenous plant auxins, which will result in controlling primary root elongation and boost lateral root formation, and finally, the equilibrium between ethylene and auxin enables plants to uptake water, ions, and nutrients more efficiently under salinity stress (Iqbal et al., 2017; Kudoyarova et al., 2019; Kumar et al., 2020; Eichmann et al., 2021; Park et al., 2021). Therefore, optimizing germination such that it can largely overcome soil salinity effects is possible by utilizing PGPB, a promising tool for a more sustainable future. However, the crop colonization ability of most beneficial bacteria varies depending upon the host, bacterial species, and salt concentration (Miljaković et al., 2022; Nigam et al., 2022; Sulastri et al., 2022; Yaghoubian et al., 2022a). Moreover, bacterial trait–environment relationships can vary from farm to farm and from region to region, which may affect the efficacy of PGPB (Goddard et al., 2001; Meena et al., 2015). The most recent approach, within market limitations, for PGPB strain technologies, is using Cell-Free Supernatants (CFSs) of beneficial PGPB, which could offer innovative alternatives in dealing with these challenging limitations, as well as promoting crop productivity (Pellegrini et al., 2020; Naamala and Smith, 2021). Such microbial-derived mixtures may include growth hormones, secondary metabolites, various signal compounds, and antioxidant enzymes, which would positively enhance plant growth (Yasmin et al., 2004; Fiodor et al., 2021; Saberi Riseh et al., 2021). Thus, CFS technologies offer the most viable hope for enhancing crop production under a challenging climate change situation, due to a range of conditions and the probability of success of microbial compounds which are less affected by variable environmental conditions (Naamala and Smith, 2020; Shah et al., 2022). Although these CFS metabolites have recently gained greater attention, a meaningful gap remains in the progression from research to implementation in existing farming systems. The most critical step is determining the type of growth medium for *in vitro* cultivation because it consistently influences microbial organic compound production. While plenty of studies primarily focus on the growth and nutritional requirements of root nodule bacteria, less attention has been focused on maximizing growth rates and increasing production of plant growth-promoting compounds. An appropriate medium contains a good source of carbon, nitrogen, mineral salts, and growth factors; nutrient media such as yeast extract mannitol (YEM) and tryptone yeast extract (TYE) medium are found to be very suitable for growing PGPB. Indeed, although there are many enrichment culture formulations, each bacterium has entirely unique nutritional requirements, and testing culture medium and new culture conditions can make it possible to find novel bacterial compounds enhancing plant growth and development (Pastor-Bueis et al., 2017; Sessitsch et al., 2019; Trianto et al., 2020; Jiao et al., 2021; Yusfi et al., 2021).

Thus, we hypothesized that growth conditions can play a vital role in supporting plant growth-promoting compound production. Therefore, the present study focused on acquiring detailed knowledge and understanding of cost-effective technologies to produce such biostimulants and reduce detrimental impacts of salinity on soybean germination.

2 Materials and methods

2.1 Experimental design

This research was conducted in three sets of experiments at the Macdonald Campus of McGill University in 2022. The first study examined bacterial growth on a set of agar culture media; the best medium was selected based on growth rate. Then, the growth of bacteria in the suspension culture of the selected medium was monitored using Cytation instrumentation (Cytation 5th Cell Imaging Multimode Reader, BioTek Instruments, Inc.). The second and third experiments examined simultaneously the effectiveness of Cell-free supernatant (CFS) harvested from bacterial suspensions of Yeast Extract Mannitol Broth (YEMB) and Tryptone Yeast Extract Broth (TYEB) media as technologies for enhancing seed germination of soybean (*Glycine max* L. var P09A62X) under saline and non-saline conditions. The treatments were determined as factorial combinations of three NaCl levels (0, 100, and125 mM) and dilution ratios of 1:100, 1:250, 1:500, and 1:1000 for the CFS (1 mL *Devosia* sp. CFS and 1, 100, 250, 500, and 1000 mL distilled water, respectively). Each experiment was organized following a completely randomized design.

2.2 Evaluation of solid and broth culture medium

The genus *Devosia* is a member of the *Alphaproteobacteria* and is a motile and gram-negative bacterium classified within the family *Hyphomicrobiaceae* of the order Rhizobiales (Talwar et al., 2020). *Devosia* sp. strain SL43 isolated from root nodules of *Amphicarpaea bracteate* is a plant growth-promoting phytomicrobiome member which was isolated from an undomesticated legume native to southwestern Quebec, through previous research in our laboratory (Ilangumaran et al., 2021); stock cultures were maintained on YEM broth slants at -80°C and subcultured every three months. A total of 4 different culture medium compositions, including YEM(A) (Yeast extract - Mannitol Agar), TYE(A) (Tryptone Yeast Extract Agar), TS(A) (Tryptic Soy Agar), and LB (A) (Luria-Bertani Agar) were used for growing the bacterium. A loopful (0.1 mL) of a suspension of *Devosia* sp. strain SL43 was streaked onto four plates containing YEM(A), TYE(A), TS(A), and LB(A) inside a laminar air flow hood and incubated at 28 ± 2°C and the visual culture characteristics of SL43 colonies on the plates was observed once daily for 10 days; each experiment was performed three times. Growth was not observed on TS(A) and LB(A) media; however, significant bacterial growth was observed for YEM(A) and TYE(A) plates which were selected for the next steps. Pure cultures of SL43 on YEM(A) and TYE(A) plates were picked off the plates with a sterile inoculating loop and inoculated into 25 mL of YEM(B) or TYE(B), which were agitated on a rotary shaker at 150 rpm and 28°C until reaching maximum growth based on the optical density of the bacterial growth which was measured spectrophotometrically at 600 nm (Ultrospec 4300 pro UV/Visible Spectrophotometer, Biochrom, Ltd, Cambridge, UK).

Bacterial growth was monitored using a microplate reader (Cytation 5 Cell Imaging Multi-Mode Reader) by incubating *Devosia* sp. strain SL43 in a set of sterilized 96-well clear bottom microplates (Corning Incorporated, NY). Each row contained the following treatments, in randomized order with 12 replications: YEM(B) (Control), TYE(B) (Control), YEM(B) (distilled water), YEM(B) (100 mM NaCl), YEM(B) (125 mM NaCl), TYE(B) (distilled water), TYE(B) (100 mM NaCl), TYE(B) (125 mM NaCl).

Briefly, for measuring bacterial growth, 200 µL of the prepared bacterial cultures in YEM or TYE broth culture (prepared with distilled water or saline solution) were injected into the microplate wells. Additionally, two rows were allocated to the YEM(B) or TYE (B) without adding any bacterial starter cultures. After covering microplates with pre-processed lids, they were placed on a rotary shaker microplate reader at 355 rpm at 28°C. The optical density (OD) of the bacterium was monitored at 600 nm. The OD of each well was read every 2 h for 7 days.

2.3 Propagation of bacteria and harvesting cell-free supernatants

For germination tests, 100 µL of SL43 broth from the final step were inoculated into a 100 mL Erlenmeyer containing 50 mL of YEM or TYE broth medium for 10 days at 28 ± 2°C; the material was considered ready for germination evaluation when the O.D. reached 1.0 (or maximum growth); at this stage, cell-free supernatant (CFS) was collected by centrifuging the liquid culture at 10,000 g for 30 min at room temperature, in order to remove the cells and other larger particles; after being centrifuged, the supernatant was filtered through a 0.22-um pore size syringe membrane (AwelTM MF 48-R, NuAire, USA) (Legesse, 2016; Sarbadhikary and Mandal, 2017).

2.4 Seed germination test

Soybean seeds were prepared through surface sterilization in 5% NaOCl (sodium hypochlorite) for one minute, followed by rinsing three times in sterile distilled water to disinfect soybean seeds; then seeds were placed on filter paper in Petri dishes. After which surface-sterilized seeds were moistened with 5 mL of cell-free supernatant of strain SL43 at various dilution levels (control, 1:100, 1:500, and 1:1000), diluted in sterile distilled water (unstressed) or 100 and 125 mM NaCl solution (stressed). Additionally, in some Petri dishes, seeds were wetted with 5 mL of sterile distilled water (unstressed) or 100 and 125 mM NaCl solution (stressed) without CFS addition, acting as the control. Then, Petri dishes were put in a thin polyethylene bag to avoid drying caused by evaporation, and Petri dishes containing the treated seeds were placed inside a growth chamber and incubated at 25 ± 2°C with a relative humidity of 70% in darkness. The seed

germination rate was observed several times per day over the following 96 h; a seed was considered germinated when the root was over 0.2 cm long. The number of germinated seeds and germination time of each seed was determined (Ghassemi-Golezani et al., 2016). In addition, root length and dry weight of seedlings were recorded 7 days after sowing. Other variables calculated are described immediately below.

2.5 Mean germination time

Computation of mean germination time (MGT) was performed according to the following formula:

$$MGT = \frac{\sum(D \times g)}{\sum n}$$

Where g is the number of seeds germinated on each day, D is the number of days from the start of the germination test, and n is the total number of seeds germinated at the termination of the experiment (Ellis and Roberts, 1981).

2.6 Germination rate

The germination rate (GR) was calculated followed Al-Mudaris (1998) as:

$$GR = \frac{\sum n}{\sum(D \times g)}$$

2.7 Seed vigor index

The seed vigor index (SVI) was computed using the formulas proposed by Yaghoubian et al. (2022c):

$$SVI = \frac{SDW}{MGT}$$

Where MGT is mean germination time and SDW is the mean value of seedling dry weight.

2.8 Statistical analysis

Data were analyzed using SAS 9.4, and differences between control and treatments were considered statistically significant at $P < 0.05$ using a Duncan's multiple test. Excel software was used to draw figures. The correlations were calculated by the correlation (CORR) procedure of the SAS 9.4, software.

3 Results

3.1 Performance of solid and broth culture media

Devosia sp. on TS and LB Agar (A) media showed no visible growth. However, it grew well on YEM and TYE (A) media at pH 7.0 as seen after incubation for 7-10 days at 28 °C. *Devosia* sp. grew more slowly on the TYE(A) medium (7-10 days) than the YEM(A) medium (5-7 days). The *Devosia* sp. colonies on the YEM(A) plates appeared a golden yellow color, shiny, mucoid in texture, irregularly shaped, and apparent in non-uniformity of size. However, TYE(A) caused *Devosia* to grow in fairly circular colonies with more uniform size. In addition, *Devosia* colonies on TYE(A) had a smaller and thinner appearance than colonies on YEM(A) (Figure 1). A set of results for *Devoisa* growth, based on optical density (OD) measurements on broth culture, is given in Figure 2. The results showed that *Devosia* grew faster and more efficiently in YEM(B) medium, under both saline and non-saline conditions than TYE(B) medium (Figure 2).

3.2 Germination trend

The seed germination trend observed during the study showed that the process was more rapid when YEM(B)-CFS was applied at all salinity levels (Figure 3). Using CFS at 1:1 dilution resulted in the quickest germination under 100 mM NaCl. In addition, under the highest salinity level, both 1:1 and 1:100 CFS dilution caused a trend to increased germination than other CFS levels. However, the

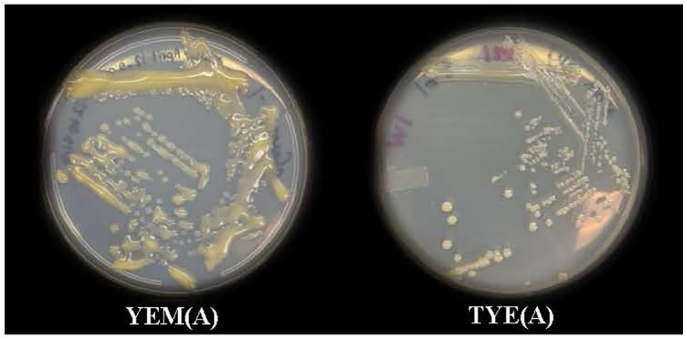

FIGURE 1
Growth of Devosia sp. (SL43 strain) in YEM(A) (Yeast Extract Mannitol Agar) and TYE(A) (Tryptone Yeast Extract Agar) media.

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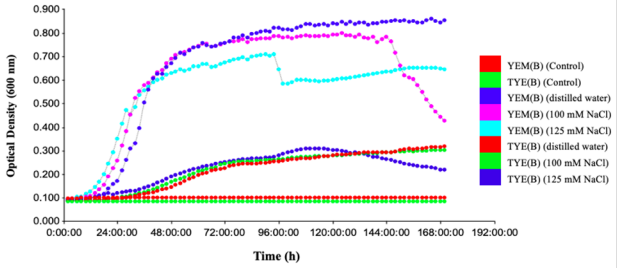

FIGURE 2
Changes in growth of *Devosia* sp. (SL43 strain) in Yeast Extract Mannitol broth (YEM-B) and Tryptone Yeast Extract broth (TYE-B) under salinity conditions. 1: YEM(B) (Control), 2: TYE(B) (Control), 3: YEM(B) (distilled water), 4: YEM(B) (100 mM NaCl), 5: YEM(B) (125 mM NaCl), 6: TYE(B) (distilled water), 7: TYE(B) (100 mM NaCl) and 8: TYE(B) (125 mM NaCl).

soybean germination trend was similar under all YEM(B)-CFS levels in non-saline conditions. The application of TYE(B)-CFS showed an increasing trend during soybean germination either in saline or non-saline conditions, and 1:250 (non-saline), 1:1 (100 mM NaCl), and both 1:100 and 1:500 (125 mM NaCl) induced the most rapid seed germination (Figure 3).

3.3 Final germination

The results indicated that *Devosia* YEM(B)-CFS at 1:1 significantly increased soybean germination in the presence of 125 mM NaCl. However, the germination promotion effects of other treatments were not statistically significant (Figure 4). In the presence of 125 mM NaCl, YEM(B)-CFS at 1:1 dilution increased the final germination of soybean by 17.6% compared to salt-stressed seeds with no CFS addition. Moreover, under moderate salinity (100 mM NaCl), different levels of YEM(B)-CFS increased final germination from 14.2 (CFS dilution 1:1) to 2.8% (CFS dilution at 1:500);

In contrast, in the presence of 100 mM and 125 mM NaCl, various TYE(B)-CFS levels had no meaningful effect on soybean germination in comparison with salt-stressed seeds. There was only a slight promoting effect from 11.4% (CFS dilution 1:1) to 2.8% (CFS dilution 1:500) under 100 mM NaCl, and from 11.7% (CFS

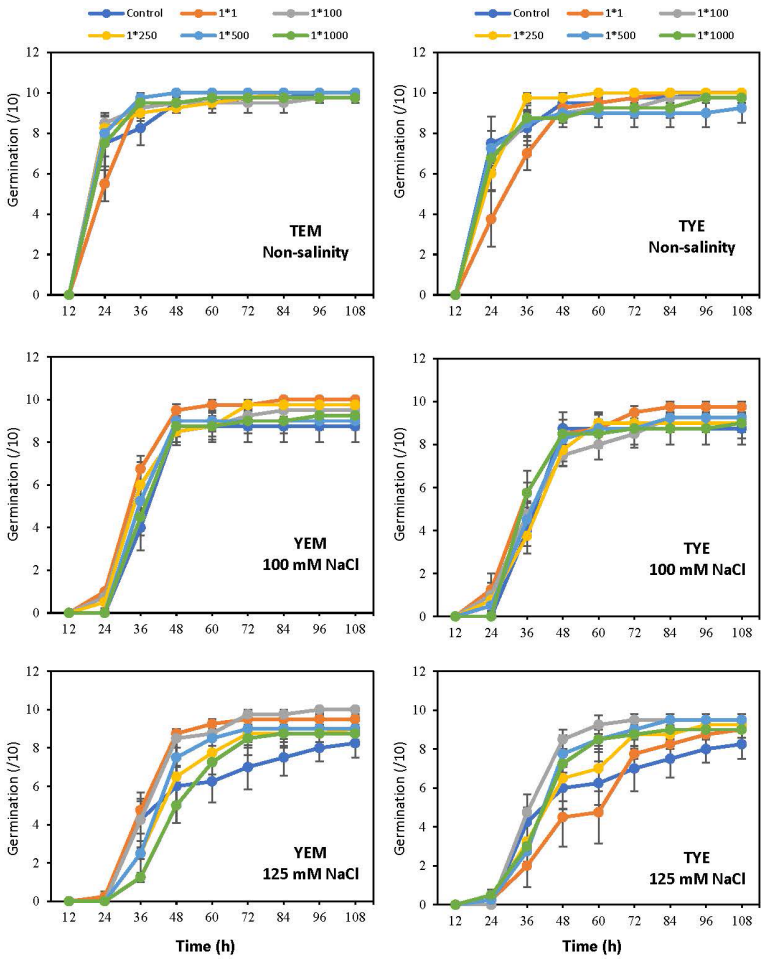

FIGURE 3
Changes in seed germination for soybean in response to salinity and dilutions of *Devosia* sp. YEM(B)-CFS and TYE(B)-CFS. Values represent the means of four replicates ± SD. 1:1, 1:100, 1:250, 1:500 and 1:1000 = 1 mL *Devosia* sp. CFS and 1, 100, 250, 500 and 100 mL distilled water respectively.

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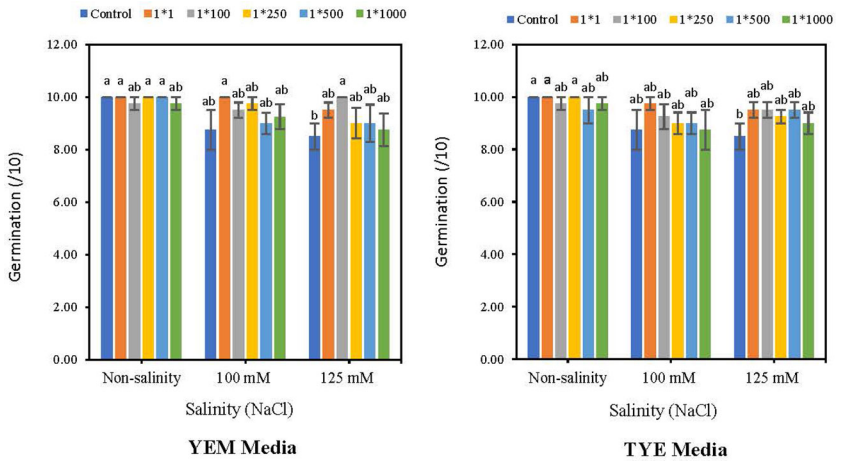

FIGURE 4
Effect of dilutions of *Devosia* sp. YEM(B)-CFS and TYE(B)-CFS and salinity on germination of soybean seedlings. Values represent the mean of four replicates ± SD. 1:1, 1:100, 1:250, 1:500 and 1:1000 = 1 mL *Devosia* sp. CFS and 1, 100, 250, 500 and 100 mL distilled water, respectively.

dilutions at 1:1, 1:100, and 1:500) to 5.8% (CFS dilution1:1000) under 125 mM NaCl, compared to stressed seeds with no additional CFS (Figure 4).

3.4 Germination rate

Figure 5 indicates that the *Devosia* CFS treatments did not significantly affect the soybean germination rate (GR) of the seeds. However, when seeds were exposed to varying levels of salinity stress, YEM(B)-CFS at 1:1 concentration showed the greatest seed germination enhancement, with a 7.4 (100 mM NaCl) and 18.6% (125 mM NaCl) increase over salt-stressed seeds without CFS application. Moreover, germination enhancement effect of YEM (B)-CFS on GR was limited under non-saline conditions, and YEM (B)-CFS at 1:500 dilutions provided the best GR, with a 9.0% increase over the control seeds.

Application of TYE(B)-CFS had no meaningful effect on soybean GR (Figure 5). The maximum enhancement effect of TYE(B)-CFS on GR was only 0.73% at the 1:250 level under the non-salinity conditions, compared to the control seeds. Similarly, GR was not affected by TYE(B)-CFS at 100 mM NaCl. However, at the highest salinity level, the soybean GR was promoted by TYE(B)-CFS from 16.5% (1:100 dilution) to 3.3% (1:250 dilution) compared to the salt-stressed seed with no additional CFS, except for the 1:1 CFS level (Figure 5).

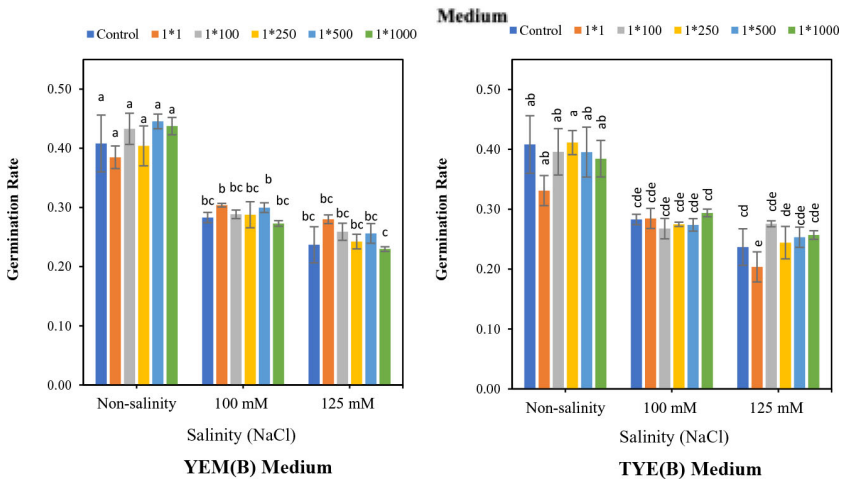

FIGURE 5
Effect of dilutions of *Devosia* sp. YEM(B)-CFS and TYE(B)-CFS and salinity on germination rate of soybean seedlings. Values represent the mean of four replicates ± SD. 1:1, 1:100, 1:250, 1:500 and 1:1000 = 1 mL *Devosia* sp. CFS and 1, 100, 250, 500, and 100 mL distilled water, respectively.

3.5 Mean germination time

Applying YEM(B)-CFS caused a meaningful decreased effect on Mean Germination time (MGT) under highest level of salinity (Figure 6). The lowest MGT was observed at 1:1 and 1:100 dilutions of YEM-CFS at 125 mM NaCl salinity levels, with 19.2 and 11.7% decreases compared to salt treatments with no addition of CFS. In contrast, under non-stress conditions and 100 mM NaCl salinity level, CFS treatments had no meaningful effect on MGT. Applying YEM(B)-CFS at 1:1 dilution, when seeds were exposed to moderate salinity, only caused a 7% reduction in MGT compared to the stressed seeds with no additional CFS. Additionally, under optimal conditions, YEM(B)-CFS at 1:100 (10.8%), 1:500 (12.4%), and 1:1000 (9.3%) caused a slight reduction in MGT compared to the control seeds.

In addition, applying TYE(B)-CFS under both saline and non-saline conditions had no significant effect on MGT. The highest level of TYE(B)-CFS induced an increase in MGT under non-saline conditions and the highest salinity level, with 19.4 and 16.0% increases, respectively. In addition, at 125 mM NaCl, applying TYE(B)-CFS shortened MGT at 1:100 (17.8%), 1:250 (3.85%), 1:500 (9.5%), and 1:1000 (11.7%) dilutions, compared to the salt-treated seeds without CFS addition (Figure 6).

3.6 Root length

Devosia YEM(B)-CFS and TYE(B)-CFS application significantly increased root length under the highest salinity levels (Figure 7). Under 125 mM NaCl condition, applying YEM(B)-CFS

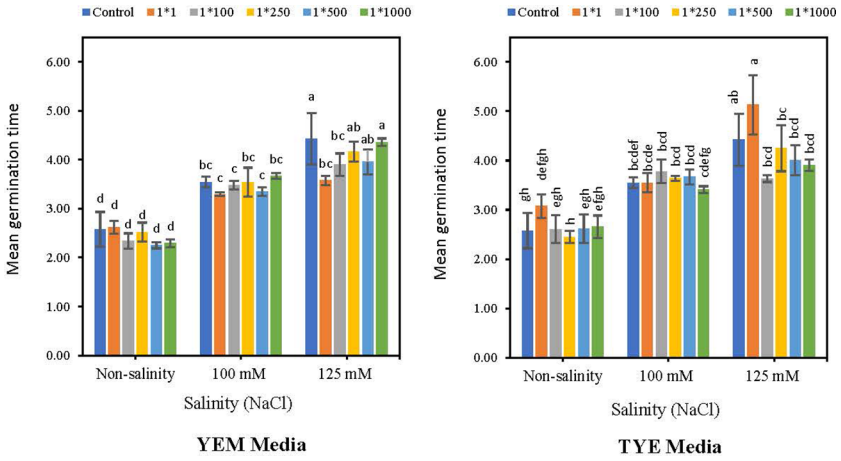

FIGURE 6
Effect of dilutions of *Devosia* sp. YEM(B)-CFS and TYE(B)-CFS and salinity on mean germination time (MGT) of soybean seedlings. Values represent the mean of four replicates ± SD. 1:1, 1:100, 1:250, 1:500 and 1:1000 = 1 mL *Devosia* sp. CFS and 1, 100, 250, 500 and 100 mL distilled water, respectively.

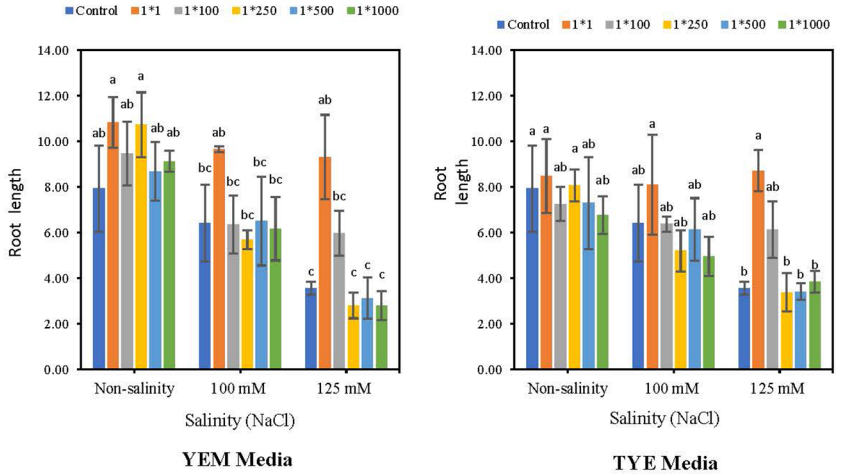

FIGURE 7
Effect of dilutions of *Devosia* sp. YEM(B)-CFS and TYE(B)-CFS and salinity on root length of soybean. Values represent the mean of four replicates ± SD. 1:1, 1:100, 1:250, 1:500 and 1:1000 = 1 mL *Devosia* sp. CFS and 1, 100, 250, 500 and 100 mL distilled water, respectively.

at 1:1 and 1:100 dilutions caused the highest increase in soybean root length, by 162.2 and 68.51%, respectively. Under moderate levels of salinity, however, only the 1:1 level of CFS showed the heightening effect with a 50.4% increase compared to the salt-treated seeds with no CFS addition.

Furthermore, applying TYE(B)-CFS at the 1:1 concentration was the best root length promoter with 6.8% (non-saline), 26.0% (100 mM NaCl), and 145.2% (125 mM NaCl) increases; however, only at the highest level of salinity the difference was statistically meaningful. In addition, TYE(B)-CFS at the 1:100 dilution caused an increase in root length under 125 mM NaCl compared to the salt-treated seeds without CFS addition, with 72.6% increase (Figure 7).

3.7 Root dry weight

Root weights of soybean seeds were boosted by applying *Devosia*-CFS grown on a YEM(B) culture medium, under both saline and non-saline conditions (Figure 8). The results indicated that the highest concentration of YEM(B)-CFS (1:1) had the greatest effect on root weight with 1.26, 2.95, and 2.39 fold increases compared to controls in 0, 100, and125 mM NaCl, respectively. All other concentrations of YEM(B)-CFS showed an elevated effect on root weight, ranging 60.6 to 92.3% under optimal conditions, and 58.9 to 95.0% under 100 mM NaCl. In addition, applying YEM(B)-CFS at 1:100 under extreme salinity levels caused a 91.5% increase in root weight, which was statistically meaningful.

Application of CFS from TYE(B) medium also increased soybean seedling root weight, the 1:1 concentration being the best, with 54.7, 108.6 and 132.3% increases under the 0, 100 mM NaCl, and 125 mM NaCl, respectively. Additionally, TYE(B)-CFS at 1:250 dilution caused a meaningful increase in soybean root dry weight compared to control seeds, by 58.9%. However, the overall results indicated that enhancement effects of *Devosia* CFS from YEM(B) culture medium was greater than from TYE(B) medium (Figure 8).

3.8 Seed vigor index

Applying YEM(B)-CFS caused an increase in seed vigor index (SVI) compared to the control in saline and non-saline conditions (Figure 9). However, the boosting effect of CFS was greater when *Devosia* grew in the YEM(B) culture medium. The highest concentration of YEM(B)-CFS (1:1) caused the greatest increases under saline and non-saline conditions with 106% (non-salinity), 336.8% (100 mM NaCl), and 318.7% (125 mM NaCl) enhancements of SVI.

Moreover, the highest SVI resulted from applying a 1:250 concentration of TYE(B)-CFS under non-saline conditions (56%). In addition, TYE(B)-CFS at 1:1 dilution was the best under 100 and 125 mM NaCl which caused a 121.0 and 112.5% increases, respectively; however, these differences were not statistically significant (Figure 9).

3.9 Correlation

The correlation coefficients of the total number of germinated seeds, GR, MGT, root length, and root dry weight were statistically significant compared with the SVI (Table 1). For both YEM(B) and TYE(B) medium, a positive correlation was found between the total number of germinated seeds ($r = 0.50$ and 0.41, $p \leq 0.01$), GR ($r = 0.79$ and 0.75, $p \leq 0.01$), root length ($r = 0.84$ and 0.71, $p \leq 0.01$), and root dry weight ($r = 0.92$ and 0.90, $p \leq 0.01$). However, MGT was negatively correlated with the SVI for both YEM(B) ($r = -0.78$ $p \leq 0.01$) and TYE(B) ($r = -0.66$, $p \leq 0.01$) (Table 1).

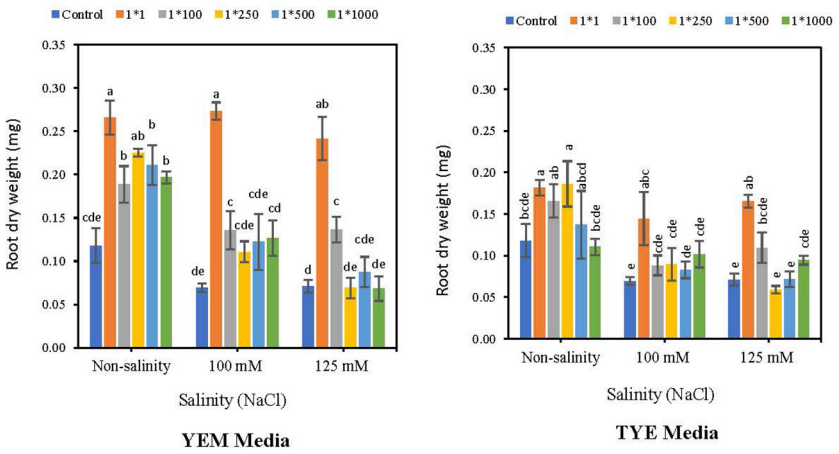

FIGURE 8
Effect of dilutions of *Devosia* sp. YEM(B)-CFS and TYE(B)-CFS and salinity on Root dry weight of soybean seedlings. Values represent the mean of four replicates ± SD. 1:1, 1:100, 1:250, 1:500 and 1:1000 = 1 mL *Devosia* sp. CFS and 1, 100, 250, 500 and 100 mL distilled water, respectively.

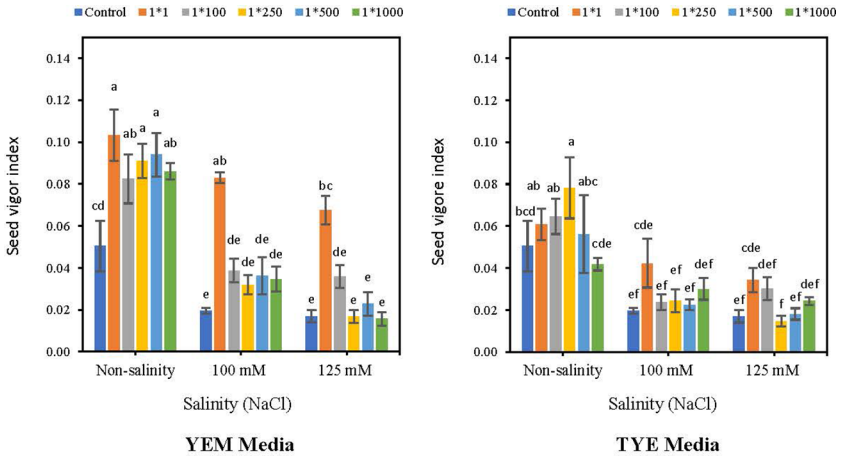

FIGURE 9
Effect of dilutions of *Devosia* sp. YEM(B)-CFS and TYE(B)-CFS and salinity on seed vigor index of soybean seedlings. Values represent the mean of four replicates ± SD. 1:1, 1:100, 1:250, 1:500 and 1:1000 = 1 mL *Devosia* sp. CFS and 1, 100, 250, 500 and 1000 mL distilled water, respectively.

4 Discussion

PGPB are reliable bioinoculants that improve plant performance, among other things through counteracting salinity stress by biosynthesizing a diverse array of bioactive compounds with the ability to activate and regulate plant physiological mechanisms (Ayuso-Calles et al., 2021). However, many reports suggested that microbial culture medium or nutrition strongly influence bacterial growth and their production of bioactive compounds, and extensive testing of bacterial growth as a function of nutrients is necessary for selecting an appropriate culture medium that can support and promote the growth and survival of microorganisms (Davis et al., 2005; Trianto et al., 2020). For this study, we assessed the effect of a range of growth media for the novel *Devosia* sp. (SL43 strain). Based on the results, we selected yeast-extract-mannitol (YEM) as the culture medium for further experiments, as it resulted in the highest growth rate and cell density either under saline or non-saline conditions, and it was generally more effective for growing *Devosia* SL43 strain than other tested media. In addition, we compared this medium and TYE, which was also effective in producing cultures of the *Devosia* SL43 strain. The results indicated that not only did the bacterium have different growth rates in each medium but also the growth patterns (colony morphology) differed visually. Many reports have indicated that bacterial growth on various media is altered in appearance and maximal growth rates for specific microorganisms, and that specific microorganisms have different growth abilities in the presence of specific nutrients or indicators (Shaneeja et al., 2014; Mohammadkazemi et al., 2015; Wang et al., 2019; Bonnet et al., 2020). Similarly, Xu et al. (2017) reported that colonies of *Devosia nitraria* sp. showed different morphologies and growth abilities in different culture media. In addition, *Devosia* sp. grown on YEM(B) medium, produced more effective results, with regard to soybean germination, than TYE(B) medium. Depending on the bacterial culture medium and nutritional growth requirements of the bacterium, compounds produced by them may exist in different quantities and qualities; it is of interest to facilitate the synthesis of the novel target compounds and validate their activities. Other authors have also demonstrated that differences in the composition of the growth medium affected the biosynthesis of bioactive compounds (Pham et al., 2019; Koim-Puchowska et al., 2021; Yusfi et al., 2021). Plants are generally very sensitive to salinity injury during the germination stage, and exposure to salt stress at early stages can retard seedling growth later, even in conditions otherwise suitable for growth. Soil salinity negatively affects the germination of seeds either by imposing osmotic stress that prevents water uptake or by hormonal imbalance (Kumar et al., 2022; Shah et al., 2022; Yaghoubian et al., 2022a). Our observations indicate that CFS treatment promoted soybean germination under salinity stress conditions (Figure 4). The ability of CFS to improve soybean germination under salt stress may result from several mechanisms, such as facilitating resource use or modulating plant hormone levels. Other researchers also reported that the germination of soybean and corn plants under salt conditions was enhanced by application of bacterial cell-free supernatants (Naamala et al., 2022; Shah et al., 2022; Yaghoubian et al., 2022c). Additionally, present results showed that using *Devosia*-CFS accelerated seed germination and stimulated hypocotyl

TABLE 1 The correlation coefficient of laboratory traits and seed vigor index of soybean.

	Seed vigor index	
	YEM(B) Medium	TYE(B) Medium
Final germination	0.5099**	0.4135**
Mean germination time (MGT)	- 0.7882**	-0.6672**
Germination rate (GR)	0.7927**	0.7554**
Root length	0.8432**	0.7112**
Root dry weight	0.9252**	0.9059**

ns and **: No significant and significant at p≤0.01, respectively.

emergence, resulting in a shorter MGT. Promoting effects of CFS may be the result of biologically active substances (hormones, antioxidants, amino acids, vitamins and microbe-to plant signal compounds) produced by the novel *Devosai* SL43 strain, which can improve water up take and regulate germination enzyme activities. It has been reported that inoculation with beneficial microbes provides a broader hormonal pool, which plays a major function in the growth and development of the system (Patten and Glick, 2002; Wang et al., 2012; Tabacchioni et al., 2021). In this sense, lipo-chitooligosaccharides (LCOs) and thuricin 17 are two of the most striking signal compounds investigated so far, both of which were discovered recently; they regulate plant responses to a variety of adverse environmental stresses. In addition, LCOs are produced by N_2-fixing rhizobacteria following isoflavone induction, triggering formation of nitrogen-fixing nodules in host legumes; thuricin 17 production is constitutive (Lyu et al., 2020). Schwinghamer et al. (2016) reported a positive plant response under saline and temperature stress conditions following from application of thuricin 17 and LCO, causing higher biomass production and root development. It has also been demonstrated that LCO strongly affects the rate and uniformity of canola seed germination under cold conditions, which is critical for early spring seeding under Canadian conditions (Schwinghamer et al., 2015). Correspondingly, Naamala et al. (2022); Yaghoubian et al. (2022b) and Sweeney et al. (2017) reported an enhancing effect of using microbe-derived bioactive compounds in stimulating germination rates of soybean, corn and *Arabidopsis thaliana*, respectively. Another stimulating effect of *Devosia*-CFS was alterations of growth/morphological traits, resulting in heavier and longer roots. Microbial-derived metabolites can influence these traits as a regulator for modulating root traits through increasing acquisition of water and nutrients, either by stimulating lateral root development or affecting osmotic balance. Several studies have demonstrated that many plant-associated microorganisms could profoundly affect root growth (Souleimanov et al., 2002; Sweeney et al., 2017). In addition, Fincheira et al. (2016) demonstrated an increase in dry weight and the number of lateral roots of *Lactuca sativa* independently of the used culture medium. Similarly, Gutiérrez-Luna et al. (2010) suggested that microbe-derived compounds can promote lateral root formation in *Arabidopsis thaliana*. Khan et al. (2011) also showed that lipo-chitooligosaccharides from *Bradyrhizobium japonicum* had a stimulus effect on root growth and development in *Arabidopsis thaliana*. The results clearly indicate that SVI was greatly affected by applying YEM(B)-CFS; SVI is a vital trait for plant establishment and uniformity, and it responded to *Devosia* CFS in a way similar to other measured variables and was positively facilitated by microbe-derived compounds under salinity stress. In general, high soil salinity decreases the SVI, either by creating lower osmotic potentials around the outside of seeds that prevent water uptake or by ionic toxicity stress (Sosa et al., 2005). The application of PGPBs has been proven to be a reliable way of helping plants deal with salinity stress by maintaining the cellular osmotic balance and ion homeostasis. The beneficial effects of PGPB in overcoming osmotic shock after exposure to salt stress can be related to osmolyte accumulation and phytohormone signaling that increases germination uniformity. Our hypothesis regarding the moderating effects of PGPBs on salinity stress is supported by formerly published results (Kang et al., 2014; Egamberdieva et al., 2017; Mishra et al., 2021). Many reports have demonstrated that using PGPB had an increasing effect on germination uniformity and vigor index, helping plants to compete more efficiently under salt stress conditions (Erman et al., 2022; Fan and Smith, 2022). Yaghoubian et al. (2022b) also showed that application of CFS from a *Bacillus* strain caused an increase in the SVI of soybean under salt stress.

5 Conclusions

Salinity can be a major abiotic stress in soybean, remarkably reducing the percentage, rate, and uniformity of seedling emergence and, therefore, final soybean production. There is now abundant research suggesting that using plant growth-promoting microbes and their bioactive metabolites is a potentially advantageous approach to improving plant health and productivity under biotic and abiotic stresses. Therefore, gaining detailed knowledge and understanding of cost-effective technologies to produce such biostimulants and biocontrol agents would be important in increasing the availability and/or accessibility of these new products in the agro-input market. This study was focused on choosing an appropriate growth medium for promoting and supporting the growth and survival of the novel *Devosia* sp. strain with the effective bioactive metabolite production, which enhances soybean seed germination under salinity conditions. Findings obtained in this study have suggested that cell-free supernatant obtained from the novel *Devosia* sp. positively improves the germination ability and uniformity of soybean under salt stress. However, increases in seed germination variables were more remarkable when the highest concentration of CFS was applied under salinity stress. Our results also showed that YEM was more appropriate as a growth medium for the *in vitro* cultivation of *Devosai* than TYE medium. The CFS produced by *Devosia* sp. (strain SL43) can successfully reduce salt stress effects on soybean germination variables. It would be interesting to study and manipulate the chemical composition of growth medium to increase bioactive product formation. Another important aspect that would be interesting to be considered is identifying and understanding molecular mechanisms of novel bacterial-derived metabolites which influence host plant physiological and morphological traits and could be a step toward enhancing microbial inoculant technology and developing practical strategies to enhance crop salt tolerance, and quite possibly tolerance to other abiotic stresses.

Data availability statement

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

Author contributions

NM conducted the research and did the initial writing. IY assisted in the research and writing. DS provided the intellectual context, the funding and editorial input. All authors contributed to the article and approved the submitted version.

Funding

The authors would like to acknowledge the support for this manuscript was provided through the Biomass Canada Cluster (BMC), which is funded through Agriculture and Agri-Food Canada's AgriScience program and the industry partner Agroworld.

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 Plant Science

TYPE Original Research
PUBLISHED 04 January 2024
DOI 10.3389/fpls.2023.1328586

OPEN ACCESS

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RECEIVED 27 October 2023
ACCEPTED 11 December 2023
PUBLISHED 04 January 2024

CITATION
He C, Zhang M, Li X and He X (2024) Seasonal dynamics of phyllosphere epiphytic microbial communities of medicinal plants in farmland environment. *Front. Plant Sci.* 14:1328586. doi: 10.3389/fpls.2023.1328586

Seasonal dynamics of phyllosphere epiphytic microbial communities of medicinal plants in farmland environment

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Introduction: The phyllosphere of plants is inhabited by various microorganisms, which play a crucial role in plant physiological metabolism. Currently, there is limited research on the dynamic effects of species and seasons on plant phyllosphere microbial community diversity and microbial interactions.

Methods: In this study, high-throughput sequencing technology was used to sequence the leaf surface parasitic microorganisms of five medicinal plants (*Bupleurum chinense*, *Atractylodes lancea*, *Salvia miltiorrhiza*, *Astragalus membranaceus*, and *Lonicera japonica*).

Results: The results showed that bacteria and fungi clustered into 3,898 and 1,572 operational taxonomic units (OTUs), respectively. Compared to host species, seasons had a more significant impact on the a diversity of bacteria and fungi. The heterogeneity of phyllosphere microbial communities was greater in winter compared to summer. Key species analysis at the OTU level and Spearman correlation analysis demonstrated significant preferences in microbial interactions under plant and seasonal backgrounds. The network connections between bacterial and fungal communities significantly increased during seasonal transitions compared to connections with plants.

Discussion: This study enhances our understanding of the composition and ecological roles of plant-associated microbial communities in small-scale agricultural environments. Additionally, it provides valuable insights for assessing the biodiversity of medicinal plants.

KEYWORDS

medicinal plants, phyllosphere, epiphytic microorganisms, seasonal dynamics, co-occurrence network

1 Introduction

"Phyllosphere microorganisms" refer to microorganisms that adhere to or parasitize the epidermal surface of plant leaves (Blakeman, 1981). Phyllosphere microorganisms originate from various sources, including wind, rain, insect air, soil, and seeds. (Chi et al., 2005; Vorholt, 2012; ZarraonaIndia et al., 2015). In reality, the phyllosphere is regarded as a challenging and unpredictable complex habitat, which is not favorable for microbial colonization due to its constant exposure to fluctuating temperature and humidity, as well as prolonged ultraviolet radiation. In recent years, there has been a growing body of research that has identified a significant abundance of microorganisms residing in the phyllosphere. The phyllosphere is considered to be a crucial ecosystem for microorganisms due to its extensive surface area, which encompasses up to 10^9 km^2 of vegetation worldwide. Additionally, it has the potential to support a vast number of microbial cells, estimated to be around 10^{26} (Vorholt, 2012).

Interactions between plants and their associated microbiomes are contingent upon the identities of the hosts (Zhu et al., 2022). The host species plays a crucial role in determining the composition and structure of microbial communities in the phyllosphere (Obrien and Lindow, 1989). Li et al. (2018) employed Illumina amplicon sequencing to investigate the microbial communities in the phyllosphere and rhizosphere of six distinct species of *Picea* spp. It was found that various plant species had distinct impacts on the diversity and composition of both phyllosphere and rhizosphere microbes. Bao et al. (2020) discovered that the community composition and diversity of phyllosphere epiphytic bacteria and fungi on urban green plants varied depending on the host species. Redford et al. (2010) conducted a study that examined the phyllosphere bacterial communities of 56 tree species. They also investigated the composition of bacterial communities in *Pinus ponderosa* at various locations. The researchers discovered significant differences in phyllosphere bacterial communities among the tree species. Interestingly, they discovered that the bacterial communities composition of *Pinus ponderosa* was similar across different sampling locations in Boulder, Colorado, USA. These results indicate that in different regions, the host species often have a greater impact on the composition of the phyllosphere microorganisms than environmental factors. The phenomenon under investigation may be attributed to the deliberate release of one or more biological signals by the host plant through leaf stomata or epidermis. These signals serve to attract beneficial microorganisms that aid in the plant's growth or act as a defense mechanism against unfavorable conditions. It is also plausible that variations in the physiological structures of plant leaves enable them to selectively attract specific microorganisms for colonization. Therefore, it is imperative to acknowledge the significance of the relationship between epiphytic microorganisms and plants.

Season is widely acknowledged as a key determinant in shaping epiphytic microbial communities. Bao et al. observed notable fluctuations in the community compositions of phyllosphere epiphytic bacteria throughout the host plants' growing season. Exogenous biodegradation pathways exhibited a notable increase in bacterial communities during in May. The findings from the network analysis revealed that the relationship between the bacterial community on leaf surfaces in May was more intricate compared to that in October, with a stronger negative correlation observed. Additionally, fluctuations in the abundance and diversity of epiphytes were observed across various seasons. (Bao et al., 2020; Bao et al., 2022). Jackson and Denney (2011) confirmed that there was seasonal pattern in the community composition of phyllosphere epiphytic bacteria on *Magnolia grandiflora* leaf layer throughout a year. Specifically, it was observed that the variation in the superbiotic bacterial community among different leaves collected during the same period was minimal. However, there was a significant difference in the bacterial community among leaves collected at different times. Notably, the bacterial community on leaves collected in August 2008 exhibited the greatest dissimilarity compared to other seasons. In addition, previous studies have demonstrated the seasonal dynamics of phyllosphere bacterial and fungal communities in *Populus deltoids*, *Ginkgo biloba*, *Pinus bungeana* and *Cunninghamia lanceolata*, respectively(Cordier et al., 2012; Peñuelas et al., 2012; Rastogi et al., 2012; Materatski et al., 2019). Therefore, it is imperative to consider different growing seasons when designing interactions between phyllosphere microbes and host plants.

Previous research on epiphytes in the phyllosphere of plants tends to focus primarily on pathogens that are of agricultural importance (Jain et al., 2019). The positive effects of the phyllosphere microbiome have, nonetheless, been substantiated by a growing body of research. Ritpitakphong et al. investigated the resistance of *Pseudomonas* sp. The efficacy of *Botrytis cinerea* control on the leaf surface of *Arabidopsis thaliana* was investigated. Specifically, under sterile conditions, the *Arabidopsis thaliana* variant bdg became as susceptible to bovines infection as the wild type (WT), while the *lacs2.3* mutant retained resistance. The resistance of *bdg* mutant to *Botrytis cinerea* was restored by adding washing solution of microbiome, which mainly include to *Pseudomonas* sp, cleaned from *lacs2.3* mutant leaf to *bdg* leaf.(Ritpitakphong et al., 2016). Busby et al. (2016) conducted an experiment where they introduced phyllosphere fungi to the leaves of *Populus trichocarpa*, resulting in a reduction in the severity of rust pathogen Melampsora × columbum infection. Phyllosphere nitrogen fixation has been identified as a significant contributor to biological nitrogen fixation in tropical ecosystems, as suggested by Cleveland et al. (1999). In Mediterranean woodland ecosystems, Rico et al. discovered that notably nitrogen-fixing bacterial populations were present in epiphytic bacteria of all *Quercus ilex* leaves (Rico et al., 2014). Therefore, phyllosphere epiphytes are microbial communities that possess significant functional significance. Predicting the functions of phyllosphere epiphytic microorganisms presents various opportunities to enhance the growth performance of host plants.

China possesses a rich and varied array of resources in the realm of traditional medicinal plants. Nevertheless, the natural regeneration rates of medicinal plants in the wild are generally low, and a significant number of these plants are currently facing the risk of extinction due to factors such as overharvesting, habitat loss, and anthropogenic activities. Industrialized plantations for medicinal plants have been established on a national scale in China. By the end of 2020, artificial cultivation of over 300 species of medicinal plants had been achieved, covering a planting area of

approximately 600 million square meters (Wang et al., 2020). Nevertheless, cultivated medicinal plants often face challenges such as insufficient levels of active components and low rates of transplant survival. In recent years, there has been a significant increase in research focusing on the correlation between medicinal plants and microorganisms, which has garnered considerable attention (Ntemafack et al., 2021). The implementation of microbiological approaches to enhance resource conservation and promote sustainable utilization of medicinal plants has emerged as a significant area of research. According to Wu et al. (2021), endophytic bacteria have been found to enhance plant growth and development, enhance their resistance to both biotic and abiotic stresses, and stimulate the production of novel compounds that may have potential medical applications. Han et al. (2021) made the discovery that the dark septate endophyte successfully colonized all 25 medicinal plants within the farming region of northern China. Chen et al. (2011) conducted a study in which they successfully isolated and identified approximately 80 culturable endophytic fungi from 10 different species of medicinal Dendrobium. The identification process involved the use of both morphological and molecular techniques. The findings of the study revealed a significant level of biodiversity among the endophytic fungi associated with Dendrobium plants. The current body of research regarding the advantageous impacts of the phyllosphere epiphytic microbial community on medicinal plants is constrained.

To examine the influence of host plants and seasonal variations on the composition of epiphytic bacterial and fungal communities in medicinal plants within agro-ecosystems, as well as to explore the associations between epiphytes and their host plants, and the functional capabilities of epiphytes, our study was conducted at the Anguo Medicine Planting Site. The abundance, diversity, and composition of phyllosphere epiphytic bacterial and fungal communities of 5 medicinal plants, viz., *Bupleurum chinense* DC., *Atractylodes lancea* (Thunb.) DC, *Salvia miltiorrhiza* Bge., *Astragalus membranaceus* (Fisch.) Bge., *Lonicera japonica* Thunb., was conducted using Illumina Miseq high-throughput sequencing (HTS) technology. Additionally, co-occurrence networks of microorganisms between plants and seasons were established. We formulated the following hypotheses: (1) The phyllosphere of various medicinal plants harbors diverse and distinct bacterial and fungal communities. (2) The composition of epiphytic communities varies based on the identity of the plant or the season. (3) Extensive intra-community interactions occur among epiphytes within the same season or plant. This research will lay the groundwork for revealing the ecological significance and functions of epiphytic communities in agricultural ecosystems, as well as the biodiversity and survival strategies of epiphytic communities in various environments.

2 Materials and methods

2.1 Study site and sample collection

The sampling sites were situated in the Anguo Medicine Planting Site (38°42′ N, 115°32′ E) within Hebei Province, China. The study area exhibits a characteristic temperate continental climate, characterized by an average monthly temperature of 12.3°C and a precipitation level of 51.6 mm. Cultivated medicinal plants at the planting site underwent two rounds of irrigation throughout their entire growing season. In addition, water-soluble fertilizer is used once a year to irrigate the roots of all plants. The amount of fertilization per time was N 180 kg/hm^2, P_2O_5 90 kg/hm^2, K_2O 180 kg/hm^2.In June and November 2021, leaf samples were gathered from five distinct species of medicinal plants, specifically *Bupleurum chinense*, *Atractylodes lancea*, *Salvia miltiorrhiza*, *Astragalus membranaceus*, and *Lonicera japonica*. Three replicated plots were designated for each species, and within each plot, three healthy plant individuals were randomly chosen, ensuring a minimum distance of 50 meters between each selection. A minimum of 100 grams of fresh and healthy leaves were harvested from each plant at a consistent height above the ground, utilizing sterile scissors. Leaf samples were promptly placed into ice boxes at a temperature of 4°C and subsequently transported to the laboratory. Subsamples of three plant individuals were collected from each plot and combined into a single sample for the purpose of extracting epiphytes.

2.2 Phyllosphere epiphytic microbial isolation

Five grams of plant leaves were weighed and put into a 50 ml centrifuge tube, with 50 mL of 0.1 M potassium phosphate buffer (PPB, pH=8.0) added. The leaf sample in tubes were washed with 1 min sonication and 10 s vortex, and repeated. Then the leaves were transferred to new tubes with 50mL of 0.1M PPB and wash again. The suspension from two washes were mixed and filtered through a 0.2μm membrane., The filter membranes with epiphytes were snap frozen in liquid nitrogen and stored at -80°C in refrigerator (Bodenhausen et al., 2013).

2.3 Sample DNA extraction

The genomic DNA from phyllosphere epiphytic microorganisms was extracted from filter membranes using the FastDNA® Spin Kit for Soil (MP Biomedicals, USA) according to user's manual. The DNA purity and concentration were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and DNA integrity was examined using 1% agarose gel electrophoresis.

2.4 PCR amplification and library creation for sequencing

The 16S V3-V4 region of epiphytic bacteria and ITS1 region of epiphytic fungi were amplified with 338F/806R (ACTC CTACGGGAGGCAGCAG/GGACTACHVGGGTWTCTAAT) and ITS1F/ITS2 (CTTGGTCATTTAGAGGAAGTAA/GCTG CGTTCTTCATCGATGC), respectively, with ABI GeneAmp® 9700 PCR thermocycler (ABI, USA). PCR reactions were

performed in a 20 μL system, which included 2 μL 10× Buffer, 2 μL 2.5 mM dNTPs, 0.8 μl each of 5 μM primers, 0.2 μL TaqPolymerase, 0.2 μL BSA, 10 ng template DNA, and ddH_2O supplemented to 20 μL. The amplification for 16S V3-V4 region of bacteria were performed under following conditions: denaturation at 95°C for 3 min; 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, 27 cycles; extension at 72°C for 10 min. For ITS1 region of fungi, PCR amplifications were conducted with same reaction system and condition, except the reaction were repeated for 35 cycles. Each amplification was replicated for three times. The replicated PCR products of a same sample were pooled, recovered using 2% agarose gel, and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). The recovered PCR products were then quantified using a Quantus™ Fluorometer (Promega, USA). The purified amplification fragments were mixed in equal amounts, and the libraries were constructed using the NEXTFLEX® Rapid DNA-Seq Kit. Shanghai Majorbio Bio-pharm Technology Co., Ltd used Illumina's MiSeqPE300 platform to carry out the final sequencing. The raw data were deposited in NCBI SRA database (PRJNA942029, PRJNA942068).

2.5 Data processing

The paired-ended raw sequences were spliced and quality controlled using software tools *fastp* (version 0.19.6) and FLASH (version 1.2. 11) with following steps: (1) filter the bases with quality values below 20 and reads containing N bases, set a window of 50 bp, truncate the bases if the average quality value within window is below 20, and finally filter the reads below 50 bp after quality control; (2) pairs of reads were spliced in accordance with the overlap between PE reads, with a minimum overlap length of 10 bp; (3) a maximum mismatch ratio of 0.2 was permitted in the overlap area of the spliced sequence, and non-conforming sequences were removed; (4) samples were demultiplexed based on the barcode. The quality controlled spliced sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity using UPARSE software (version 7.1). All sequences with mitochondrial and chloroplast annotations were stripped off. The samples were rarefied due to the minimum sum of sequences in all samples to reduce the effect of sequencing depth on the subsequent analysis of alpha- and beta-diversity data. The taxonomic placement of epiphytic bacteria and fungi were annotated according to Silva 16S rRNA gene database (v 138) and UNITE databases (Version 8.0), respectively, using the RDP classifier (version 2.11) with 70% confidence threshold. The community composition for each sample was analyzed at various species taxonomic levels. The BugBase, the FAPROTAX (Louca et al., 2016) manual construction database, and the FUNGuild (Fungi Functional Guild) database were used to perform bacterial phenotypic predictions, functional predictions, and ecological functional predictions, respectively. Relative abundance is the percentage of abundance of a species in a community that is the sum of the abundance of all species, all references below are to RA.

2.6 Statistical analysis

The Majorbio Bio Cloud platform (https://cloud.majorbio.com) was used for all data analysis. Alpha diversity indices were estimated using Wilcoxon rank sum test for inter-group variance analysis of alpha-diversity *via* the mothur software (Schloss et al., 2009). Non-metric multidimensional scaling (NMDS) based on the bray-curtis distance algorithm, was used to examine the similarity of microbial community structure between samples, and PERMANOVA non-parametric test was used to test the significance of differences in microbial community structure between groups.

Network analysis was used to predict patterns of interaction between phyllosphere epiphytic bacteria and fungi. Spearman correlation tests were performed, and networks were constructed using OTU based on the top 50 of abundance. The total abundance of the top 50 OTUs of bacteria is 40.8%, while that of fungi is 43.6%. Correlations with absolute values of correlation coefficient (rho) greater than 0.8 and p-values less than 0.01 were retained for network analysis (Wang et al., 2017). Co-occurrence networks were analyzed using Cytoscape (version 3.8.2), and the number of nodes and edges, clustering coefficients and network density were analyzed using the built-in application network analyzer (Shannon et al., 2003; Assenov et al., 2008). In addition, modules and highly interconnected nodes and central taxa, were analyzed using MCODE (Bader and Hogue, 2003). Sobs refers to observed richness. Shannon is one of the indices used to estimate the diversity of microorganisms in a sample, with higher Shannon values indicating higher community diversity. Shannoneven is a measure of homogeneity based on the Shannon index. The Student's T test, uses t-distribution theory to infer the probability of a difference occurring and thus compare whether the difference between two means is significant. The Kruskal-Wallis H test, is a method of extending the Wilcox rank sum test for two independent samples to a non-parametric test for multiple (≥ 3) independent samples.

3 Results

3.1 Epiphytic microbial community composition

A total of 1,272,729 reads were obtained for bacterial sequences, while 1,900,620 reads were obtained for fungal sequences, using high-throughput sequencing. After implementing quality control measures to eliminate low-quality sequences, a total of 1,189,582 reads for bacterial sequences and 1,752,919 reads for fungal sequences were obtained, meeting the necessary criteria for further analysis. The sequences in both datasets were clustered, resulting in a total of 3,537 bacterial OTUs and 1,450 fungal OTUs, respectively. The rarefaction curves of the Sobs indices for phyllosphere epiphytic bacteria and fungi at the OTU level demonstrated a plateau phenomenon as the number of sampled reads increased. This observation suggests that the sampling and sequencing strategy utilized in this study were adequate for conducting diversity analysis (Supplementary Figure S1).

A comprehensive analysis of phyllosphere epiphytic bacteria from five medicinal plants during both summer and winter seasons resulted in the identification of a total of 3,537 OTUs. These OTUs belonged to 38 different phyla, 101 classes, 232 orders, 390 families, 859 genera, and 1,601 species. Meanwhile, the assemblage of phyllosphere epiphytic fungi consisted of a total of 1,450 OTUs, which encompassed 9 phyla, 34 classes, 89 orders, 205 families, 437 genera, and 780 species.

The composition of phyllosphere epiphytic bacteria at the phylum level (Figure 1A) exhibited two predominant phyla during both summer and winter across the five plants studied. These phyla were Actinobacteriota (Relative Abundance=32.61%-52.40%) and Proteobacteria (RA=20.28%-53.70%). The Firmicutes group exhibited the third highest abundance during the summer months, with a relative abundance ranging from 11.89% to 25.94%. However, their presence significantly decreased in November, with a relative abundance ranging from 0.04% to 5.34%. In contrast, Bacteroidota (RA=5.37%-10.94%) emerged as the third most prevalent group during the winter season across all plant species. The order Micrococcales (RA=11.73%-43.22%) was found to be the dominant epiphytic bacterial community in both summer and winter across all host species (Figure 1B). The Rhizobiales taxonomic group exhibited a seasonal turnover, with relative abundance (RA) ranging from 3.62% to 6.09% in summer, and significantly higher values of 11.77% to 31.03% in winter.

In both summer and winter, the composition of phyllosphere epiphytic fungal communities in host plants was found to be similar at the phylum level (Figure 1C). The dominant phyla in these communities were Dothideomycetes (RA=23.73%-76.40%) and Tremellomycetes (RA=20.20%-66.69%). An exception was observed in the summer community on L. maackii, where Leotiomycetes exhibited predominance with a relative abundance (RA) of 64.58%. In contrast, Dothideomycetes and Tremellomycetes had RAs of 20.81% and 14.09%, respectively. At the order level (Figure 1D), the fungal communities of all host plants in summer, with the exception of L. maackii, were dominated by Filobasidiales (RA=13.12% - 64.33%), Capnodiales (RA=18.82% - 44.44%), and Pleosporales (RA=11.79% - 31.95%). The fungal communities associated with L. maackii during the summer season were found to be predominantly composed of Erysiphales (RA=64.56%), Filobasidiales (RA=13.62%) and Capnodiales (RA=18.46%). Meanwhile, during the winter season, the Tremellales (RA=10.64% - 36.42%), along with the Pleosporales (RA = 10.22% - 59.11%) and Capnodiales (RA = 6.42% - 17.99%), exhibited dominance in the epiphytic fungal communities.

The study revealed that the prevalence of bacterial OTUs was significantly greater during the summer in comparison to the winter across all host plants (Figure 2A). During the summer, B. chinense demonstrates the highest OTU richness among the species, whereas *S. miltiorrhiza* displays the lowest OTU richness. In the winter, the plant species *A. membranaceus* displays the highest level of abundance, whereas *L. japonica* exhibits the lowest level of abundance. A comprehensive analysis revealed that a total of 139 bacterial OTUs exhibited consistent occurrence across all host plants and throughout both seasons, as illustrated in Figure 2C. When analyzing the data with respect to seasons, it was observed that there were 871 bacterial OTUs were present during the summer, whereas only 185 were detected during the winter (Figure 2E). In the summer, the number of host-specific OTUs observed among different plant species. The species *S.miltiorrhiza* exhibited the lowest number of OTUs, with a count of 38, whereas *A. membranaceus* had the highest number of OTUs, totaling 235. *Lonicera japonica* demonstrated the lowest count of distinct OTUs, amounting to 34, while *A.membranaceus* displayed the highest count of unique OTUs, reaching 494 during the winter (Figure 2E). The summer and winter communities of five host species, namely *B.chinense*, *A.lancea*, *S.miltiorrhiza*, *A. membranaceus*, and *L. japonica*, displayed shared OTUs of 1,020, 258, 606, 1,202, and 355, respectively (Figure 2G). Pie charts were employed to visually depict the distribution of OTUs within the intersecting subsets of a particular plant species during both the summer and winter (Figure 2G). We have observed that different plant species display diverse bacterial OTU throughout various seasons.

The abundance of epiphytic fungi OTUs in winter is significantly higher than in summer, in contrast to the phenomenon observed in bacteria. In the summer, *S. miltiorrhiza* exhibits the highest OTU abundance, while *L. japonica* has the lowest. In winter, A. membranaceus exhibits the highest OTU abundance, while *L. japonica* shows the lowest (Figure 2B). Analysis of the petal diagram reveals a total of 87 OTUs across all plant species (Figure 2D). In the analysis of season-specific OTUs, the results indicate that during the summer, *S. miltiorrhiza* has the highest number of specific OTUs, while *L. japonica* has the lowest. In winter, *A. membranaceus* has the highest number of specific OTUs (Figure 2F). In summer and winter, *B. chinense*, *A. lancea*, *S. miltiorrhiza*, *A. membranaceus*, and *L. japonica* have 236, 196, 374, 290, and 139 shared OTUs, respectively (Figure 2H).

3.2 Epiphytic microbial alpha-diversity

The alpha diversity of epiphyte OTUs was assessed based on host plants and seasons using Sobs, Shannon, and Shannoneven indices. In the context of bacterial communities, we have observed a lesser degree of variation in diversity among summer communities. Significant differences were observed between *B. chinense* and *S. miltiorrhiza*, *A. lancea* and *S. miltiorrhiza*, and *A. lancea* and *A. membranaceus*, as indicated by the Sobs index. No significant disparity in host plant selection during the summer was observed by Shannon and Shannoneven indices. However, a significant influence of the host was observed on the diversity of epiphytic bacteria, as evidenced by the diversity indices which showed statistically significant differences ($p < 0.05$) among plants during the winter (Figure 3A). *B. chinense*, *A. lancea*, and *L. japonica* exhibited notable variations in bacterial diversity between the summer and winter, as indicated by the Shannon index.

In the fungal communities, notable variations were observed in the Sobs index, Shannon index, and Shannoneven index, both among the host plants during the summer and among the host plants during the winter (Figure 3B). Furthermore, *B. chinense*, *A. lancea*, *S. miltiorrhiza*, and *L. japonica* exhibited notable variations

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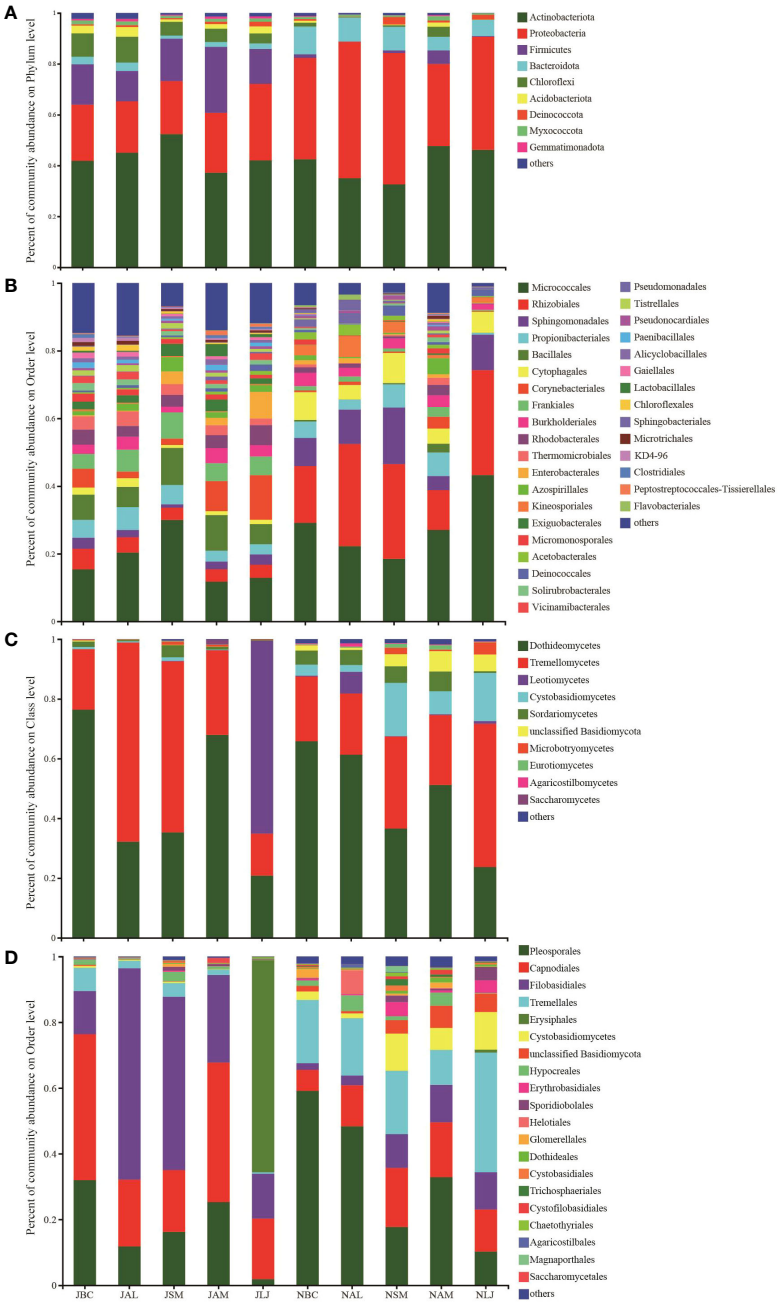

FIGURE 1
Relative abundance of epiphytic bacterial **(A, B)** and fungi **(C, D)** in medicinal plants.Represents < 0.01% of the total reads of epiphytic bacterial and fungi were all assigned to "Others". J, June-Summer; N, November-Winter; BC, *Bupleurum chinense*; AL, *Atractylodes lancea*; SM, *Salvia miltiorrhiza*; AM, *Astragalus membranaceus*; LJ, *Lonicera japonica*.

in fungal populations across seasons, as indicated by the diversity indices.

3.3 Comparison of the similarity of phyllosphere epiphytic microbial communities

The composition of the epiphytic bacterial community exhibited significant variations among different plants (F=0.9639, P=0.001) and across different seasons (F=0.8209, P=0.001), as determined by NMDS and ANOSIM tests (Figures 4A, C). The PERMANOVA analysis demonstrated that plant species accounted for 85.42% of the variation in the composition of the phyllosphere epiphytic bacterial community (P=0.001), while seasons explained 43.89% of the variation (P=0.001) for seasons (Table 1).

The ANOSIM results indicated that the community composition of phyllosphere epiphytic fungi was significantly influenced by both seasons (F=0.5769, P=0.001) and plants (F=0.9521, P=0.001) (Figures 4B, D). The composition of the

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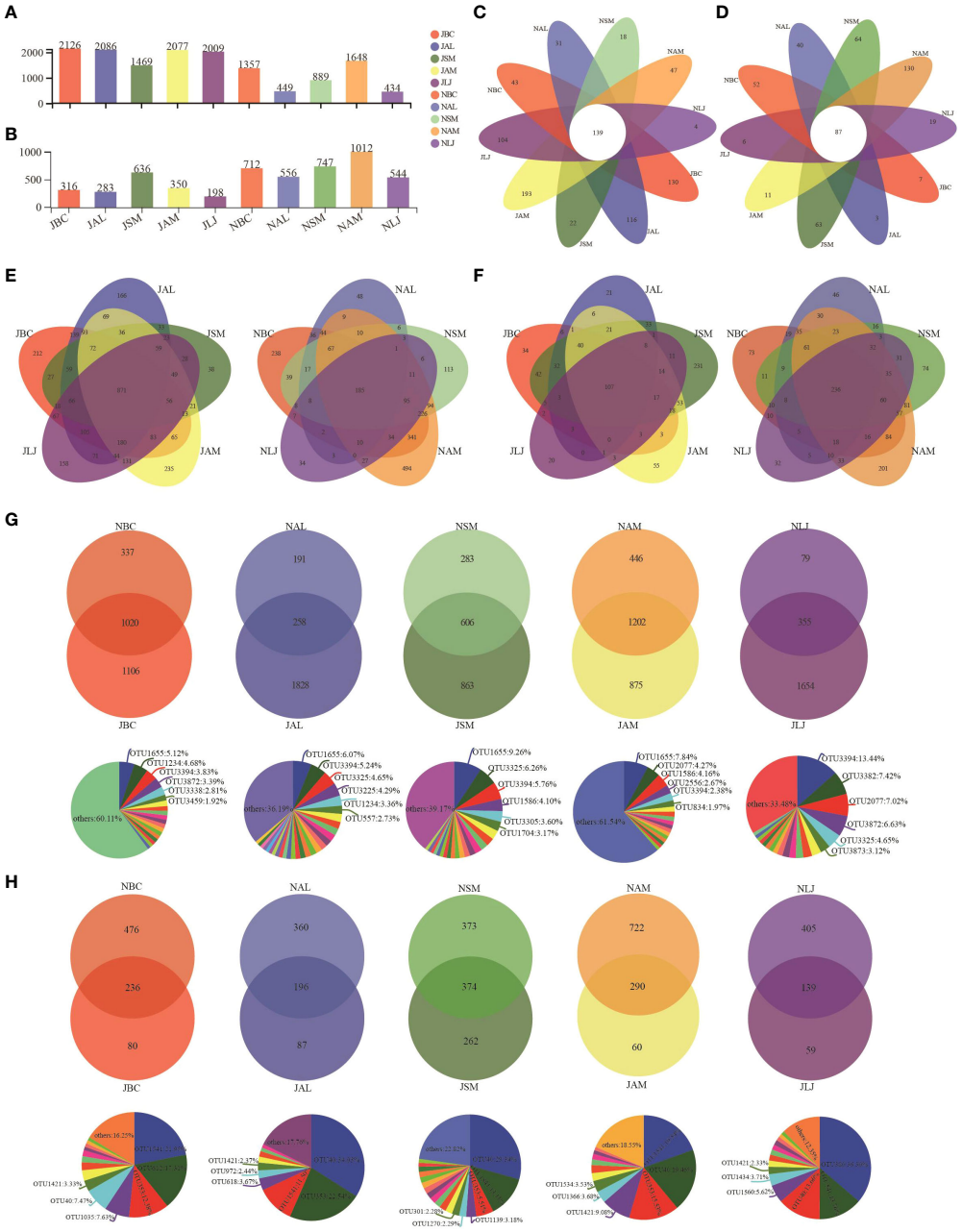

FIGURE 2
Distribution of the epiphytic bacterial **(A, C, E, G)** and fungi **(B, D, F, H)** OTUs in different plants **(E, F)** and in different seasons **(G, H)**. J, June-Summer; N, November-Winter; BC, *Bupleurum chinense*; AL, *Atractylodes lancea*; SM: *Salvia miltiorrhiza*; AM, *Astragalus membranaceus*; LJ, *Lonicera japonica*.

epiphytic fungal community was significantly influenced by the plant species (91.24%, P=0.001) and the season (29.87%, P=0.001), as determined by PERMANOVA analysis (Table 1).

3.4 Analysis of differential phyllosphere epiphytic microbial communities

The colonization patterns of epiphytic bacterial and fungal taxa exhibited notable variations across different host plants in our study. ANOSIM tests conducted among various plant species revealed significant differences in the abundance of 15 most prevalent taxa in both summer and winter communities (Figure 5). In the summer, the unclassified *Paracoccus* (OTU172) exhibited a significant increase in abundance within *B. chinense*. Unclassified *Marmoricola* (OTU3414), uncultured *Frankiales* (OTU1369), uncultured *Planomicrobium* (OTU824), and *Nocardioides* sp. were identified in the sample. (OTU3276) exhibited a significant enrichment in *A. lancea*. Unclassified *Arthrobacter* (OTU1655), uncultured *Skermanella* (OTU2556), and unclassified *Enterobacteriaceae* (OTU2146) exhibited significant enrichment in *S. miltiorrhiza*. The presence of

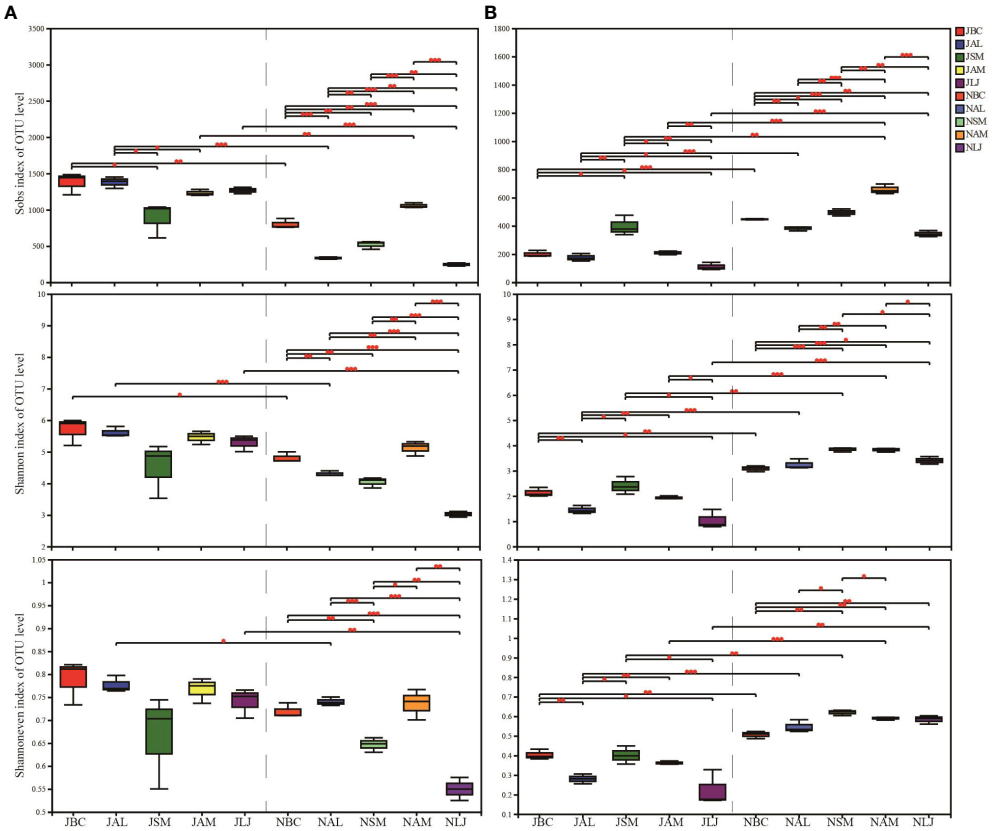

FIGURE 3
OTU richness, Shannon and Shannoneven of phyllospheric epiphytic bacterial **(A)** and fungi **(B)**. * P < 0.05, ** P < 0.01 and *** P < 0.001. J, June-Summer; N, November-Winter; BC, *Bupleurum chinense*; AL, *Atractylodes lancea*; SM, *Salvia miltiorrhiza*; AM, *Astragalus membranaceus*; LJ, *Lonicera japonica*.

Uncultured *Rubellimicrobium* (OTU1558) was significantly higher in *L.japonica* (Figure 5A). In the winter, the *A. lancea* species exhibits a significantly higher abundance of unclassified *Microbacterium* (OTU3225) and *Quadrisphaera granulorum* (OTU3335). *S. miltiorrhiza* was found to be colonized by *Methylobacterium brachiatum* (OTU3325), *Methylobacterium komagatae* (OTU 2908), and an uncultured *Novosphingobium* (OTU3305), which were present in high abundance. The presence of unclassified *Arthrobacter* (OTU1655) was significantly higher in *A. membranaceus*. Unclassified *Curtobacterium* (OTU 3394), *Methylobacterium adhaesivum* (OTU 3872), *Microterricola viridarii* (OTU3382), unclassified *Sphingomonas* (OTU3873), and *Methylorubrum extorquens* (OTU3657) exhibited significant differences in their presence within *L. japonica* (Figure 5B).

Similarly, the colonization patterns of phyllosphere epiphytic fungi with high abundance varied significantly across different host plants. In the summer, we discovered the presence of unclassified Alternaria (OTU353), *Vishniacozyma* sp. (OTU14), *Vishniacozyma tephrensis*, and *Paraphoma* sp. (OTU1209) exhibited enrichment in *B. chinense*. *Filobasidium* sp. (OTU40) and *Filobasidium globisporum* (OTU1420) exhibited higher abundance in *A. lancea*. *Naganishia* sp. (OTU286, OTU51) and *Gibberella intricans* (OTU257) were found to be enriched in *S. miltiorrhiza*. *Cladosporium aggregatocicatricatum* (OTU335) and *Epicoccum nigrum* (OTU1366) were found to be abundant in *A. membranaceus*. *Erysiphe lonicerae*(OTU380) was found to be abundant in *L. japonica* (Figure 5C). In the winter, there was a notable increase in the presence of *Didymella rosea* (OTU1035) in *B. chinense*. *A. lancea* was colonized by enriched *unclassified Alternaria* (OTU353). *Cladosporium delicatulum* (OTU1541), an unclassified *Basidiomycota* (OTU1534), *Symmetrospora coprosmae* (OTU430), *Epicoccum nigrum* (OTU1366), and an unclassified *Didymella* (OTU1270) exhibited higher abundance in *A. membranaceus*. *Filobasidium* sp. (OTU40), *Dioszegia zsoltii* (OTU1560), *Symmetrospora symmetrica*(OTU1139), and *Bullera alba* (OTU1434), exhibited a significant enrichment in *L. japonica* (Figure 5D).

Significant variations in colonization patterns were observed between seasons for both bacterial and fungal taxa in our study. Through the implementation of Veen analysis to assess the diversity of epiphytic bacteria and fungi across various plant species, our findings indicate that there is only one shared bacterial species among all the species examined. However, there are distinct bacterial species that are unique to each of the following plant species: *A.lancea* (3 unique species), *S.miltiorrhiza* (1 unique species), *A.membranaceus* (6 unique species), *L.japonica* (2 unique species), and *B.chinense* (2 unique species). Among fungi, the total number of species shared by all species is 1,

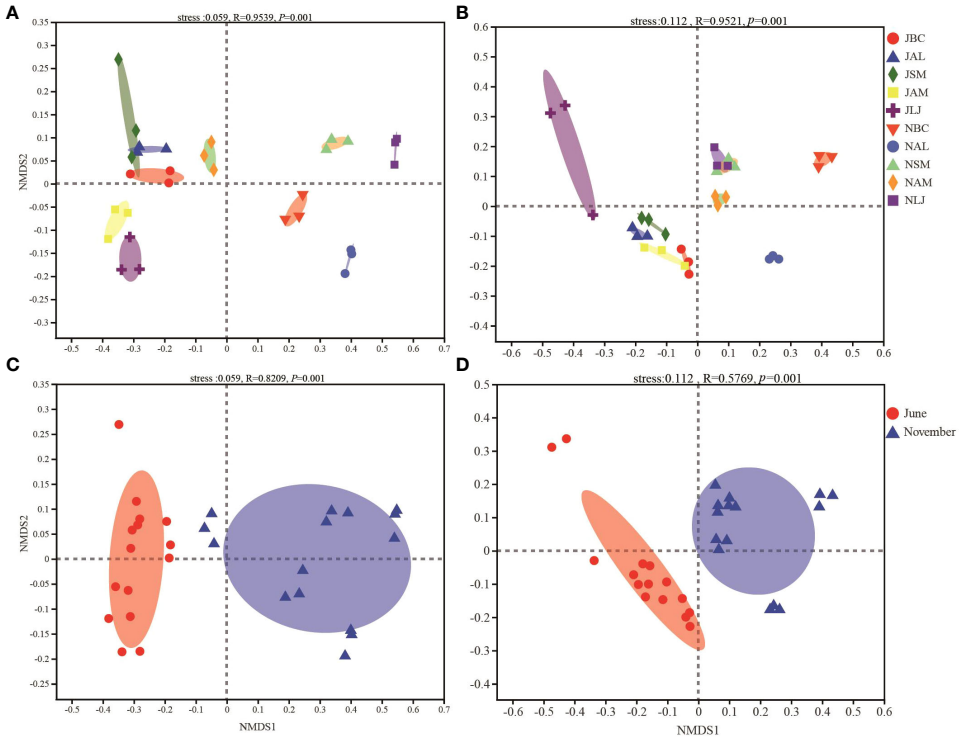

FIGURE 4
Nonmetric multidimensional scaling (NMDS) ordination of the bacterial **(A, C)** and fungi **(B, D)** community composition among different plant species and seasons. J, June-Summer; N, November-Winter; BC, *Bupleurum chinense*; AL, *Atractylodes lancea*; SM, *Salvia miltiorrhiza*; AM, *Astragalus membranaceus*; LJ, *Lonicera japonica*.

whereas *A. lancea*, *S.miltiorrhiza*, *A. membranaceus*, *L. japonica*, and *B. chinense* have 6, 3, 5, 5, and 8 unique species, respectively (Figures 6A, B).

Specifically, we found that the 15 most abundant taxa were depicted separately based on the host plants (Figure 6C). Of these 15 bacterial taxa, only *Sphingomonas panni* (OTU3459) occurs in all host plants. *A. membranaceus* has the most uniquely different bacterial taxa, including *Rhodococcus erythropolis* (OTU2077),unclassified *Methylobacterium* (OTU854), *Bacillus aryabhattai* (OTU2151), uncultured *Tumebacillus* (OTU 1592), unclassified *Mycobacterium* (OTU2313) and *Bacillus simplex* (OTU3465). *S. miltiorrhiza* exhibited the lowest number of distinct bacterial taxa, with only one identified as *Novosphingobium* sp. P6W(OTU3374).

Among the 15 abundant fungal taxa with seasonal differentiation in each plant species, only one taxon, viz., unclassified *Cladosporium* (OTU1421), was presented among all host plants (Figure 6D). *B. chinense* exhibits a high abundance of distinctive fungal taxa, such as *Didymella rosea* (OTU1035), *Cryptococcus aureus* (OTU1563), unclassified *Vishniacozyma* (OTU501), *Paraphoma* sp. (OTU1209), *Vishniacozyma tephrensis* (OTU560), unclassified *Lectera* (OTU284), unclassified *Neosetophoma* (OTU788) and *Vishniacozyma carnescens* (OTU301). *S. miltiorrhiza* exhibits the lowest number of distinct fungal taxa, including unclassified *Cryptococcus* (OTU818), *Naganishia* sp. (OTU286), and *Saitozyma flava* (OTU874).

3.5 Correlation network analysis

Correlation network analysis using Spearman correlation was conducted to examine the variations in network structures of epiphytic communities among different plants and across seasons. This analysis was based on the abundance of the top 50 most abundant OTUs (Figure 7). The networks of communities consisting of epiphytic bacteria from all five host plants exhibited a decrease in the number of nodes, edges, and clustering coefficients during the summer compared to the winter. Conversely, the network

TABLE 1 Effects of plant species and seasons on microbial community structure based on PERMANOVA.

	Bacterial		Fungi	
	R^2(%)	Pr(>*F*)	R^2(%)	Pr(>*F*)
Plant species	85.41	0.001	91.24	0.001
Season	43.89	0.001	29.87	0.001

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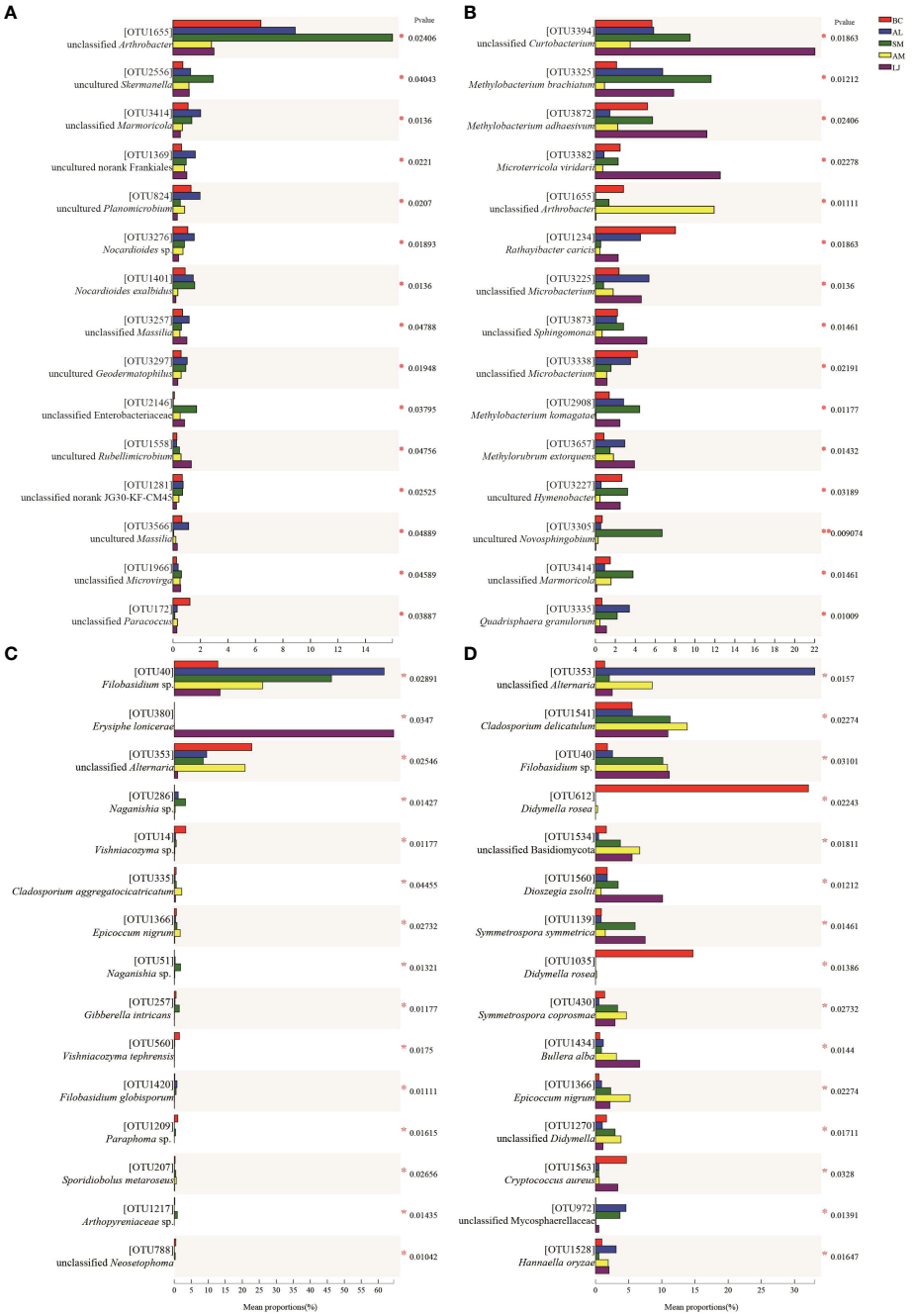

FIGURE 5
Anosim tests the richness differences of the 15 most abundant in phyllospheric epiphytic bacterial **(A, B)** and fungal **(C, D)** communities between different plant species in summer **(A, C)** and winter **(B, D)**. BC, *Bupleurum chinense*; AL, *Atractylodes lancea*; SM, *Salvia miltiorrhiza*; AM, *Astragalus membranaceus*; LJ, *Lonicera japonica*. *indicates the significant difference at P < 0.05. **indicates the significance difference at P < 0.01.

densities and centrality coefficients were higher in the summer than in the winter (Figures 7A, B; Table 2). The epiphytic bacterial community networks within a specific host exhibited distinct network characteristics across different seasons (Figures 7C–G; Table 2). For instance, the bacterial communities in *B. chinense* exhibited the highest number of edges, network density, and centrality coefficient. On the other hand, *A. lancea* had the highest number of nodes but the lowest clustering coefficient. *S.miltiorrhiza*, in contrast, had the lowest centrality coefficient. *A. membranaceus* had the fewest nodes, edges, and lowest network density. Lastly, *L. japonica* displayed the highest clustering coefficient.

Community networks comprising epiphytic fungi from all five host plants exhibited higher edge, clustering coefficients, network density, and centrality coefficients during the summer compared to the winter (Figures 7H, I; Table 2). The network characteristics of epiphytic fungal communities associated with a specific host

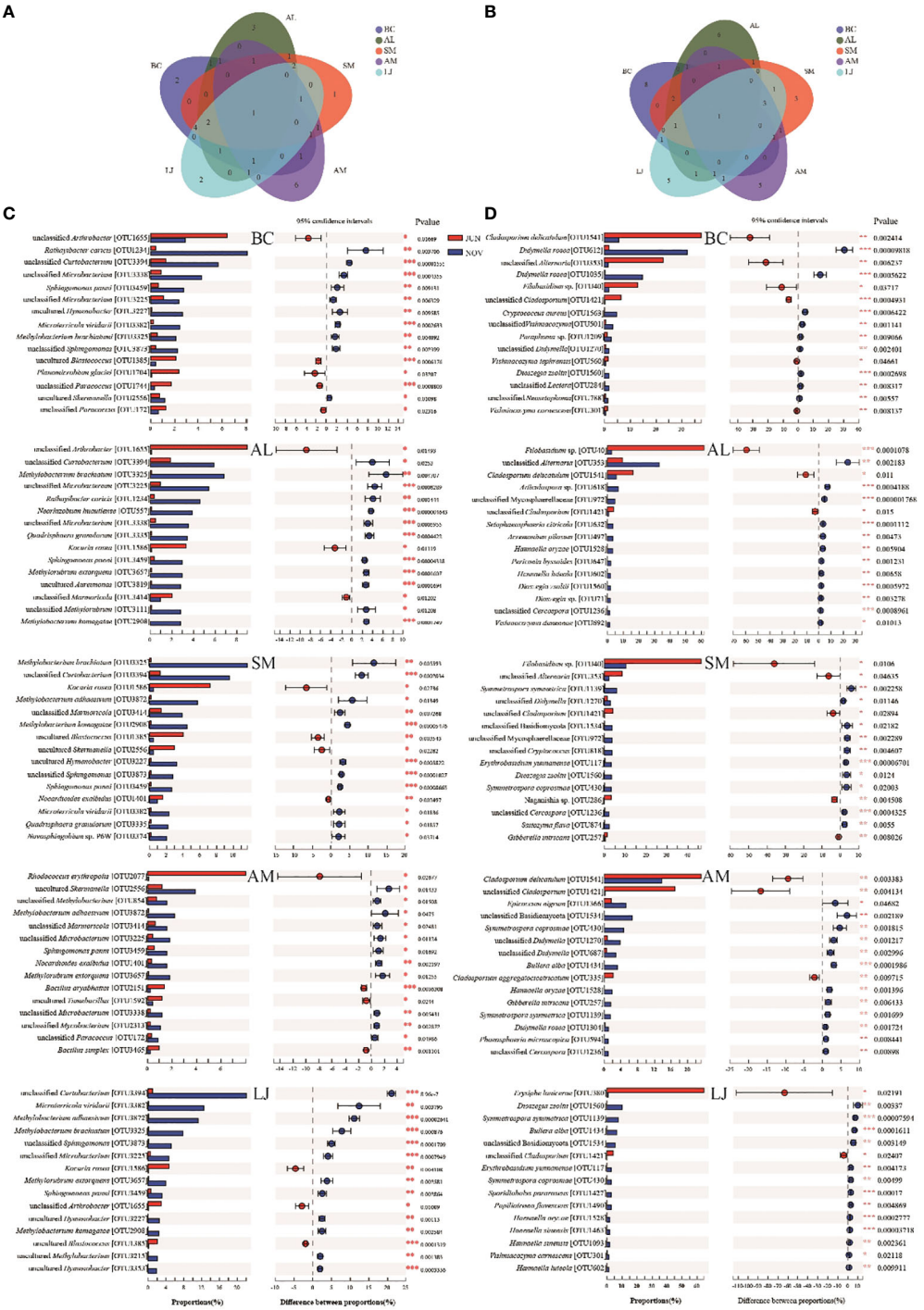

FIGURE 6
The distribution of the 15 most abundant epiphytic bacterial **(A, C)** and fungal **(B, D)** species in phyllospheric of different plant species in summer and winter. The * symbol indicates the significant difference P < 0.05. The ** symbol indicates the significance level intervenes between P < 0.01. The *** symbol indicates difference is very significant, P < 0.001.

exhibited variations across different seasons (Figures 7J–N, Table 2). Among the studied plant species, *B. chinense* exhibited the lowest number of nodes, edges, and centrality coefficients. On the other hand, *A. lancea* displayed the highest number of nodes, edges, clustering coefficients, and network density. *S. miltiorrhiza* had the highest number of nodes, the fewest edges, and the lowest network density. *A. membranaceus* had the highest number of nodes and the fewest clustering coefficients. Lastly, *L. japonica* exhibited the highest number of centrality coefficients.

Both positive and negative correlations were observed in the epiphytic bacterial and fungal networks in our study (Figure 7). The community who possessed advanced degrees were identified as

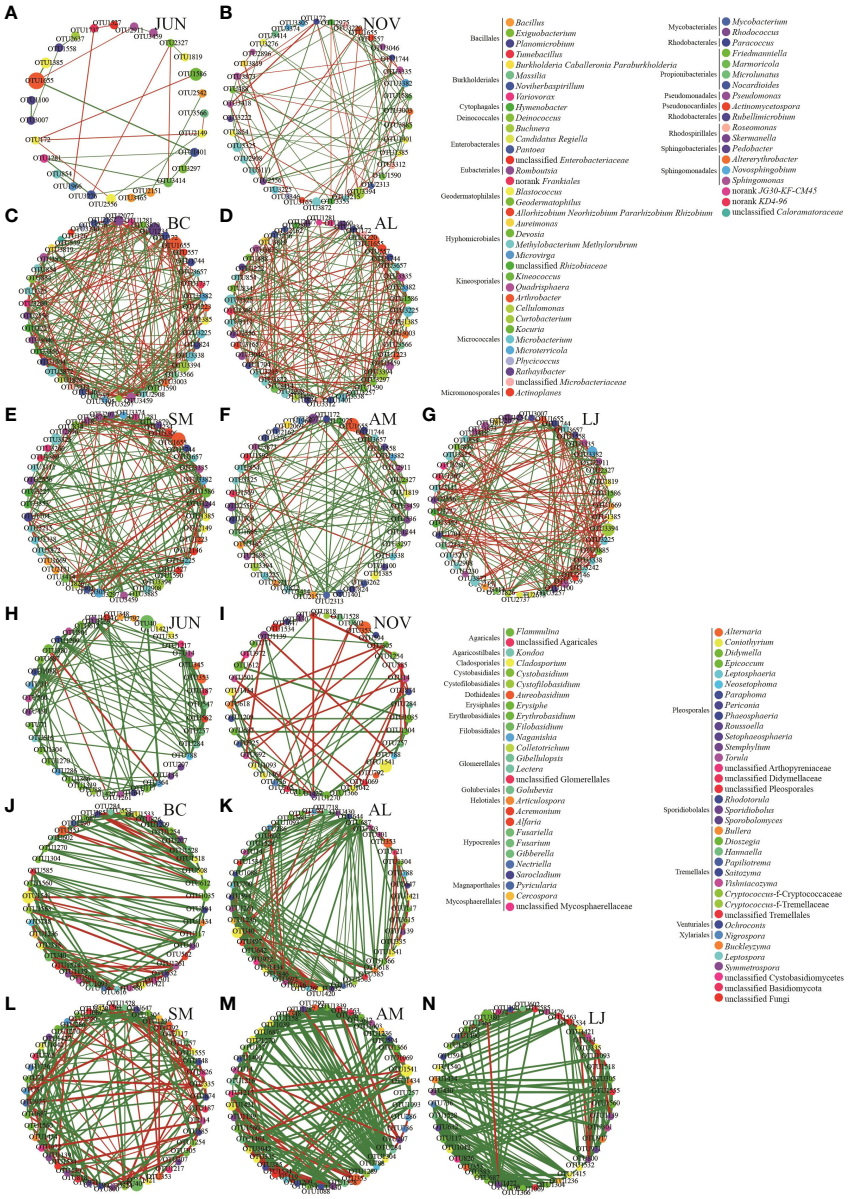

FIGURE 7
Co-occurrence networks of microbial taxa in the bacterial **(A–G)** and fungi **(H–N)** communities.Note: Nodes represent bacterial OTUs, different node colors are used to distinguish different bacterial genus, MCODE Rank is a module with significant relationships calculated based on networks between plants, and the relative abundance of OTU is represented by the node size. Edges represent significant interaction between OTUs, green edges indicate a positive correlation, red edges indicate negative correlation, and the width of each edge reflects the Spearman correlation coefficient between nodes.

keystone taxa within the network (Table 3). In addition, it is evident that negative correlations have significantly increased within the fungal winter community. In our study, we observed predominantly positive associations between taxa in fungal communities across seasons in all five host plants (Figures 7J–N).

The top 10 phyllosphere epiphytic bacterial or fungal OTUs with the highest degrees were recognized as keystone taxa (Tables 3, 4). The composition of keystone taxa in bacterial or fungal communities varied as a result of plant species and seasonal changes. The majority of associations involving keystone fungal taxa exhibited positive interactions, indicating that the fungal communities were predominantly influenced by positive interactions (Table 4). Nevertheless, the interaction patterns within bacterial communities exhibited a certain level of ambiguity, as a considerable number of negative associations were observed (Table 3).

Unclassified *Arthrobacter* (OTU 1655, *Micrococcales*) was identified as a bacterial keystone taxa in both seasons (Figures 7A, B). It accounted for 25.46% and 11.86% of the OTUs of the bacterial network nodes, respectively, among the *Frankiales*, *Micrococcales*, *Rhodobacterales*, and *Sphingomonadales*. These taxa were found among plants in both summer and winter. In contrast, none of the bacterial taxa were found in all five species that formed the inter-seasonal co-occurrence network. However,

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TABLE 2 Structural attributes of networks of phyllospheric epiphytic bacteria(B) and fungi(F) for plant species and seasons.

		Jun	Nov	JBC-NBC	JAL-NAL	JSM-NSM	JAM-NAM	JLJ-NLJ
Number of nodes	B	27	39	45	48	46	42	47
	F	41	42	43	47	47	47	46
Number of edges	B	26	84	225	189	178	120	187
	F	95	59	141	204	141	186	185
+	B	16	52	123	93	119	96	88
	F	92	35	99	162	89	148	168
–	B	10	32	102	96	59	24	99
	F	3	24	42	42	52	38	17
Avg. number of neighbors	B	2.364	4.629	10.419	7.875	8.045	5.950	8.267
	F	6.087	3.067	6.558	8.681	6.222	7.915	8.043
Network diameter	B	6	13	6	8	7	10	7
	F	6	8	10	7	6	5	7
Characteristic path length	B	2.636	4.328	2.316	2.874	2.807	4.050	2.995
	F	2.419	3.124	3.815	2.898	3.083	2.744	2.923
Clustering coefficient	B	0.212	0.531	0.627	0.577	0.630	0.600	0.663
	F	0.538	0.331	0.628	0.656	0.625	0.507	0.646
Network density	B	0.236	0.136	0.248	0.168	0.187	0.153	0.188
	F	0.277	0.219	0.156	0.189	0.141	0.172	0.179
Network heterogeneity	B	0.487	0.453	0.413	0.41	0.409	0.548	0.412
	F	0.539	0.577	0.392	0.538	0.293	0.345	0.377
Network centralization	B	0.200	0.136	0.189	0.136	0.121	0.163	0.136
	F	0.344	0.242	0.111	0.212	0.137	0.138	0.162
Connected components	B	5	2	2	1	2	2	2
	F	4	7	1	1	2	1	1
Analysis time (sec)	B	0.047	0.047	0.047	0.050	0.043	0.0447	0.031
	F	0.063	0.043	0.032	0.053	0.043	0.046	0.037

Micrococcaceae, *Rhizobiales*, and *Bacillariophyceae* were present in four different species. Among these, *Micrococcaceae* had the highest representation in the inter-seasonal network nodes in *B. chinense*, *S. miltiorrhiza*, and *L. japonica*, accounting for 9.52%, 22.63%, and 2.23% of the nodal OTUs, respectively. *Rhizobiales* were found to be present in *A. lancea* and *A. membranaceus*, with *A. membranaceus* accounting for the highest percentage of nodes OTU at 9.72%, followed by *A. lancea* at 7.52%. This finding indicates that the presence of Micrococcus taxa and *Rhizobiales* taxa is significant in establishing a bacterial network that connects medicinal plants across different seasons (Table 3).

Pleosporales, *Tremellales*, and *Cystobasidiomycetes* were identified as significant fungal keystone taxa during both seasons. Among these, *Tremellales* exhibited the highest proportion of OTUs in the summer node, accounting for 1.20%. On the other hand, *Cystobasidiomycetes* displayed the highest proportion of OTUs in the winter node, accounting for 4.86%. In addition, the presence of *Tremellales* was observed in the interseasonal symbiotic network of all five species. The predominant group of OTUs found in *B. chinense* was *Tremellales*, accounting for 4.37% of the total. The predominant fungal taxa in *A. lancea* and *L. japonica* were *Pleosporales*, accounting for 3.12% and 4.05% of the total nodes, respectively. In contrast, *Capnodiales* represented the highest percentage (4.51%) in *S. miltiorrhiza*, while *A. membranaceus* exhibited a prevalence of *Cystobasidiomycetes*, accounting for 2.72% of the total nodes. Thus, various keystone taxa are responsible for connecting inter-seasonal fungal networks among different host plant species (Table 4).

For the analysis of bacterial communities, the MCODE algorithm identified one module in the summer and five modules in the winter that exhibited statistical significance (Supplementary Figures S2A, B). This suggests that the network associations among

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TABLE 3 The ten keystone species in phyllospheric epiphytic bacteria networks for plant species and seasons.

	Order	Genus	OTU	degree	+	-	Abundance (%)
Jun	Propionibacteriales	*Marmoricola*	OTU3414	4	3	1	3.91
	Propionibacteriales	*Nocardioides*	OTU1401	4	4	0	3.09
	Frankiales	*Geodermatophilus*	OTU2327	3	1	2	1.50
	Micrococcales	*Arthrobacter*	OTU1655	3	2	1	25.46
	Rhodobacterales	*Paracoccus*	OTU172	3	0	3	1.56
	Thermomicrobiales	norank JG30-KF-CM45	OTU1281	3	3	0	1.92
	Propionibacteriales	*Nocardioides*	OTU3276	3	2	1	3.15
	Frankiales	*Blastococcus*	OTU2149	3	1	2	1.87
	Sphingomonadales	*Sphingomonas*	OTU2911	2	1	1	1.50
	norank KD4-96	norank KD4-96	OTU1737	2	0	2	1.44
Nov	Azospirillales	*Skermanella*	OTU2556	9	6	3	1.98
	Rhodobacterales	*Paracoccus*	OTU1744	8	3	5	0.70
	Micrococcales	*Arthrobacter*	OTU1655	7	4	3	5.47
	Cytophagales	*Hymenobacter*	OTU2975	7	3	4	1.00
	Rhodobacterales	*Paracoccus*	OTU172	7	4	3	0.74
	Sphingomonadales	*Sphingomonas*	OTU3873	7	2	5	4.39
	Cytophagales	*Hymenobacter*	OTU3353	7	4	3	1.30
	Corynebacteriales	*Mycobacterium*	OTU2313	7	3	4	0.58
	Frankiales	*Blastococcus*	OTU1385	7	6	1	0.85
	Micrococcales	*Microterricola*	OTU3382	6	3	3	6.39
JBC-NBC	Corynebacteriales	*Rhodococcus*	OTU2077	18	2	16	4.70
	Micrococcales	*Curtobacterium*	OTU3394	17	11	6	6.81
	Micrococcales	*Microterricola*	OTU3382	16	10	6	2.71
	Rhodobacterales	*Paracoccus*	OTU172	16	5	11	1.83
	Cytophagales	*Hymenobacter*	OTU3227	16	10	6	2.71
	Cytophagales	*Hymenobacter*	OTU3356	16	10	6	0.73
	Rhizobiales	*Methylorubrum*	OTU3872	16	10	6	6.01
	Rhizobiales	*Methylorubrum*	OTU2908	16	10	6	1.38
	Burkholderiales	*Variovorax*	OTU3260	15	9	6	1.53
	Bacillales	*Planomicrobium*	OTU1704	15	5	10	2.31
JAL-NAL	Rhizobiales	*Methylorubrum*	OTU3111	14	8	6	2.43
	Micrococcales	*Kocuria*	OTU1586	13	4	9	2.91
	Kineosporiales	*Quadrisphaera*	OTU3335	13	8	5	2.95
	Rhodobacterales	*Paracoccus*	OTU1744	13	4	9	1.59
	Rhizobiales	*Methylorubrum*	OTU3325	13	8	5	5.87
	Bacillales	*Planomicrobium*	OTU1704	13	5	8	0.78
	Kineosporiales	*Kineococcus*	OTU1590	13	8	5	1.68
	Rhodobacterales	*Paracoccus*	OTU172	12	9	3	0.77
	Rhizobiales	*Methylorubrum*	OTU3872	12	5	7	1.42

(Continued)

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TABLE 3 Continued

	Order	Genus	OTU	degree	+	-	Abundance (%)
	Acetobacterales	*Roseomonas*	OTU3312	12	7	5	0.80
JSM-NSM	Micrococcales	*Kocuria*	OTU1586	13	4	9	5.59
	Micrococcales	*Microterricola*	OTU3382	13	8	5	1.72
	Micrococcales	*Cellulomonas*	OTU1826	13	4	9	1.31
	Sphingomonadales	*Novosphingobium*	OTU3305	13	8	5	4.90
	Micrococcales	*Microbacterium*	OTU3225	13	8	5	0.86
	Micrococcales	*Arthrobacter*	OTU1655	12	4	8	12.61
	Micrococcales	*Rathayibacter*	OTU1234	12	7	5	0.54
	Sphingomonadales	*Novosphingobium*	OTU3374	12	7	5	1.55
	Sphingomonadales	*Sphingomonas*	OTU3873	12	7	5	2.09
	Bacillales	*Planomicrobium*	OTU1704	12	3	9	4.32
JAM-NAM	Rhizobiales	*Methylorubrum*	OTU3657	12	11	1	2.16
	Propionibacteriales	*Microlunatus*	OTU1665	12	11	1	0.92
	Rhizobiales	*Methylorubrum*	OTU3872	12	11	1	2.67
	Corynebacteriales	*Rhodococcus*	OTU536	12	11	1	1.25
	Sphingomonadales	*Sphingomonas*	OTU2911	10	8	2	1.04
	Rhodobacterales	*Paracoccus*	OTU172	10	10	0	1.37
	Sphingomonadales	*Sphingomonas*	OTU3873	10	10	0	0.89
	Rhizobiales	*Methylorubrum*	OTU854	10	10	0	2.69
	Corynebacteriales	*Mycobacterium*	OTU2313	10	10	0	1.44
	Sphingomonadales	*Sphingomonas*	OTU3459	10	10	0	2.40
JLJ-NLJ	Rhodobacterales	*Paracoccus*	OTU1744	14	8	6	1.31
	Bacillales	*Planomicrobium*	OTU1704	14	7	7	0.55
	Burkholderiales	*Massilia*	OTU3257	14	8	6	0.91
	Rhodobacterales	*Rubellimicrobium*	OTU1100	14	8	6	0.76
	Sphingomonadales	*Sphingomonas*	OTU2911	13	9	4	0.47
	Deinococcales	*Deinococcus*	OTU3885	13	4	9	1.36
	Bacillales	*Bacillus*	OTU1669	13	7	6	0.47
	Kineosporiales	*Quadrisphaera*	OTU3335	11	4	7	0.87
	Micrococcales	*Arthrobacter*	OTU1655	11	6	5	2.23
	Azospirillales	*Skermanella*	OTU2556	11	6	5	0.89

plants were more intricate during the winter compared to the summer. In addition, the MCODE analysis predicted a total of 5 modules for *B. chinense*, 5 for *A.lancea*, 4 for *S.miltiorrhiza*, 3 for *A.membranaceus*, and 6 for *L.japonica* across seasons for each respective host plant (Supplementary Figures S2C–G). The phyllosphere epiphytic fungal network exhibited two statistically significant modules during the summer and five modules during the winter (Supplementary Figures S3A, B). Modules were also identified within the epiphytic fungal network of five different plant species (*B.chinense*, *A.lancea*, *S.miltiorrhiza*, *A.membranaceus*, and *L.japonica*) across multiple seasons. The number of modules observed in each species were as follows: 6 in *B. chinense*, 5 in *A.lancea*, 6 in *S.miltiorrhiza*, 5 in *A.membranaceus*, and 5 in *L.japonica* (Supplementary Figures S3C–G). Our findings indicate the presence of intensive networks within phyllosphere epiphytic microbial communities across different seasons in all the medicinal plants examined. Furthermore, the networks of both epiphytic bacterial and fungal communities among plants were found to be more intricate during the winter compared to the summer.

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TABLE 4 The ten keystone species in phyllospheric epiphytic fungi networks for plant species and seasons.

	Order	Genus	OTU	degree	+	-	Abundance (%)
Jun	Pleosporales	*Paraphoma*	OTU1209	13	13	0	0.39
	Cystobasidiomycetes	*Symmetrospora*	OTU1139	11	11	0	0.11
	Pleosporales	*Neosetophoma*	OTU788	10	10	0	0.20
	Pleosporales	unclassified	OTU1217	9	9	0	0.29
	Hypocreales	*Gibberella*	OTU257	9	9	0	0.60
	Hypocreales	*Alfaria*	OTU562	9	9	0	0.07
	unclassified Basidiomycota	unclassified Basidiomycota	OTU1534	8	8	0	0.15
	Pleosporales	*Paraphoma*	OTU1088	8	8	0	0.15
	Hypocreales	*Gibberella*	OTU547	8	8	0	0.13
	Tremellales	*Vishniacozyma*	OTU14	7	7	0	1.20
Nov	Cystobasidiomycetes	*Symmetrospora*	OTU1139	6	3	3	4.86
	Pleosporales	unclassified	OTU765	6	5	1	0.68
	Sporidiobolales	*Sporidiobolus*	OTU1427	6	3	3	1.20
	Tremellales	unclassified	OTU585	5	1	4	0.53
	Erythrobasidiales	*Erythrobasidium*	OTU305	5	3	2	0.54
	Helotiales	*Articulospora*	OTU618	5	2	3	2.14
	Tremellales	*Saitozyma*	OTU874	4	2	2	0.72
	Tremellales	*Vishniacozyma*	OTU14	4	1	3	1.47
	Tremellales	*Hannaella*	OTU602	4	1	3	1.27
	Tremellales	*Vishniacozyma*	OTU692	4	1	3	0.46
JBC-NBC	Pleosporales	*Leptospora*	OTU616	11	4	7	0.16
	Trichosphaeriales	*Nigrospora*	OTU736	11	10	1	0.16
	Pleosporales	*Torula*	OTU1261	10	5	5	1.15
	Erythrobasidiales	*Erythrobasidium*	OTU117	10	9	1	0.26
	Tremellales	*Cryptococcus*	OTU1563	10	8	2	2.65
	Tremellales	*Hannaella*	OTU1533	10	8	2	0.14
	Tremellales	*Dioszegia*	OTU1560	10	9	1	1.00
	Tremellales	*Hannaella*	OTU1518	10	8	2	0.35
	Tremellales	unclassified	OTU585	10	9	1	0.23
	Agaricales	unclassified	OTU508	9	7	2	0.37
JAL-NAL	Venturiales	*Ochroconis*	OTU644	18	17	1	0.15
	Pleosporales	*Leptospora*	OTU700	18	17	1	0.26
	Pleosporales	*Didymella*	OTU687	15	14	1	0.72
	Pleosporales	*Paraphoma*	OTU1088	15	14	1	0.17
	Pleosporales	*Setophaeosphaeria*	OTU632	15	13	2	1.84
	Capnodiales	*Cercospora*	OTU1236	15	13	2	0.80
	Pleosporales	*Torula*	OTU1261	15	13	2	0.13
	Hypocreales	*Acremonium*	OTU497	14	12	2	1.81
	Agaricostilbales	*Kondoa*	OTU846	14	12	2	0.50

(Continued)

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TABLE 4 Continued

	Order	Genus	OTU	degree	+	-	Abundance (%)
	Tremellales	*Vishniacozyma*	OTU692	14	12	2	0.73
JSM-NSM	Tremellales	*Saitozyma*	OTU748	12	10	2	0.45
	Capnodiales	*Cladosporium*	OTU335	9	1	8	0.41
	Tremellales	*Hannaella*	OTU1528	9	7	2	0.45
	Cystofilobasidiales	*Cystofilobasidium*	OTU824	9	5	4	0.44
	Capnodiales	unclassified	OTU972	8	6	2	2.49
	unclassified Basidiomycota	unclassified	OTU1534	8	4	4	2.58
	Sporidiobolales	*Rhodotorula*	OTU190	8	3	5	0.48
	Tremellales	*Vishniacozyma*	OTU14	8	1	7	0.52
	Dothideales	*Aureobasidium*	OTU792	7	5	2	0.42
	Capnodiales	*Cercospora*	OTU1236	7	5	2	1.61
JAM-NAM	Pleosporales	*Didymella*	OTU1304	14	14	0	0.92
	Tremellales	*Hannaella*	OTU1463	13	10	3	0.25
	Pleosporales	unclassified	OTU1217	13	10	3	0.57
	Pleosporales	*Coniothyrium*	OTU1042	12	11	1	0.63
	Cystobasidiomycetes	*Symmetrospora*	OTU430	12	9	3	3.27
	Agaricales	*Flammulina*	OTU767	12	11	1	0.31
	Pleosporales	*Leptosphaeria*	OTU1119	12	11	1	0.30
	Hypocreales	*Fusarium*	OTU1158	11	11	0	0.64
	Dothideales	*Aureobasidium*	OTU348	11	11	0	0.33
	Pleosporales	*Neosetophoma*	OTU788	10	10	0	0.30
JLJ-NLJ	Sporidiobolales	*Sporidiobolus*	OTU1427	15	14	1	2.04
	Pleosporales	unclassified	OTU1069	14	14	0	0.43
	Tremellales	*Hannaella*	OTU1528	14	14	0	1.43
	Pleosporales	*Setophaeosphaeria*	OTU632	14	14	0	0.20
	Cystobasidiomycetes	*Symmetrospora*	OTU800	13	11	2	0.15
	unclassified Basidiomycota	unclassified Basidiomycota	OTU1534	13	11	2	3.83
	Pleosporales	*Didymella*	OTU687	11	11	0	0.85
	Pleosporales	*Didymella*	OTU1270	11	11	0	0.97
	Pleosporales	*Epicoccum*	OTU1366	11	11	0	1.60
	Erythrobasidiales	*Erythrobasidium*	OTU117	11	11	0	2.08

3.6 Functional prediction

The BugBase microbiome analysis tool was employed to forecast phenotypes for the bacterial communities residing on the phyllosphere as epiphytes. The findings demonstrated consistent trends in the phenotypic composition of phyllosphere epiphytic bacterial communities among plants in both seasons (Figure 8A). The phenotypic composition of bacterial communities on each host species exhibited notable variations across different seasons. Aerobic, mobile element-containing, Gram-positive, and pathogenic phenotypes were found to be prevalent among phyllosphere bacteria. The Kruskal-Wallis tests showed that mobile element containing and aerobic were phenotypes with significant differences between plants in both summer and winter (Supplementary Figures S4A, B). Furthermore, there were significant variations observed in the anaerobic, biofilm forming, facultatively anaerobic, Gram positive, and pathogenic phenotypes among the five host plants during the winter. Meanwhile, the phenotypes of epiphytic bacterial communities exhibited notable seasonal variations in our study (Supplementary Figure S4C). Epiphytic bacteria with mobile element containing

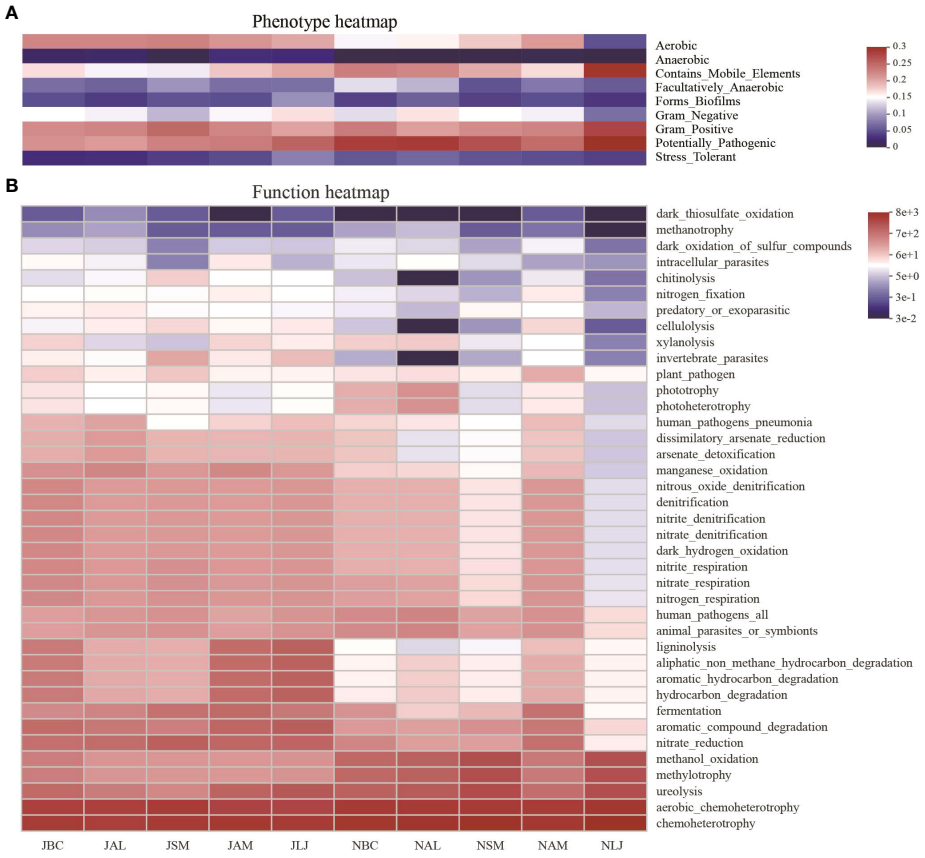

FIGURE 8
Phenotypic **(A)** and functional prediction **(B)** of phyllospheric epiphytic bacterial communities. J, June-Summer; N, November-Winter; BC, *Bupleurum chinense*; AL, *Atractylodes lancea*; SM, *Salvia miltiorrhiza*; AM, *Astragalus membranaceus*; LJ, *Lonicera japonica*.

phenotype were significantly different in abundance between seasons for all five plants, and the abundance of bacteria possessing this phenotype was significantly higher in summer in four plants except in *A. membranaceus*.

A comprehensive analysis was conducted using the FAPROTAX database to predict a total of 39 bacterial ecological functions within the phyllosphere epiphytic bacterial communities. The ecological functions of bacterial communities across different plant species exhibited seasonal variations. Specifically, during the winter season, the bacterial communities of five plant species demonstrated a higher level of function heterogeneity compared to those observed during the summer (Figure 8B). The ecological functions of bacterial communities were found to be primarily dominated by chemoheterotrophy, accounting for 23.96% to 38.09% of the overall abundance of the bacterial community. Additionally, aerobic chemoheterotrophy (RA=17.11%-28.86%). In accordance with the results obtained from the heat map analysis, it was observed that the functions of aerobic chemoheterotrophy and chemoheterotrophy were the predominant features in the composition of epiphytic bacterial communities. The results of the Kruskal-Wallis tests revealed that eight out of the nine dominant functions displayed statistically significant variations in abundance among plants during the winter season. However, no significant differences were observed in the abundance of these functions during the summer (Supplementary Figures S5A, B).

Seasonal comparisons on abundances of bacteria possessing the top 9 dominant functions indicated that certain types of ecological function showed accordant patterns in seasonal changes in all five host species, and some with statistical significance (Supplementary Figure S5C). For example, there were more bacteria with nitrate reduction in summer than winter for all five host species, and the differences were significant in four plants except AM. The study revealed a higher abundance of chemoheterotrophic bacteria during the winter season in five plant species, specifically *B. chinense*, *A.lancea*, and *L.japonica*. These differences in abundance were statistically significant. The prevalence of methanol oxidation bacteria exhibited a consistent increase during the winter season, with significantly higher levels observed in *A.lancea*, *S.miltiorrhiza*, and *L.japonica*.

Based on their mode of nutrition, the epiphytic fungi were categorized into three primary trophic groups as per the FUNGuild prediction: pathotrophs (including phagotrophic fungi phagotrophs), symbiotrophs, and saprophytes. The findings indicated that during the summer, the epiphytic fungal community in LM was predominantly comprised of pathotrophs (RA=64.63%) and pathotroph-saprotroph-symbiotrophs (RA=20.42%). In contrast, the fungal communities in the other four plants during the summer were primarily saprotrophs (RA=14.79%-63.08%) and pathotroph-saprotroph-symbiotrophs (RA=31.83%-75.06%). The pathotroph-saprotroph-symbiotrophs (RA=27.23%-62.70%) was as the dominant functional group of

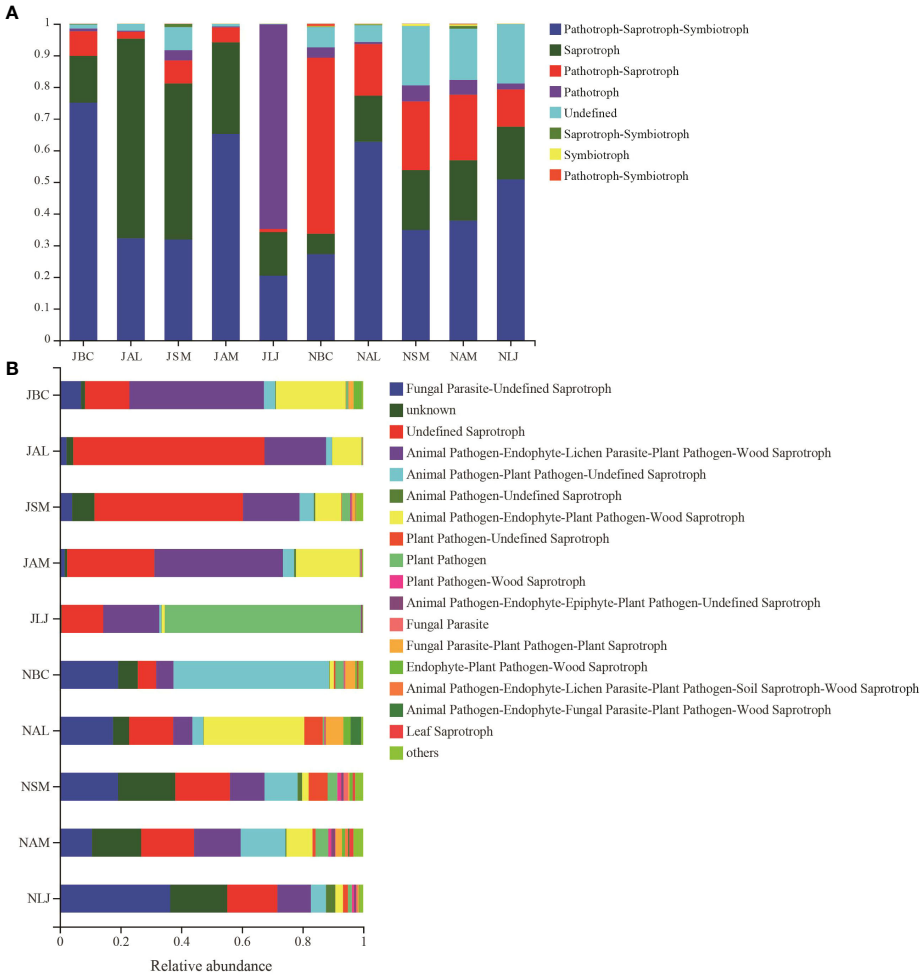

FIGURE 9
Functional prediction of phyllosphere epiphytic fungal community base on FUNGuild database. **(A)** Basic functional classification of different epigenetic fungi; **(B)** multiple detailed functional classification of different epigenetic fungi J, June- Summer; N, November-Winter; BC, *Bupleurum chinense*; AL, *Atractylodes lancea*; SM, *Salvia miltiorrhiza*; AM, *Astragalus membranaceus*; LJ, *Lonicera japonica*.

epiphytic fungi in winter. Notably, there was a significant decrease in the abundance of fungal saprotrophs (RA=6.43%-19.06%) and a corresponding increase in pathotroph-saprotrophs (RA= 11.80%-55.62%) across all host species during the winter, as compared to the summer communities (Figure 9A).

The epiphytic fungi were further classified into twelve distinct functional guilds. The findings of the study revealed that the dominant types of epiphytic fungi on *L. japonica* during the summer were primarily plant pathogens (RA = 64.62%). Additionally, a significant proportion of the fungi belonged to the categories of Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Wood Saprotroph (RA=18.44%) and Undefined Saprotroph (RA=13.74%). On the other hand, the epiphytic fungi found on the other four plants were predominantly categorized as Undefined Saprotroph (RA=14.64%-63.05%), Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Wood Saprotroph (RA=18.63%-44.35%), and Animal Pathogen-Endophyte-Plant Pathogen-Wood Saprotroph (RA=8.58%-22.95%). The diversity of ecological functions performed by epiphytic fungi on five plant species was found to be higher during the winter season. Specifically, there was an increase in the presence of Animal Pathogen-Plant Pathogen-Undefined Saprotroph fungi (RA=3.58%-51.27%) and Fungal Parasite-Undefined Saprotroph fungi (RA =10.64%-36.42%) compared to the summer. Conversely, there was a decrease in the abundance of Undefined Saprotroph fungi (RA=6.03%-18.02%) and Animal Pathogen-Endophyte-Lichen Parasite-Plant Pathogen-Wood Saprotroph fungi (RA=5.73%-15.36%).Animal Pathogen-Endophyte-Plant Pathogen-Wood Saprotroph fungi exhibited a decline from summer to winter in all four species (RA=1.40%-8.61%), with the exception of *A. lancea* (RA=33.18%) (Figure 9B).

4 Discussion

4.1 Taxonomic composition of community

In this study, the dominant taxa in the epiphytic bacterial communities of medicinal plants were Actinobacteriota and Proteobacteria. This finding is in line with previous research that

investigated phyllosphere epiphytic bacteria using both culture-dependent and -independent approaches.The Actinobacteriota and Proteobacteria were found to be the most prevalent in all of the examined locations. However, their abundance was observed to be higher in 2014 as compared to 2016. In the year 2014, the Actinobacteriota constituted 93.4% and 86.8% of the epiphytic bacterial communities found on plants in rural and urban areas, respectively (Espenshade et al., 2019). Leff et al. (2015) proposed that the elevated rates of reproduction observed in numerous members of the Proteobacteria phylum are the primary factor contributing to the substantial proportion of isolates from this group. Meanwhile, the Firmicutes have been identified as a dominant bacterial group during the summer. This finding is consistent with a previous study by Wei et al. (2022), which reported Firmicutes as the dominant group of phyllosphere epiphytic bacteria. Unlike fungi, bacteria are unable to penetrate the cuticle of plant tissues through mycelium. However, Firmicutes, which are nitrogen-fixing bacteria, are capable of supplementing nitrogen acquisition and adapting to epiphytic niches (Zehr et al., 2003). Furthermore, Bacteroidota, which are also the prevailing phylum found in marine macrophytes, constitute a relatively significant portion of the bacterial community that inhabits winter epiphytic environments (Chen et al., 2022). Bacteroidota bacteria play a crucial role in the degradation of biopolymers, facilitating the growth of colonizing bacteria by creating an aerobic environment within the surface biofilm (Dang et al., 2011). At the order level, *Micrococcales* is a main constituent group of epiphytic bacteria, which has not been reported for phyllosphere epiphytic microorganisms to our knowledge. *Micrococcales* played an important role in networks of epiphytic bacterial communities across plants and across seasons. The ability to degrade biological macromolecules (e.g. cellulose and lignin) may account for the dominance of *Micrococcales* (Liu et al., 2020). The genus *Curtobacterium* has been identified as a significant pathogenic bacterium in economically important crops (Evseev et al., 2022). However, we have identified an unclassified member of the genus *Curtobacterium* (OTU 3394) present on all five medicinal plants during both seasons. The investigation of the interaction between taxa and host plants, as well as the potential ecological consequences of their presence, requires further examination.

The epiphytic fungal community was dominated by *Dothideomycetes* (Ascomycota) and *Tremellomycetes* (Basidiomycota), which is consistent with findings from previous studies conducted on both tropical and temperate plants (Coleman-Derr et al., 2016; Yao et al., 2019; Bao et al., 2022). The *Dothideomycetes* is recognized as the largest and most diverse in terms of ecological and functional characteristics (Haridas et al., 2020; Hongsanan et al., 2020). This group encompasses various species that are known to be pathogens of both humans and plants, as well as endophytes and epiphytes. In addition, *Dothideomyetes* has been widely documented as one of the important taxa related to leaves (Qian et al., 2018; Xiong et al., 2021). *Pleosporales* represents the most extensive order within the class *Dothideomycetes* (Yu et al., 2022). Its constituents exhibit a wide range of ecological roles, including epiphytic, endophytic, and parasitic associations with various host plants (Mapook et al., 2016). *Pleosporales* emerges as a prominent fungal order within the epiphytic fungal community in our study. It encompasses keystone taxa that exhibit the highest abundance within the epiphytic fungal networks of the five medicinal plants under investigation. *Tremellales* exhibited a preference for cold seasons in our study. Taxa belonging to *Tremellales* were identified as keystone taxa in all five medicinal plant species within the epiphytic fungal networks, regardless of the season. The concept of keystone species has been proposed to have a significant impact on the stabilization of microbial communities (Vetrovsky et al., 2020). Our findings indicate that the presence of *Pleosporales* and *Tremellales* taxa significantly contribute to the preservation of network structure and stability within epiphytic fungal communities on medicinal plants in agroecosystems.

4.2 Epiphytic bacterial and fungal community construction in phyllosphere

Plants exert a filtering influence on the microbial communities they are associated with (Bringel and Couee, 2015), and the species of the host plant play a significant role in shaping the compositions of these communities (Kim et al., 2012; Yao et al., 2019). Alpha diversity indices of epiphytic bacteria and fungi in this study showed significant differences among all five plant species. Šigutová et al. (2023) demonstrated that the diversity of bacteria was significantly impacted by the species of the host plant, whereas the composition of the fungal community was more strongly influenced by the host species. Bodenhausen et al. (2014) The study found that the host alleles with the greatest influence on the microbiota were lacs2 and pec1, compared to wild-type Arabidopsis. These mutations affect the formation of the cuticle, leading to a significant increase in bacterial abundance, suggesting that different bacteria can benefit from the modified cuticle to varying degrees. In addition, ein2, which is involved in ethylene signaling, was found to be a major host factor regulating the composition of the epigenetic microbial community. In a study conducted by Kang et al. (2022), the diversity indices and community structure of epiphytic fungi in the phyllosphere of bamboo were examined during the spring and autumn seasons. The results revealed significant differences in both the diversity indices and community structure among different bamboo species as well as between the two seasons. Li M. et al. (2022) found that phyllosphere fungal communities of subtropical trees varied with host species identity and seasonality, and that host species identity had a greater effect on phyllosphere fungal community assembly compared to seasonality. The findings from the NMDS and PERMANOVA analyses revealed significant variations in the communities of phyllosphere epiphytic microorganisms of medicinal plants across different plant species and seasons. Additionally, the ANOSIM analysis demonstrated that the phyllosphere epiphytic microorganisms differed between species, plants, and seasons. The dissimilarities in leaf hairiness among plant species were found to be responsible for the variations in phyllosphere epiphytic bacterial communities. Moreover, the level of hairiness indirectly influenced the contact area and habitat of microorganisms with plants, thereby impacting the diversity and structure of phyllosphere microbial communities (Bai et al., 2022).

Previous research has also associated seasonal variations with alterations in the composition of the epiphytic microbial community (Jackson and Denney, 2011; Warshan et al., 2016). We observed that the alpha-diversity indices of bacterial and fungal communities exhibited a greater degree of variation between seasons as opposed to variations between plants. Seasonal variations exhibited significant impacts on the richness of epiphytic bacteria and fungi, aligning with findings from previous research (Vokou et al., 2019; Wei et al., 2022). In the current study, it was observed that there was a higher richness of bacterial OTUs during the summer compared to the winter. Conversely, a greater diversity of fungal OTUs was found during the winter as opposed to the summer. Šigutová et al. (Šigutová et al., 2023) conducted a study to investigate the impact of season on the composition of epiphytic bacterial communities. The results revealed that there were significant variations in the bacterial communities between different months, with the most pronounced differences observed between April and the other months. Zhang et al. (2022) employed NMDS plots utilizing Bray-Curtis distances and conducted PERMANOVA analysis to characterize the bacterial community structure across all samples of Medicago sativa. The study revealed notable differences between samples in each season, indicating significant distinctions. Zheng (2011) discovered that the diversity of the microbial community in the phyllosphere of Pinus exhibited the highest diversity during autumn, followed by summer and spring. In contrast, Thompson et al. (1993) demonstrated that the diversity of the epiphytic fungal community in the phyllosphere of Beta vulgaris was lower in spring compared to autumn. The variation in leaf characteristics and environmental factors between young and old leaves, along with the influence of host plant species, play crucial roles in shaping leaf-associated communities and account for the observed differences (Šigutová et al., 2023). Overall, our study provides confirmation of significant seasonal fluctuations in the composition of phyllosphere epiphytic bacteria and fungi (Jumpponen and Jones, 2010; Gomes et al., 2018; Postiglione et al., 2022).

In this study, it was observed that both host species and season played a significant role in influencing the presence of epiphytic microorganisms. However, it is worth noting that the limitations of the planting site may have affected the season's impact on interleaf epiphytic microorganisms at a smaller habitat scale, resulting in a weaker host selectivity.

4.3 Symbiotic patterns of epiphytic bacterial and fungal communities

Network analysis, utilizing correlation tests, has been increasingly employed in recent years to enhance comprehension of the interactions among community members within microbial communities (Qian et al., 2018; Xiong et al., 2021). In our study, the co-occurring networks formed by the fungal or bacterial communities continued to exhibit seasonal variations. For instance, during the summer, there was a decrease in the number of interactions observed in the bacterial network compared to the winter. Conversely, the opposite trend was observed for fungi, with an increase in the number of interactions during the summer. Liu et al. (2022) elucidated substantial fluctuations in microbial networks across distinct seasons within a comprehensive investigation of lake ecosystems. The researchers discovered that the autumn season displayed the highest level of complexity and resilience in the network. This conclusion was ascribed to the phenomenon of environmental filtering and its associated interspecies interactions, wherein certain taxonomic groups exhibited distinct characteristics specific to different seasons. Interestingly, it was also found that bacteria and fungi exhibited a higher occurrence of interspecies interactions and positive correlations when there was a decrease in overall diversity.

The networks of each plant exhibited a higher number of connections compared to the networks of each season. This suggests that the interactions between epiphytic bacteria and fungi primarily took place among individuals of the same host species, rather than between individuals of different host species. For each plant species examined, there was a predominantly positive correlation between the types of bacterial and fungal network interactions observed in *B.chinense*, *S.miltiorrhiza*, and *A.membranaceus*. This finding suggests that mutualistic symbiosis between microorganisms plays a dominant role in these plants. On the other hand, there were numerous negative correlations observed in the bacterial network interaction types for *A.lancea* and *L. japonica*. In contrast, fungi exhibited a predominance of positive correlations. These findings suggest that in these plants, there exist not only mutually beneficial symbiotic relationships between microorganisms, but also frequent negative interactions, including antagonism, competition, and parasitism.

The stability of a microbial community is contingent upon its modular structure and the presence of keystone taxa (Liu et al., 2022). In the present study, the formation of modules was frequently observed within networks comprising bacterial and fungal communities. We observed that the network structure of winter networks exhibited a higher degree of modularity compared to summer networks. Additionally, we noted a decrease in connectivity among fungal modules during the winter. This observation implies that the selection of connections between plant hosts may exhibit a stronger preference for a specific season. As modular structures have been found to provide protection to communities against secondary extinctions that occur after disturbances, they also enhance the stability of the entire network (Stouffer and Bascompte, 2011). In the context of inter-seasonal networks, the level of modularity observed in individual plants was higher compared to inter-plant networks. This finding suggests that epiphytic communities, which consist of different periods of the same host, are more resilient to secondary species extinctions caused by disturbances compared to communities with different hosts within the same period. We conducted an investigation into the impact of host species and seasonal fluctuations on the stability of epiphytic communities. Our findings revealed that the majority of keystone taxa, characterized by high nodes, were species with low relative abundance. Conversely, community members with high abundance displayed limited or no mutualistic relationships. This observation suggests that the dynamics of microbial communities are primarily influenced by infrequent taxa with low population sizes.

4.4 Ecological functions of epiphytic bacterial and fungal communities

Any characteristic that is associated with the growth, reproduction, or survival of a plant and has the potential to influence its fitness is commonly referred to as a functional trait (Violle et al., 2007). The plant microbiome is proposed to be a functional characteristic of plants, as the diversity of bacterial communities on leaves has been found to be correlated with host growth and mortality rates (Kembel et al., 2014). In the current study, an examination of bacterial populations indicated that the relative abundance of Gram-negative bacteria remained stable and dominant across different seasons. The proportion of Gram-positive bacteria exhibited a peak during the summer months and subsequently experienced a gradual decline throughout the winter. The presence of an outer lipid membrane in Gram-negative bacteria can be attributed to this phenomenon, which presents a challenge for penetration. Consequently, Gram-negative bacteria exhibit an additional layer of protection that is not present in Gram-positive bacteria. Additionally, chemoenergetic heterotrophic bacteria serve a pivotal function as the principal component of epiphytic bacteria. These bacteria employ plant organisms as a source of carbon and energy to synthesize their own organic compounds. Phyllosphere epiphytic fungi predominantly demonstrate saprophytic trophic characteristics, leveraging their saprophytic nature to break down plant epidermal cells and obtain the necessary nutrient resources necessary for their sustenance. Based on the classification of trophic types into 13 functions, the high occurrence of indeterminate saprophytic fungi implies that the relationship between epiphytic fungi and the plant organism is predominantly parasitic.

In the present study, it was discovered that diverse epiphytic microbial communities thrive in even the most challenging phyllosphere environments. Furthermore, the composition and species diversity of both epiphytic bacterial and fungal communities exhibited significant variations across seasons and among different medicinal plant species. The influence of season on the composition of epiphytic microbial communities in the phyllosphere of medicinal plants is more pronounced than its impact on the host species. The presence of epiphytic microbes in the phyllosphere is dependent on a consistent microbiota across different plant species during various seasons. Additionally, the association between different plant species during different seasons is characterized by distinct microbiota. Lemanceau et al. (2017) discovered that various plant species possess distinct microbial communities. Within the context of functional redundancy in plant-associated microbial communities, there is a distinct subset known as the "core microbiome." This core microbiome plays a critical role in maintaining the health of the host plant by carrying essential genes. The dissimilarities observed in the epiphytic microbial communities of medicinal plants were more pronounced during the winter compared to the summer. The significant correlation between the host plant's significance in the phytosphere and the microbial community of the phytosphere underscores the necessity for a thorough examination of the interactions between the host plant and the epiphytic microbial community of the phytosphere. This study also establishes a theoretical foundation for the potential application of foliar fungicides in medicinal contexts.

Data availability statement

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

Author contributions

CH: Conceptualization, Formal Analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. MZ: Formal Analysis, Investigation, Writing – review & editing. XL: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. XH: Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Key R&D Program of China (No. 2022YFC3501503,No.2022YFC3501501).

Acknowledgments

We greatly appreciate the support of the Anguo Medicine Planting Site for their invaluable assistance on this experiment. We thank the students Li Han for sampling and laboratory work.

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/fpls.2023.1328586/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 08 February 2024
DOI 10.3389/fpls.2023.1295674

OPEN ACCESS

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RECEIVED 17 September 2023
ACCEPTED 27 December 2023
PUBLISHED 08 February 2024

CITATION
Coffman L, Mejia HD, Alicea Y, Mustafa R, Ahmad W, Crawford K and Khan AL (2024) Microbiome structure variation and soybean's defense responses during flooding stress and elevated CO_2. *Front. Plant Sci.* 14:1295674. doi: 10.3389/fpls.2023.1295674

Microbiome structure variation and soybean's defense responses during flooding stress and elevated CO_2

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Introduction: With current trends in global climate change, both flooding episodes and higher levels of CO_2 have been key factors to impact plant growth and stress tolerance. Very little is known about how both factors can influence the microbiome diversity and function, especially in tolerant soybean cultivars. This work aims to (i) elucidate the impact of flooding stress and increased levels of CO_2 on the plant defenses and (ii) understand the microbiome diversity during flooding stress and elevated CO_2 (eCO_2).

Methods: We used next-generation sequencing and bioinformatic methods to show the impact of natural flooding and eCO_2 on the microbiome architecture of soybean plants' below- (soil) and above-ground organs (root and shoot). We used high throughput rhizospheric extra-cellular enzymes and molecular analysis of plant defense-related genes to understand microbial diversity in plant responses during eCO_2 and flooding.

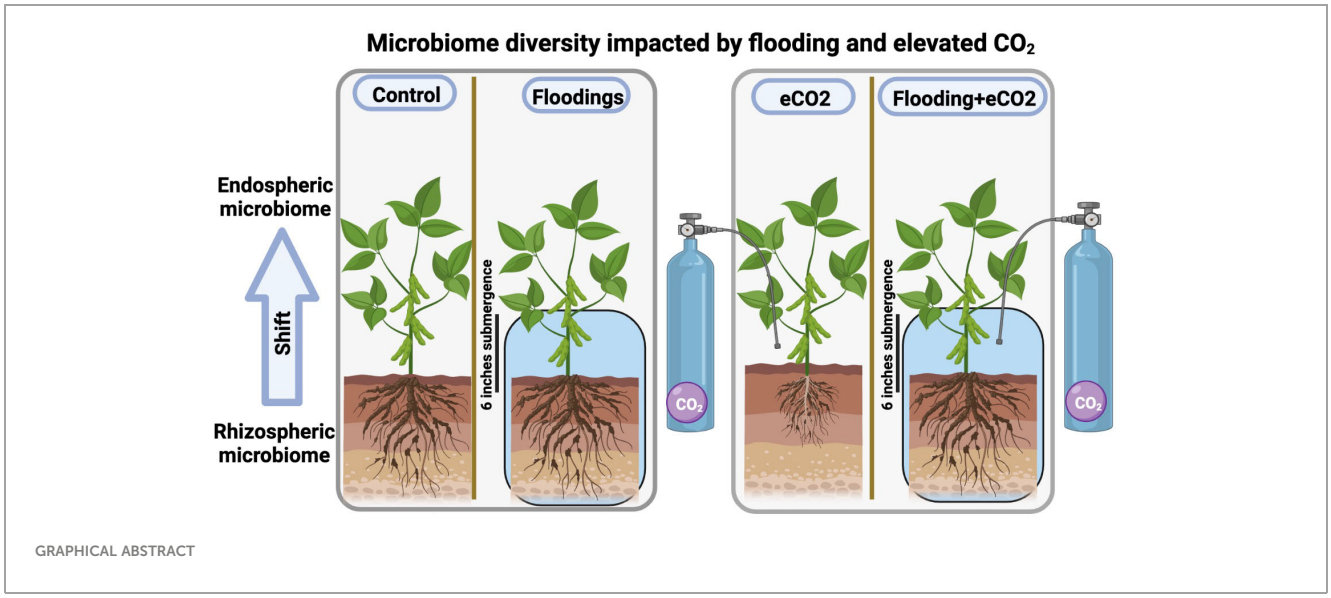
Results: Results revealed that bacterial and fungal diversity was substantially higher in combined flooding and eCO_2 treatments than in non-flooding control. Microbial diversity was soil>root>shoot in response to flooding and eCO_2. We found that sole treatment of eCO_2 and flooding had significant abundances of *Chitinophaga, Clostridium*, and *Bacillus*. Whereas the combination of flooding and eCO2 conditions showed a significant abundance of *Trichoderma* and *Gibberella*. Rhizospheric extra-cellular enzyme activities were significantly higher in eCO_2 than flooding or its combination with eCO_2. Plant defense responses were significantly regulated by the oxidative stress enzyme activities and gene expression of *Elongation factor 1* and *Alcohol dehydrogenase 2* in floodings and eCO_2 treatments in soybean plant root or shoot parts.

10.3389/fpls.2023.1295674

Conclusion: This work suggests that climatic-induced changes in eCO_2 and submergence can reshape microbiome structure and host defenses, essential in plant breeding and developing stress-tolerant crops. This work can help in identifying core-microbiome species that are unique to flooding stress environments and increasing eCO_2.

KEYWORDS

microbiome, diversity, flooding stress, climatic CO2, gene expression, oxidative stress, soybean



GRAPHICAL ABSTRACT

Introduction

Climate change decreases plant productivity and threatens food security (Ahmad and Prasad, 2011). Climate changes are interconnected and multifaceted. Greenhouse gas emissions, specifically CO_2, are increasing, leading to changes in global temperature and rainfall patterns. The IPCC reported that with global warming of 1.5°C, there will be more flooding in coastal and low-lying cities and local areas experiencing increased frequency and intensity of rain. In 2019 alone, flooding along three major rivers caused roughly $20.3B in damage, affecting agriculture and infrastructure [NOAA National Centers for Environmental Information (NCEI), 2018]. The increased amount of water available or excess submergence is hazardous to plant growth and productivity.

Flooding broadly comes in two forms: waterlogging, where water is on the soil surface and only plant roots are surrounded by water. The other form is called submergence, where the whole plant can either be underwater/fully submerged or partially submerged (Jia et al., 2021). Hypoxia is caused in both cases by a lack of oxygen in the plants (Loreti and Perata, 2020). Submergence, studied here, causes excessive hypoxia (Lee et al., 2011). It exacerbates subsidiary stresses such as pathogenesis, herbivory (Hsu and Shih, 2013), and soil nutrient balance (Hurkman, 1992; Degenhardt et al., 2000; Zhu, 2001; Yang et al., 2008; Valliyodan et al., 2016). Hypoxia induces the production of reactive oxygen species (ROS; superoxide O_2^-, singlet oxygen $_1O^2$, hydrogen peroxide H_2O_2) that damage the functional proteins, lipids, carbohydrates, and nucleic acid in plants (Boyarshinov and Asafova, 2011; Boogar et al., 2014). While other factors, such as soil nutrient availability, can influence soil microbiome during flooding, the overwhelming factor is the lack of oxygen (Unger et al., 2009). A study has shown that soil type, soil moisture, and field slope can influence bacterial movement in flooded soils, but this would be specific (Callahan et al., 2017b) and is outside of the scope of this study.

Crop plant flooding events are estimated to decrease yields by 50%–80% (Mittler and Blumwald, 2010; Nanjo et al., 2014; Cooke and Leishman, 2016; Sasidharan et al., 2017). Flooding's impact on the agriculture economy costs more than $5.5 billion in the United

States, whereas climate change impacts are estimated to range up to $1.5 trillion globally. Soybeans are in the top 5 important food crops around the world (Savary et al., 2019), which is mostly due to their essential amino acid composition and complete protein content (Michelfelder, 2009). There have been many studies investigating the physiological and/or biochemical effects of flooding on soybeans (Khan et al., 2021; Komatsu et al., 2021; Staniak et al., 2023; Wang and Komatsu, 2018; Zhou et al., 2021), but few studies have investigated the shifts of its microbial communities (Lian et al., 2023; Yu et al., 2022). For example, it has been shown that flooding stress creates signaling for cell death and proteolysis in the root tips (Yanagawa and Komatsu, 2012; Nanjo et al., 2013), along with diminished root elongation and hypocotyl pigmentation (Hashiguchi et al., 2009). Soybeans and other legumes are potentially more sensitive to flooding due to lack of oxygen, having a negative impact on nitrogen fixation in the root systems (Shimamura et al., 2002; Yamauchi et al., 2013; Souza et al., 2016). However, soybeans generate aerenchyma throughout the plant, termed "secondary" aerenchyma, to cope with flooding stress (Shimamura et al., 2003).

Plant molecular response pattern to stress triggers the gene expression profile, and biosynthetic pathways enable signal transduction to produce biochemical metabolites and enzymes that increase the defense responses of plants (Ahuja et al., 2010; Godoy et al., 2021; Razi and Muneer, 2021). For example, *SnRK1* directly binds to the promoter regions of hypoxia-inducible genes in response to submergence (Park et al., 2020). In plants, the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are crucial players during low oxygen conditions (Jardine and McDowell, 2023; Strommer and Garabagi, 2009). However, more needs to be understood about how these molecular signaling events correspond to microbial symbionts also affected by climatic conditions.

CO_2, on the other hand, is essential to plant photosynthesis; however, it can negatively impact plant growth and physiology (Gojon et al., 2023). The interaction of water and CO_2 is well known. The elevated CO_2 (eCO_2) produces weak carbonic acid, which causes root cell wall acidification (Tan and Zwiazek, 2019). This impacts the root architecture and changes the rhizospheric soil chemistry, where any change in the rhizosphere can also influence microbial community structures. Furthermore, eCO_2 mainly lowers the nitrogen content of plant tissues, possibly through specific inhibition of nitrate uptake and assimilation (Tausz-Posch et al., 2020). The altered nutrient status of plants grown at eCO_2 is one likely cause of the acclimation of photosynthesis to eCO_2 that prevents complete stimulation of biomass production in response to "CO_2 fertilization" (Cotrufo et al., 1998). The high natural genetic variability of the eCO_2 impact on plant nutrient status can be exploited as a promising strategy to breed future crops better adapted to a high-CO_2 world (Tausz et al., 2017). eCO_2 and flooding separately drastically impact the agricultural production system. Water has a lower gas exchange rate than air, reducing gas exchange in the soil while already in a higher CO_2 environment, limiting oxygen availability more. Elevated CO_2 levels have the potential to be either beneficial or detrimental. Thus, eCO_2 and flooding-induced hypoxia can impact the plant's ability to tolerate stress and influence the associated microbial communities, which has not been fully elucidated (Jones et al., 2018).

Microbes, conversely, improve plant growth, productivity, and resistance against pathogenicity and abiotic stresses (D'hondt et al., 2021; Lyu et al., 2021). Recently, the plant-associated microbiome has been coined as a "second genome" highly variable in diversity, abundance, and composition (Pfeiffer et al., 2017). Some recent studies have explained the role of the microbiome in drought and heat stress conditions (Jorquera et al., 2016; Citlali et al., 2018; Delgado-Baquerizo et al., 2018; Mandakovic et al., 2018; Araya et al., 2020; Astorga-Eló et al., 2020; Khan et al., 2020b); however, how microbial communities respond to eCO_2 and hypoxia-induced flooding has not been fully explained. Stressors such as flooding can cause a shift in a plant's root exudates, the main mode of communication for the rhizospheric microbiome (Vives-Peris et al., 2020; Martínez-Arias et al., 2022). It is established that abiotic stress changes root exudates, influencing the microbiome (Vargas et al., 2020; Martínez-Arias et al., 2022). Developing "secondary" aerenchyma can release oxygen to aid beneficial microbes during abiotic stressors such as flooding (Bodelier, 2003). Gaining popularity recently is the phyllosphere which encompasses the aboveground portions of the plant from the leaves, stems, fruits, and flowers (Bashir et al., 2022). The phyllosphere microbiome composition can shift by host, season, pollution, and location (Bao et al., 2020; Qian et al., 2020; Sohrabi et al., 2023). Still, a knowledge gap exists on how phytomicrobiome, populations, and function can improve crop stress tolerance (Khan et al., 2015; Khan et al., 2020b; Trivedi et al., 2020). Increasing our mechanistic understanding and real-world understanding of microbiome–plant interactions under flooding stress offers enormous potential for increasing the resilience of plants in such conditions (Van Der Heijden and Hartmann, 2016; De Vries et al., 2020).

Looking at the current focus on plant–microbe interactions, there is also a significant need to harness stress tolerance mechanisms to improve plant growth in extreme conditions and focus on increasing plant yields (Hussain et al., 2018). Since the two factors—i) increased eCO_2 and ii) floodings—are extremely important to plant life, it is expected that flooding more strongly influences the rhizosphere microbiome while eCO_2 significantly influences the phyllosphere microbiome. Here, we hypothesize that flooding and eCO_2 exposure can influence the microbiome diversity in the rhizosphere and phyllosphere of soybean plants. Both factors can also influence the microbial abilities to produce rhizospheric enzymes and plant stress tolerance by regulating oxidative stress and stress-related gene expressions. However, these adaptive mechanisms at the molecular, biochemical, and metabolite levels vary across different species of plants, their growth conditions, and exposure to stress factors. This work will provide new insights into how increased flooding and elevated carbon dioxide levels caused by global warming will have a novel impact on plant stress response and microbiome structure. While this study only scratches the surface of plants' responses, it provides new questions for future studies. For this purpose, in the current study, we aim to i) elucidate the impact of flooding stress and increased eCO_2 on the plant defenses and ii) understand the changes in microbial communities' structure during flooding stress.

Results

Flooding and eCO_2 exposure impact plant growth and oxidative stress enzymes

The results showed that the treatments impacted plant growth and development compared with control plants. Morphologically, flooding stress caused 27% fewer leaves and 38% higher internode length than the control. Overall, the sole or combined treatments of flooding and/or eCO_2 have significantly ($p< 0.05$) hindered the plant growth (shoot and root length, biomass, number of leaves, and internode distances) compared with non-flooded control plants (Supplementary Table 1). A similar negative impact was also observed for the photosynthetic pigments in the combined flooding and/or eCO_2 treatments. We found that chlorophyll contents (chl-*a* and chl-*b*) were significantly lower ($p< 0.05$) in flooding and flooding + eCO_2 compared with control soybean plants. Combined flooding and eCO_2 interaction was significant ($p< 0.05$). Both control and flooding showed insignificant quantities of carotenoids, whereas the eCO_2 treatment with or without flooding was significantly lower than the rest (Supplementary Figure 1).

The flooding stress causes significant oxidative stress, evidenced by the increased antioxidant enzyme activities. PPO (polyphenol oxidase) activities were significantly higher ($p< 0.01$; 26.2%) in the leaf part during flooding stress compared with other treatments and control soybean plants (Figure 1). The PPO activities were comparatively reduced in eCO_2 and in combination with flooding stress. The peroxidase (POD) activities were non-significantly higher in the leaf during different treatments than in the control. POD activity was also non-significantly regulated in root parts across different treatments compared with the control (Supplementary Figure 2). However, this was still insignificant compared with the control. In the case of superoxide dismutase (SOD), it was significantly increased ($p< 0.05$; 21.4% to 29.1%) in the leaf parts of plants treated with flooding both in ambient CO_2 and eCO_2 applications as compared with the control.

On the contrary, the antioxidant enzyme activity in root parts was exponentially lower in all treatments (Figure 1). In the case of H_2O_2 scavenger, catalase activities were significantly higher ($p< 0.001$; 31%) in flooding stress than eCO_2 with or without flooding stress conditions and control plants. The catalase enzyme activities were significantly lower in the root parts. However, we observed a similar trend of increased catalase activities in flooding stress conditions (Figure 1). Contrarily, the root parts treated with eCO_2 with flooding stress have shown significantly ($p< 0.05$) higher catalase activities than control plants (Figure 1). We also assessed the contents of reduced glutathione in the root and shoot parts of different treatments. We found that reduced glutathione is significantly higher in root than leaf parts during other treatments. The root parts treated with eCO_2 with flooding stress have shown significantly ($p< 0.05$; 18%) higher glutathione content than control plants (Figure 1).

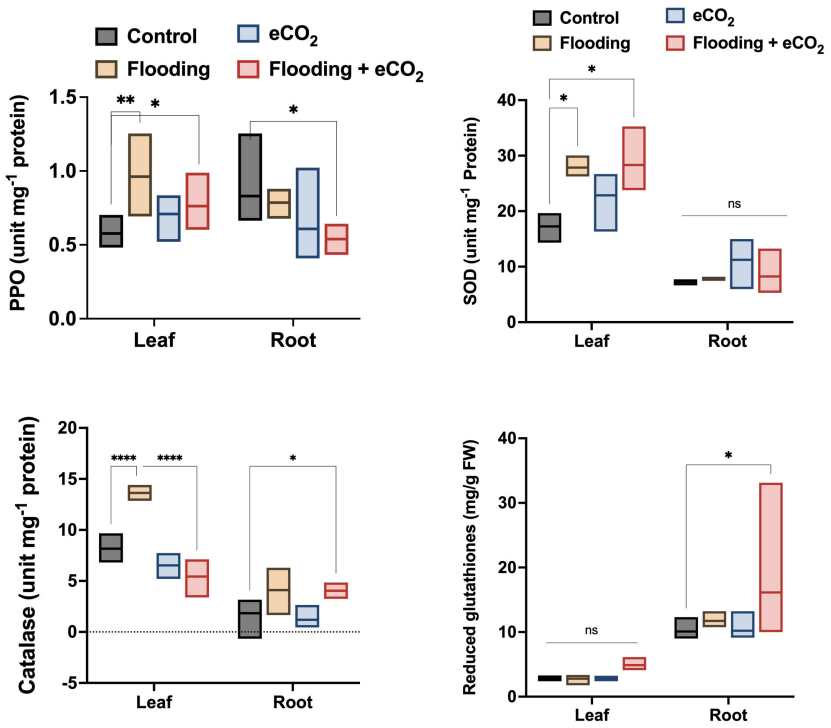


FIGURE 1
Influence of eCO_2 and flooding on the oxidative stress-related enzymes and biochemicals. PPO, SOD, CAT, and Glut were assessed from the leaf and root parts of the soybean plants treated with eCO_2, flooding, and eCO_2 + flooding and compared with non-flooded control plants. The values in the bar are the mean values of three replicates and show standard deviation. The bars showing *, **, and **** are significantly different ($p<0.05$) in their content compared with the control as analyzed by two-way ANOVA.

Flooding and eCO_2 regulate microbiome diversity

Since flooding stress has significantly influenced plant growth and oxidative stress enzyme activities, we hypothesized that it would also impact the diversity and abundance of microbial communities across different treatments. For this purpose, an in-depth amplicon sequencing of 16S rRNA and ITS regions of different treatments (control, flooding, eCO_2, and flooding + eCO_2) was performed, followed by bioinformatics analysis. We obtained 1.93 million reads and 1.41 million reads for soil's bacterial and fungal communities, with post-filtration of sequences assigned to chloroplast, mitochondria, and archaea. Similarly, we obtained 3.9 and 3.7 million reads from the shoot/root parts of the plants (Supplementary Tables 2–5). We observed 1.2 to 1.3 million bacterial amplicon sequence variants (ASVs) and 0.9 to 1.2 million fungal ASVs. ASV methods first infer biological sequences from a sample and distinguish sequence variants that differ by more than one nucleotide then analyze amplification and sequence errors (Callahan et al., 2017a). We observed that bacterial and fungal ASVs were significantly ($p< 0.01$; 28%) higher in combined flooding and eCO_2 treatments. The bacteria and fungi ASVs were 1.3 and 1.24 million for flooding + eCO_2. This was followed by eCO_2 treatment which had a moderate impact on microbial ASVs (Supplementary Table 6).

Flooding stress showed lower ASVs than eCO_2 treatments in fungal communities. In the different organs of the plants, the root/shoot parts of flooding + eCO_2 showed higher ($p< 0.01$; 22%) ASV compared with control and other treatments. This was true for both bacterial and fungal ASVs. This suggests that combining flooding and eCO_2 treatments significantly increases microbial communities' abundances compared with control and sole flooding/eCO_2 treatments (Supplementary Table 6).

Overall, the results showed significantly higher ($p< 0.05$; ~6) Shannon diversity indices in the root parts than in the shoot parts (~0.5) (Supplementary Table 7). Among the treatments for the rhizospheric soil, the results showed significantly higher (35.2%) bacterial diversity in eCO_2 treatments compared with the control. This was followed by flooding and flooding + eCO_2 treatments with 29.8% and 19.1% higher bacterial diversity than control, respectively (Figure 2). Contrarily, the fungal diversity averaged ~3.5% for all treatments, insignificantly higher in flooding and flooding + eCO_2 (Figure 2). In the endospheric microbiome, bacterial diversity was the highest in flooding and flooding + eCO_2 treatments in the root parts (Figure 2). Conversely, the fungal diversity significantly reduced (121.8%) across all treatments compared with the control in roots. In the case of the shoot, a very low bacterial diversity was observed with a Shannon value of 0.70, 0.78, and 0.52 for the control, eCO_2, and flooding + eCO_2, respectively (Figure 2). Interestingly, bacterial diversity was significantly higher in flooding (1.70) compared with other treatments. Overall, flooding and eCO_2 caused a significant ($p< 0.05$; 105.6% and 28.9%, respectively) increase in bacterial diversity compared with control, suggesting that both impact the microbiome structure. In contrast to bacterial diversity, fungal diversity in the shoot was significantly higher ($p< 0.05$; 23.6%) in flooding stress conditions compared with the control (Figure 2). In bacterial microbiomes, the control treatment is distributed unevenly across principal coordinates in rhizospheric soil samples compared with other treatments. Not surprisingly, samples with similar community diversity were observed for eCO_2 and flooding + eCO_2. The root and shoot samples were clustered adjacent throughout the microbial diversity, with replicates of flooding and flooding + eCO_2 (Supplementary Figure 3).

The rhizospheric soil showed that combined factors of flooding+ eCO_2 had significantly enriched ASVs than control vs. eCO_2 or control vs. flooding for bacterial and fungal diversity (Supplementary Table 8). In the case of the root endosphere, a relatively different trend of upregulated ASV enrichment was observed in control vs. flooding than in control vs. flooding + eCO_2 for bacterial communities. The relative fungal abundances were significantly higher in control vs. flooding + eCO_2 than in the other treatments in the root part. A similar trend of increased bacterial ASV enrichment was observed for shoot endosphere in control vs. flooding + eCO_2 than other treatments (Supplementary Table 8).

Microbiome players in flooding and eCO_2

Bacterial biomes distribution in treatments

Proteobacteria, *Actinobacteria*, *Bacteroidota*, and *Firmicutes* were the significantly abundant phyla across all treatments in the rhizospheric soil. *Proteobacteria* were highly abundant ($p > 0.05$; 79%) in control, followed by 51% abundance in flooding. In *Proteobacteria*, the significant abundant families were Caulobacteraceae, Rhizobiaceae, Xanthobacteraceae, Sphingomonadaceae, Burkholderiaceae, Comamonadaceae, Pseudomonadaceae, and Rhodanovacteraceae (Supplementary Table 9; Figure 3). Of these eight families, Caulobacteraceae had 4% abundance in control and flooding and 5.5% and 7% in eCO_2 and flooding + eCO_2, respectively. Sphingomonadaceae was ~14% abundant across all treatments compared with the control (~8%). Pseudomonadaceae, on the other hand, had significantly higher abundances of 52%, 37%, 20%, and 18% in control, flooding + eCO_2, flooding, and eCO_2. Overall, the eCO_2 treatment showed higher abundances of these families. Similarly, in the case of phyla *Bacteroidota*, the relative abundance (22%) was significantly higher in eCO_2 compared with other treatments (11% to 13%). Chitinophagaceae family abundances were substantially lower in control (7%) compared with 10%–11% in flooding and flooding + eCO_2 treatment. Contrarily, Chitinophagaceae was 20% abundant in eCO_2 (Figure 3; Supplementary Figure 4). The relative abundance of *Actinobacteriota* phylum stayed relatively consistent, with a percentage between 2% and 5%. The *Actinobacteriota* comprised 21 families, and their abundances were significantly lower (>1%). The phyla *Firmicutes* was considerably higher in flooding (33%) than in control (5%). *Firmicutes* were composed of two Bacillaceae and Clostridiaceae families. The Bacillaceae was 2% abundant in eCO_2 and control, whereas it was ~4% in flooding + eCO_2 and flooding. However, Clostridiaceae accounts for a large abundance in flooding treatment at 26% abundance. Of the other treatments, Clostridiaceae has the lowest abundance in the control, with only 1.4% abundance, followed by eCO_2, then flooding + eCO_2 with 6% and 8%, respectively (Supplementary Table 9; Figure 3).

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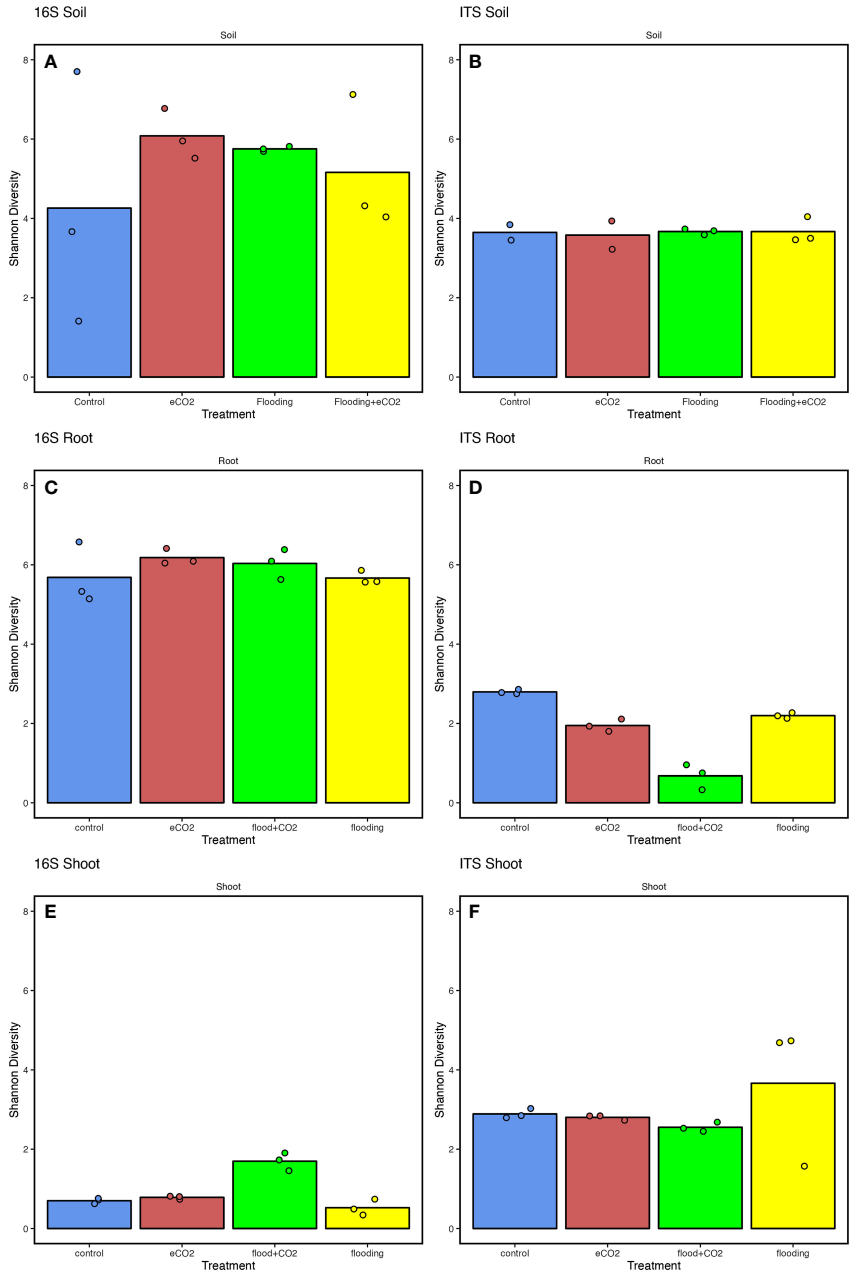

FIGURE 2
The microbiome diversity indices of soybean plants are treated with flooding stress with or without exposure to eCO_2. The results are compared with non-flooded control soybean plants, represented in blue. Treatment with eCO_2, flooding with eCO_2, and flooding are represented with red, green, and yellow, respectively. **(A, B)** The bacterial (16S) and fungal (ITS) Shannon diversity indices of rhizospheric soil across treatments compared with the control. **(C, D)** The bacterial and fungal diversity of root parts of soybean plants treated with flooding stress and eCO_2. **(E, F)** The bacterial and fungal diversity of shoot parts of soybean plants exposed to flooding and eCO_2 compared with control plants. The data analyzed represent three replicates for each treatment (control, floodings, eCO_2, and flooding + eCO_2).

Two significantly abundant phyla (*Firmicutes* and *Proteobacteria*) were in the root and shoot. The *Firmicutes* were highly prevalent in control (71%) and eCO_2 (66.5%). Out of the three *Firmicutes* families, the Bacillaceae was 70% abundant in control, 66% in eCO_2, 42% in flooding + eCO_2, and 31.5% in flooding. This was followed by many unidentified having less than 1% abundance in control and eCO_2 but ~7% in flooding and flooding + eCO_2. In contrast, *Proteobacteria* was the abundant phyla in both flooding + eCO_2 and flooding, 51% and 61%, respectively. The data showed that there are nine families of *Proteobacteria*. The highly abundant families were unidentified and had an 8% abundance in eCO_2 treatment, 4% in control, and less than 1% in flooding + eCO_2 and flooding. The family Parvularculaceae had 3% abundance for eCO_2 and control but less than 1% for flooding and flooding + eCO_2. Sphingomonadaceae was also low in flooding and flooding + eCO_2 at roughly 2% abundance compared with the 4% and 5% of control and eCO_2, respectively. Contrarily, Alcaligenaceae and Pseudomonadaceae were significantly abundant families (42% and 15% flooding and control and 31% and 17% flooding + eCO_2,

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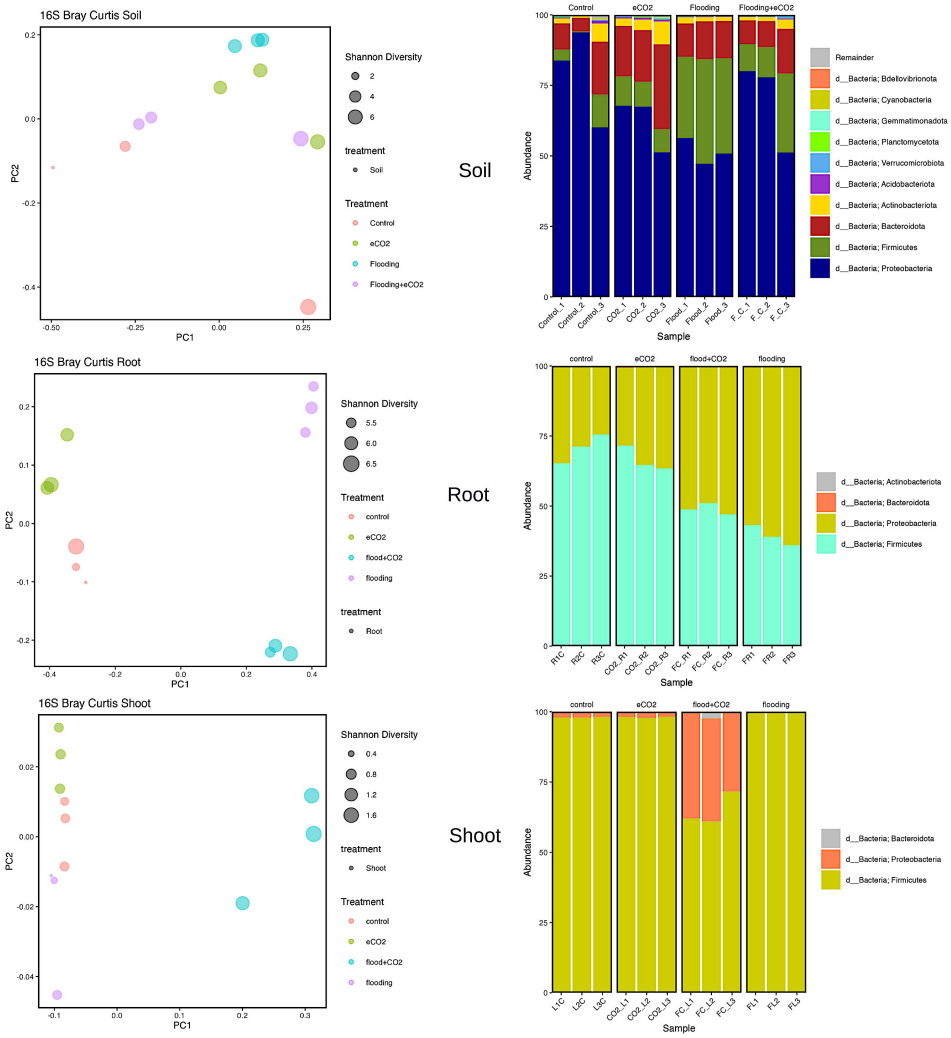

FIGURE 3
Bacterial biome diversity and phyla abundance across different treatments. The Bray–Curtis statistical analysis was used to determine bacterial microbiome variation during flooding, eCO_2, and flooding + eCO_2 and compared with the control. The bacterial biome of the host organ in terms of rhizosphere and endosphere was analyzed.

respectively). Both control and eCO_2 had a 13% abundance of Alcaligenaceae. eCO_2 had only a 1% abundance of Pseudomonadaceae, while the control had approximately 4%. The shoot had a higher (65%) diversity of the phyla *Firmicutes* followed by *Proteobacteria* (34%). The relative abundance of *Firmicutes* increases to 98% flooding stress (Supplementary Table 9).

Fungal biome distribution in stress

Our results showed that two major fungal phyla (*Ascomycota* and *Basidiomycota*) were significantly abundant. Rhizospheric soil analysis showed an increase (92%) in *Ascomycota* during flooding compared with the control (41%). Flooding + eCO_2 showed a rise in *Ascomycota* phyla to 78%. The most abundant *Ascomycota* families are Aspergillaceae, Thermoascaceae, Trichocomaceae, and Didymellaceae (Supplementary Table 10; Figure 4). The Aspergillaceaea family increases by approximately 5% in abundance during flooding with or without increased eCO_2. The opposite is true for Thermoascaceae, which increased to approximately 43% during flooding stress and 38% with flooding + eCO_2 compared with the control and eCO_2 (11% and 15%, respectively). The Trichocomaceae family remained in approximately 1%–3% abundance across all treatments. We found that *Basidiomycota* was less abundant in flooding. *Basidiomycota* is 19% for control and 12% for increased eCO_2; when flooding occurs, the abundance reduces to 3% with eCO_2 and 1% without elevated eCO_2 (Figure 4; Supplementary Figure 5). Rhynchogastremataceae is abundant in control and decreases with stress. The highest relative abundance was found in flooding (1%), then flooding + eCO_2 (2%), with eCO_2 (10%) being the least affected. There are unidentified fungal species with no assignment to phyla for approximately 42% presence in control and eCO_2 treatments and reduced to half during flooding + eCO_2 stress to 20% and a more significant drop in flooding of 6% (Supplementary Table 10).

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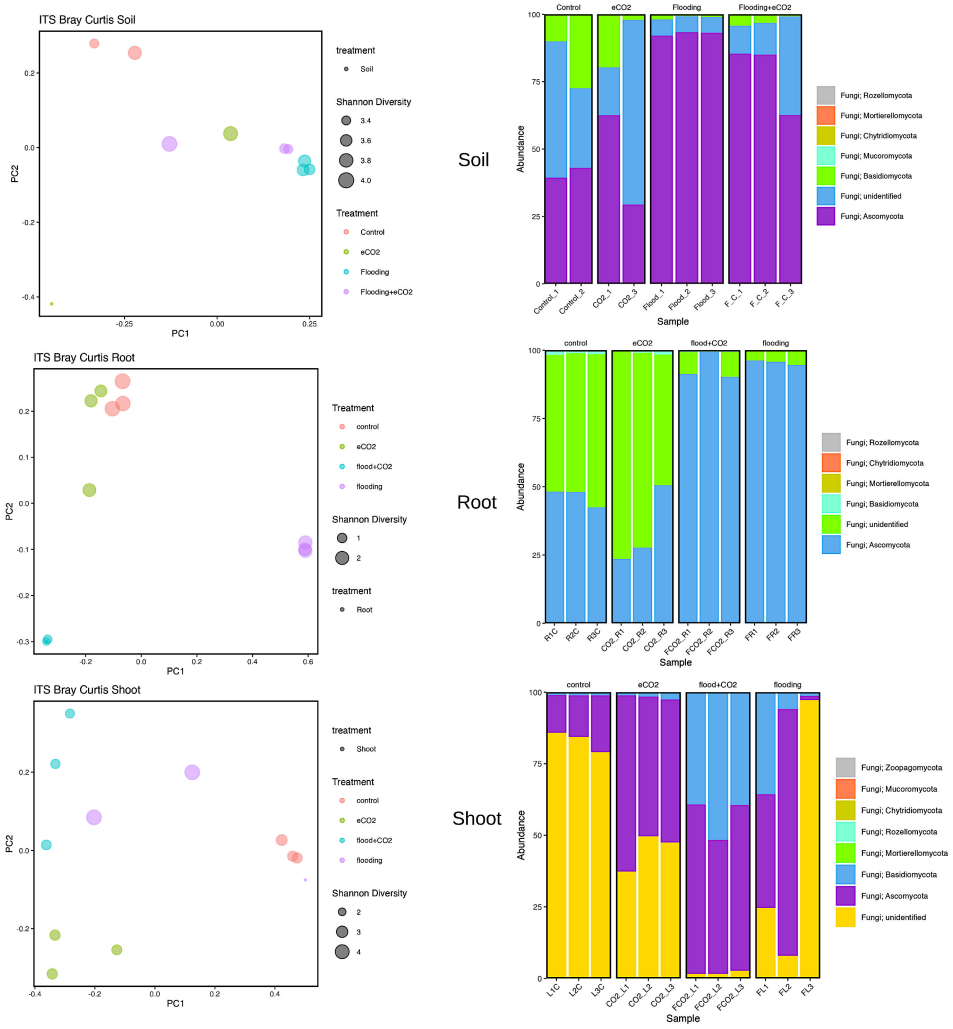

FIGURE 4
Fungal biome diversity and phyla abundance across different treatments. The Bray–Curtis statistical analysis was used to determine bacterial microbiome variation during flooding, eCO_2, and flooding + eCO_2 and compared with the control. The bacterial biome of the host organ in terms of rhizosphere and endosphere was analyzed.

Similarly, the root had 94% and 96% abundance of *Ascomycota* in flooding + eCO_2 and flooding, respectively, followed by unidentified microbes. The most abundant *Ascomycota* families were Didymellaceae, Hypocreaceae, Nectriaceae, and Ophiostomatac. Didymellaceae were present in the control treatment at approximately 5% abundance and less than 1% in the eCO_2 with and without floodings. The flooding treatment showed a 30% abundance of the family Didymellaceae. Hypocreaceae was 90% abundant in flooding + eCO_2. The Nectriaceae family was significantly abundant (59%) during flooding, while in the flooding + eCO_2, it was negligible. Both eCO_2 and control had Nectriaceae at 2% and 4% lower levels, respectively. The Ophiostomatac family is in control at 4% abundance and essentially 0% in all other treatments (Supplementary Table 10; Figure 4).

In the case of shoot, the *Ascomycota* was 16% in control, 43% in flooding, 55% in flooding + eCO_2, and 53% in abundance in eCO_2. The prominent families in *Ascomycota* were Cladosporiaceae, Didymellaceae, Pleosporaceae, Aspergillaceae, Thermoascaceae, Trichocomaceae, Hypocreaceae, and Nectriaceae. The Cladospriaceae was abundant (18%) in flooding + eCO_2; in sole flooding, it was 1.5% compared with control and other treatments. The Didymellaceae shows a decrease in eCO_2 with disregard to flooding stress. There is 4% and 6% abundance during flooding and control. Pleosporaceae has 4% abundance with flooding stress but essentially zero abundance for all other treatments. Aspergillaceae and Thermoascaceae species were abundant in all treatments ranging from 1% to 5%. Trichocomaceae is present in roughly 0% abundance for control and flooding + eCO_2 but has 1% abundance in eCO_2 and 3.5% abundance in flooding stress. The Hypocreaceae family is abundant for all stress treatments, from 2% abundance in control to 11% in flooding, 25% in flooding + eCO_2, and 42% in eCO_2. Nectriaceae is more prevalent in control and flooding treatments at 2% and 4%, while eCO_2 and flooding + eCO_2 were absent. Finally, *Basidiomycota* has low abundance in both control and eCO_2 with a max of 2% abundance, followed by 14% in flooding conditions and 43% in flooding+ eCO_2 levels. The family Podoscyphaceae has a 10% abundance during flooding stress but

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nearly none in all other treatment conditions. The family Rhynchogastremataceae has less than 1% abundance in control, eCO_2, and flooding stress, but when flooding + eCO_2 are both present, it is noted that it makes up 43% of the total microbial abundance (Supplementary Table 10).

Genera-level abundance across treatments

In the case of bacterial genera, the most abundant was *Chitinophaga*, with approximately 16% relative abundance in rhizospheric soil of eCO_2 and between 5% and 8% abundance for other treatments. During flooding, the two highly abundant genera were *Clostridium sensu stricto 1* (14%) and *Clostridium sensu stricto 13* (10%). The main genus of the family Caulobacterales was *Asticcacaulis*, which is most abundant in eCO_2 (Figure 5). Several genera of the family Sphingomonadaceae were also found. For example, *Novosphingobium* was the most abundant genus present during eCO_2 (6.5% eCO_2 and 7% flooding + eCO_2). However, it was 3.5% in flooding compared with 2% relative abundance in control. The *Sphingobium* was also higher during flooding stress (7%) than that of eCO_2 (3.5% eCO_2 and 4% flooding + eCO_2). The control had the lowest relative abundance of 2%. The other genera were *Burkholderia–Caballeronia–Paraburkholderia* higher in eCO_2 at 5%, but flooding and flooding + eCO_2 had less than 2% relative abundance. Both control and flooding treatments had less than 1% of *Burkholderia–Caballeronia–Paraburkholderia* present. The root bacterial genera *Bacillus* was the most abundant ranging from 31.5% to 70% across all treatments. The control and eCO_2 treatments had less than 1%; for the Alcaligenaceae family, all relative abundance was represented by the genus *Pigmentiphaga*. Of the Pseudomonadaceae family, the genus of representation was *Pseudomonas*. Shoot 16S had two highly abundant genera: *Bacillus* and *Pseudomonas* (Figure 5).

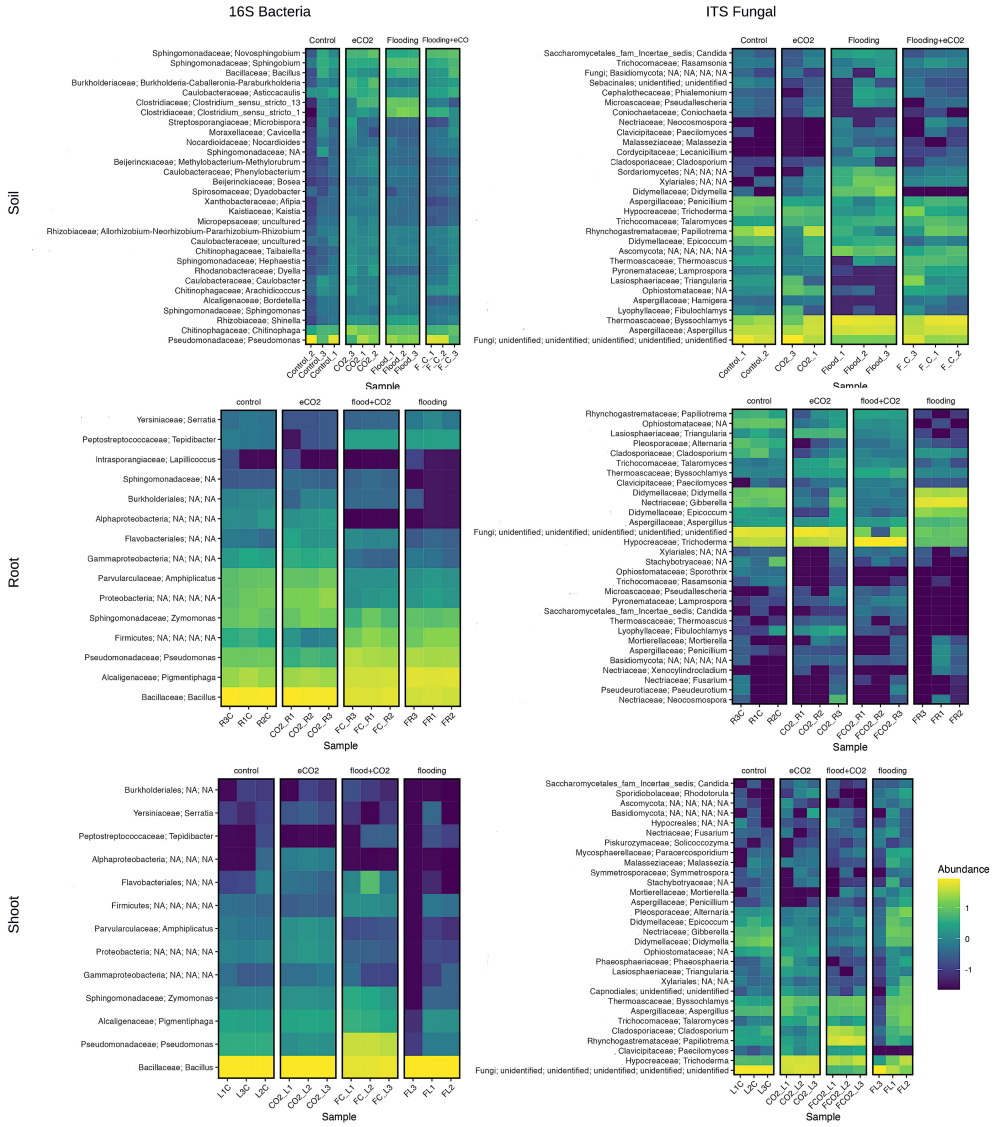

FIGURE 5
Genus-level microbiome diversity and abundance during flooding and eCO_2 treatments. The heatmap shows the top 20 microbiome species in the rhizosphere (soil) and endosphere (root and shoot).

Rhizospheric soil had two genera of the Didymellaceae family, which had high relative abundance: Didymella with 5% relative abundance in flooding and Epicoccum with 2% relative abundance in control. Aspergillaceae had three genera, one having a 0% relative abundance. The other two genera were *Penicillium*, with almost 4% relative abundance in control. *Aspergillus* had 27%–30% relative abundance during flooding regardless of eCO_2 exposure. There were only two genera for Thermoascaceae, with *Byssochlamys* being the most abundant and *Thermoascus* having less abundance. Of the family Lasiophaeriaceae, the genus *Triangularia* was present (1%) in eCO_2 and 3% in abundance in flooding + eCO_2 treatments. The genus *Papilotrema* had high abundance in the control treatment at 18%, falling to 9% in the increased eCO_2 treatment. *Papilotrema* declined in the flooding + eCO_2 treatment to 2.5% and finally to less than 1% in the flooding treatment (Figure 5).

In the endosphere, the roots of two major genera from *Didymella* and *Epicocum* belong to the Didymellaceae family. Importantly, the genus *Gibberella* had 4% abundance in control, lowering to 1% in eCO_2 treatment and 0% in flooding + eCO_2 treatment; however, the relative abundance increased to 59% during flooding treatment. For the shoot part, the genus *Cladosporium* showed high abundance in flooding + eCO_2 treatment at 18% and 1.5% in flooding. The abundance of *Cladosporium* for both eCO_2 and control was less than 1%. *Trichoderma* is a highly abundant species, showing an increase of 42% in eCO_2, 25% in flooding + eCO_2, and 11% in flooding. *Trichoderma* only had a 2% relative abundance in the control treatment. *Papiliotrema* is a genus with a high abundance of 43% in the flooding + eCO_2 treatment, while other treatments had less than 1% relative abundance (Figure 5).

Differential abundance of taxon in treatments

The interactions of different microbiome species and clustering show that flooding and eCO_2 strongly influence microbial species (Figure 6). For example, *Firmicutes* and *Proteobacteria* significantly

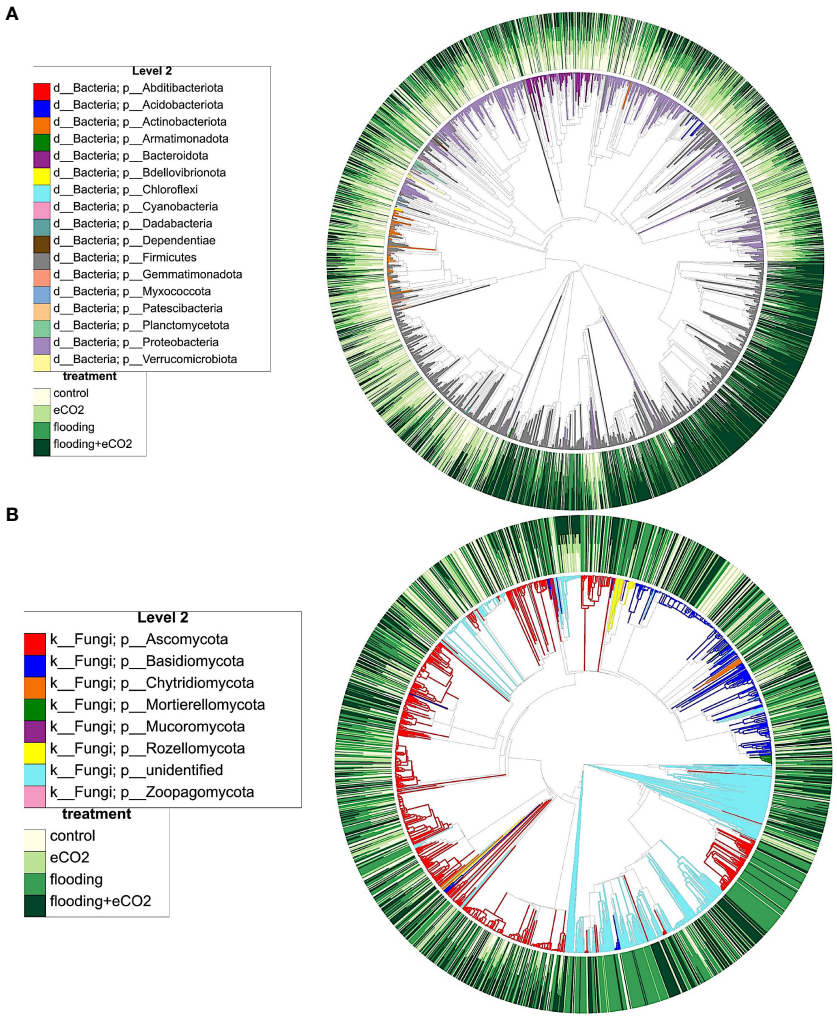

FIGURE 6
Phylogenetic clustering and interaction of different microbiome players from key phyla, their distribution during flooding, and eCO_2 treatments. **(A)** shows the bacterial and **(B)** shows fungal phylogenetic clustering. The color distribution depicts the abundance pattern of OTUs across different treatments and their interactions. The outer circle shows the abundance levels (from light yellow to dark green), and the inner circle shows the dominance of specific microbiome players in different conditions.

cluster in response to both factors. A similar clustering was evident in the Ascomycota and Basidiomycota (Figure 6). To understand the taxa distribution and differential abundance in response to flooding and eCO_2, we carried out ANCOM-BC2 (Lin and Peddada, 2020). The results showed that out of 288 taxa, 21 genera were differentially abundant in bacterial soil samples. Of the 21 genera, 20 taxa were differentially abundant in flooding + eCO_2 stress (Supplementary Table 11; $p< 0.05$). While flooding stress only had one taxon of differential abundance, the family Lachnospiraceae. Increased eCO_2 stress conditions had only two taxa of differential abundance: one from an uncultured genus and the other from Candidimonas.

Bacterial root samples had 15 taxa; only 7 were found differentially abundant. All seven differentially abundant taxa were present in flooding treatment, and all but two were in flooding + eCO_2 treatment. The order Burkholderiales and the family Sphingomonadaceaea were differentially abundant in flooding but not in flooding + eCO_2. The class Alphaproteobacteria was differentially abundant across all treatments except increased eCO_2. Of the 13 taxa analyzed for differential abundance in bacterial shoot samples, only six were differentially abundant. eCO_2 stress only had one taxon of differential abundance, the class Alphaproteobacteria, which is shared with the bacterial root samples. Two differentially abundant taxa were present in the flooding + eCO_2 treatment: Pseudomonadaceae and Amphiplicatus. It is noted that Amphiplicatus was differentially abundant in both root and shoot bacterial ASVs for both flooding and flooding + eCO_2 treatment (Supplementary Table 11).

In the case of fungal ASV, 16 of 95, 16 of 70, and 8 of 147 were found differentially abundant taxa in soil, root, and shoot samples, respectively. All but one taxon was differentially abundant in soil ITS samples for flooding treatment, except the genus *Epicoccum* which was differentially abundant in flooding + eCO_2. Seven differentially abundant taxon for fungal shoot samples were from flooding + eCO_2. Two genera were differentially abundant in flooding conditions, Plectosphaerella and Paecilomyces; the latter was also differentially abundant in flooding + eCO_2. eCO_2 treatment had one differentially abundant genus of *Fibulochlamys* (Supplementary Table 12).

Influence of flooding and eCO_2 on microbial enzymes in the rhizosphere

We performed an analysis of the soil enzymes, viz., β-D-cellubiosidase (BDC), α-glucosidase (AG), β-glucosidase (BG), N-acetyl-β-glucosaminidase (NAG), and phosphatase (Phos), after eCO_2, flooding, and eCO_2 + flooding stress compared with non-flooded control. Our results showed that during flooding stress, the BDC activities were significantly ($p< 0.05$) reduced as compared with the control (Figure 7). In the case of eCO_2 treatments with or without flooding, the BDC activities were non-significant compared with the control. The AG and BG enzymatic activities were non-significant during flooding stress (Supplementary Figure 3). However, among treatments, only the eCO_2 application showed significantly ($p< 0.001$) higher activities of BG than AG compared with the control and other treatments (Figure 7). Overall, BG and AG showed lower enzyme activities during flooding stress. Phos enzyme activities were also significantly ($p< 0.001$) reduced during flooding stress compared with control soybean plants. Contrarily, the Phos activities were significantly ($p< 0.001$) increased by eCO_2 compared with the control. Interestingly, these activities substantially reduced twofold in the combined treatment of flooding + eCO_2 compared with the control (Figure 7).

To understand the molecular effect of CO_2 and flooding stress, we investigated the relative expression of mRNA genes involved in CO_2 and flooding stress and the oxidative defense system of soybean seedlings using qRT-PCR (Figure 8). The genes were chosen based on their relationship to oxidative defense, flooding, or elevated levels of CO_2, with some being specific to *Glycine max*. Superoxide dismutase (*SOD1*), peroxidase (*POD*), catalase (*CAT1*), and ascorbate peroxidase (*APX*) are all oxidative defense genes that help reduce the damage of ROS during stress. Submergence-1b and -1c (*Sub1b* and *Sub1c*), alcohol dehydrogenase (*Adh-2*), and elongation factor 1 (*Elf-2b*) are genes related to flooding stress in plants. At the same time, pyruvate decarboxylase 1 (*PDC1*) catalyzes the first step in anaerobic fermentation.

The results showed that the relative expression of oxidative defense-related genes such as *SOD1* and *APX1* was significant ($p< 0.001$) in both the eCO_2 and flooding stress alone and combined stress. The relative expression of the *SOD1* gene in flooding + eCO_2 was the highest (6.72-fold) compared with flooding alone (4.4-fold), eCO_2 alone (5.7-fold), and control. Similarly, the *POD* gene's relative expression was higher in flooding + CO_2 (5.8-fold) than in others (Figure 8). Interestingly, the *CAT1* gene expression was highly significant (6.5-fold) in flooding stress compared with eCO_2 (4.03-fold), flooding + eCO_2 (2.02-fold), and control. Furthermore, the flooding stress-associated genes were also investigated in which the *sub1b* gene was upregulated (1.3-fold) in combined flooding + eCO_2 stress compared with control. Similarly, the *adh-2* gene showed the highest expression (3.9-fold) in combined flooding + eCO_2 stress as compared with the control, whereas the *elf1b* gene showed the highest expression (3.4-fold) in flooding stress compared with eCO_2 stress (2.2-fold), flooding + eCO_2 (2.0-fold), and control. Interestingly, the *PDC1* gene, which is associated with eCO_2 stress, showed the highest relative expression (6.0-fold) in combined flooding + eCO_2 stress compared with the eCO_2 (1.8-fold), flooding (1.19-fold), and control (Figure 8).

Discussion

This study showed that flooding and eCO_2 significantly impact the soybean plant growth attributes (shoot/root lengths and biomass) and photosynthetic pigments. In addition, these stress factors increase oxidative stress by regulating the antioxidant enzyme activities significantly compared with control or sole flooding and eCO_2 treatments. Elevated CO_2 alone did not show significant variation from the control except when considering the microbial activity. This study revealed that flooding in the presence of eCO_2 influences the abundance of bacterial and fungal microbiome communities compared with control treatments and

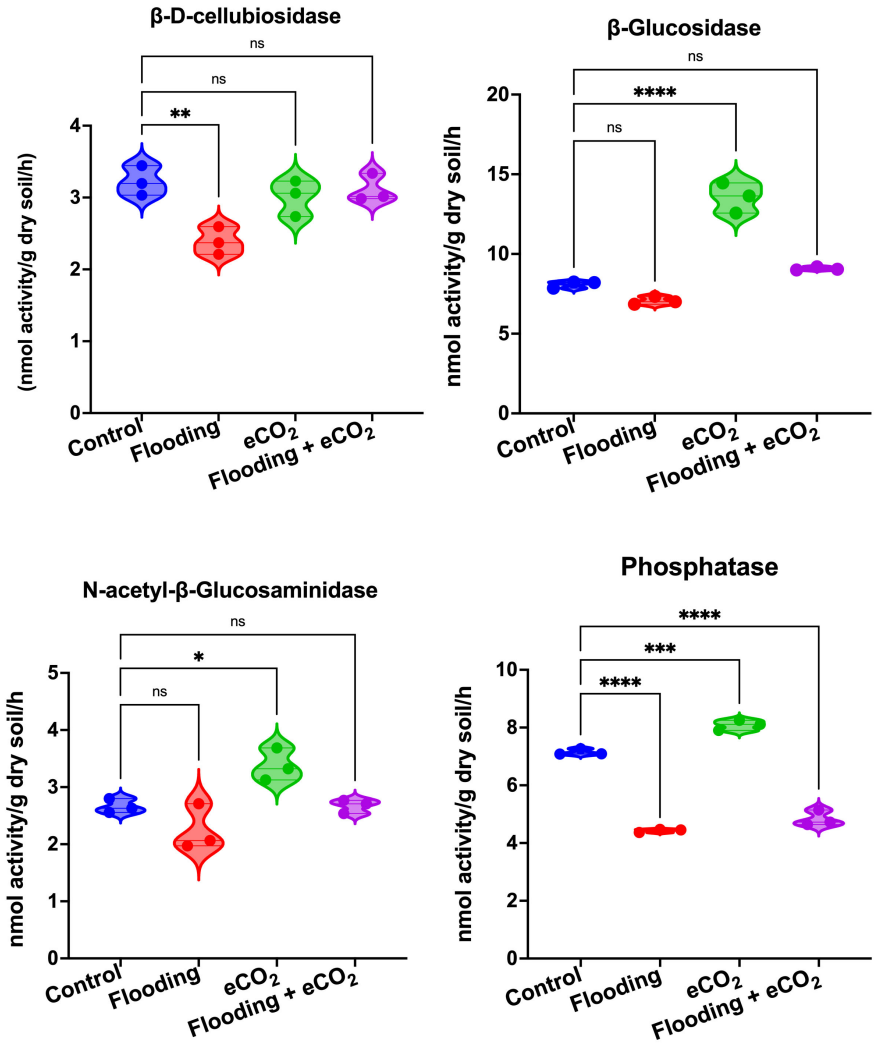

FIGURE 7
Extracellular enzymatic activities in rhizospheric soil of soybean plants treated with flooding and eCO_2. The treatments were compared with the control (non-flooding). The values represent the mean values of three replicates and show standard deviation. The bars showing *, **, *** and **** are significantly different ($p<0.05$) in their content compared with the control as analyzed by two-way ANOVA analysis. "ns" shows that values are insignificant compared with control treatments.

influences oxidative stress reactions. Changes in water level are a significant driver for plant growth and microbiome diversity. Previous studies showed that soybean is extremely sensitive to abiotic stress conditions (Trépanier, 2019; Longley et al., 2020). *Glycine max* is rich in oil and proteins (Hasanuzzaman et al., 2021). The flooding stress negatively affects its growth, development, and yield (Mustafa et al., 2015; Li et al., 2020). The resulting exudation of metabolites in the rhizosphere has also been argued for changes in symbiotic microbes (Sugiyama, 2019). The change in soil chemistry due to lack (drought) and abundance (high moisture) of water exacerbates the abundance of microbial communities (Jiao et al., 2023). Our results showed that flooding and eCO_2 accelerated bacterial and fungal diversity.

Investigating further, we noticed a significant shift of microbial ASVs from the rhizosphere into the phyllosphere. Since both flooding and eCO_2 created an abnormal growth condition, we propose a driving shift in the microbial community. Previous studies showed that plant cell division and gibberellic acid synthesis increase during flooding to escape hypoxia and expose the leaf to submergence (Kim et al., 2016). Microbial ASV abundances in the phyllosphere rather than in the rhizosphere suggest a similar phenomenon with microbial community structure.

We found significant variations across sole and combined treatments while looking at bacterial and fungal phylum distribution and its impact on their diversity due to flooding and eCO_2. The family *Actinobacteria* was negatively impacted by soil moisture, while *Proteobacteria*, specifically *Betaproteobacteria* and *Gammaproteobacteria*, showed positive aggregation from soil moisture. In the case of eCO_2, the microbial communities were not significantly affected compared with combined treatments. Microbiome richness across endophytic root bacterial and fungal communities appears resilient to the two factors. We showed that stress conditions increase bacterial richness in soil samples, but it caused a decrease in the endophytic root fungal community

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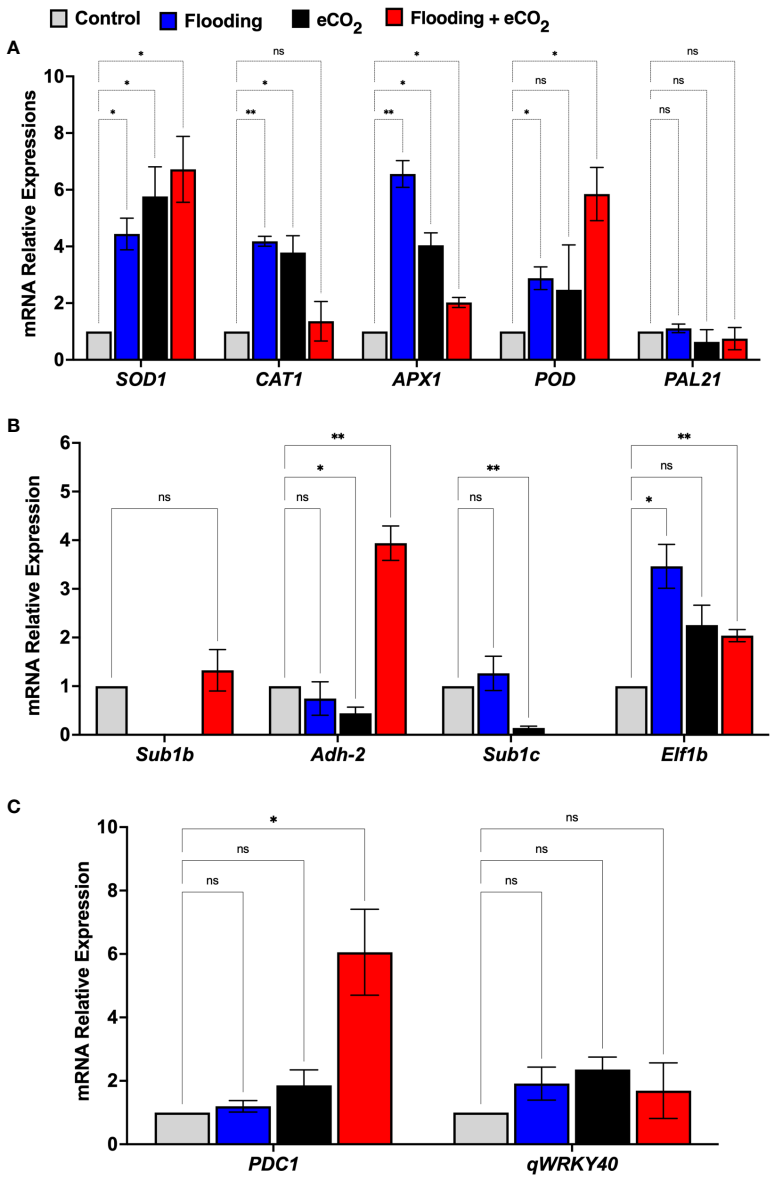

FIGURE 8
mRNA gene expression related to oxidative stress **(A)**, flooding **(B)**, and eCO_2 **(C)** of soybean plants treated with flooding and eCO_2. The treatments were compared with the control (non-flooding). The values represent the mean values of three replicates and show the standard deviation of relative expression to housekeeping genes and control. The bars showing * and ** are significantly different ($p<0.05$) in their content compared with the control as analyzed by two-way ANOVA analysis. "ns" shows that values are insignificant compared with control treatments.

richness. The combined factors of flooding + eCO_2 showed a significantly higher ($p< 0.01$) impact on microbial abundances. Some dominant bacteria phyla in flooding + eCO_2 were *Bacteroidota*, *Firmicutes*, and *Proteobacteria.* Interestingly, we noticed a significant diversity of *Firmicutes* in the soil rhizosphere, but the same was significantly lower ($p< 0.05$) in the root endosphere. *Firmicutes* are known to be anaerobic species, which is likely why they play a large role during flooding stress (Martínez-Arias et al., 2022). Contrarily, *Proteobacteria* were more abundant in flooding + eCO_2, which are known to play a crucial role in abiotic stress environments (Vaishnav et al., 2018).

The phylum *Bacteroidota* remained stable in the soil rhizosphere during flooding + eCO_2 stress but increased relative abundance due to flooding and eCO_2 separately. Only two genera were found in the soil, root, and shoot samples: *Bacillus* and *Pseudomonas*. In addition, we found *Novosphingobium* sp., a rhizosphere-associated bacteria known to promote rice growth through N_2 fixation and production of indole-3-acetic acid and siderophores in the rhizosphere (Krishnan et al, 2017; Vaishnav et al., 2018). Similarly, other dominant genera, such as *Sphingomonas* and *Bacillus*, have been previously shown to secrete gibberellins (Asaf et al., 2018). We hypothesize that a consortium of gibberellin-producing strains and their abundances in flooding + eCO_2 could improve the plant cell division process in escaping the flooding condition. The plant growth-promoting characteristics, production of auxin and siderophores, and the

solubilization of phosphate/silicate by bacteria can be key characteristics of plant stress tolerance in wheat (Moreira et al., 2016). These are also the key drivers of reshaping microbiome structure, as previously shown by Longley et al. (2020), where soil compositions lower microbial Shannon diversity.

Flooding and eCO_2 also impacted fungal communities heavily, shifting the rhizosphere from *Basidiomycota*, *Ascomycota*, and unidentified to a loss of diversity, with *Ascomycota* almost completely dominating the biome. While the shift to the phylum *Ascomycota* occurred in flooding treatments irrelevant to eCO_2 levels, the shift in the community during flooding with and without eCO_2 levels varied significantly. We found three fungal genera upregulated in the rhizosphere during only flooding stress: *Didymella*, *Epicoccum*, and *Gibberella*. Of note, none of the previously mentioned fungal genera were significantly present with CO_2 and flooding, with *Trichoderma* representing 90% of the fungal genera. The relative abundance of *Trichoderma* did not change when elevated CO_2 levels were the only environmental factor compared with the control. *Trichoderma* is a well-known plant mutualist that offers a wide range of benefits to the host plant (Woo et al., 2023). The three genera discovered combined with flooding and eCO_2 conditions are mostly known for their plant disease-causing species. For example, *Didymella* has been shown to cause leaf blight in maize and stem and leaf rot in legumes (Chen et al., 2015; Wille et al., 2019). *Gibberlla* and *Epicoccum* both have pathogenic species and others that can act as biological control agents. This information needs to be clarified as to whether these microbial shifts are solely caused by abiotic stress. It is more likely that an interplay of abiotic and biotic stressors will occur. A study has shown that when a plant experiences fungal infection, the plant can recruit beneficial genera (Gao et al., 2021). A network analysis would need to be performed to understand if what we see is pathogenic.

The phyllosphere sees the opposite shift where flooding induces microbial shifts, with the propagation of the *Basidiomycota* phylum. Combination stress of flooding and eCO_2 increased genera. When eCO_2 levels also occur, the unidentified fungal phylum is suppressed. A similar pattern to the rhizosphere in the phyllosphere can be seen where the fungal genera present have been studied and are seen to play both a pathogenic and beneficial role. *Didymella*, *Papilioterma*, and *Gibberella* are upregulated in the shoot of flooding and eCO_2 stress. The line between pathogenic and beneficial is not easily elucidated due to the plant–microbe, plant–environment, and microbe–microbe feedback loops. The large presence of an *unidentified* fungal phylum represents a knowledge gap in our current fungal database. Research into the line between fungal pathogens and beneficiaries is emergent; as such, it is difficult to draw clear conclusions about the microbiome from this study alone. There was no evident sign of devastating infection upon plant harvest.

The shape of the microbiome correlates with the enzyme flux in the rhizospheric environment with soybean plants. The soil enzymes BDC, AG, BG, NAG, and Phos showed significant reduction after flooding and eCO_2 + flooding stresses compared with control. Studies have shown that high microbial activities in the rhizosphere often correspond to increased activities of enzymes. This also correlates to the lower microbiome diversity in the soil part during stress conditions. However, BG, NAG, and Phos were significantly higher in eCO_2 than in other treatments. These have been recently correlated with high β-diversity in the rhizosphere of wheat plants (Jin et al., 2022). A recent study showed that Phos directly correlates to the relative abundances of Bacteroidetes, Gemmatimonadetes, and *Funneliformis* in bacterial and fungal communities, respectively (Jin et al., 2022). We show that elevated CO_2 does not mitigate the negative impacts that flooding has on rhizospheric microbial activity. The mechanisms of enzymatic activities and the influence of eCO_2 have not been fully explored. Due to rising CO_2 levels, investigating the role of carbon dioxide in these mechanisms is imperative.

At the same time, the soybean root-secreted metabolites play a pivotal role in shaping the microbial community structure in the rhizosphere (Sugiyama, 2019). Isoflavonoids are prominent rhizodeposits in soybean that help defend and enable symbiotic associations with rhizobia (White et al., 2017). Daidzein and genistein are isoflavonoids produced by soybean into the rhizosphere to communicate with rhizobia, establish nodulation, and play a role in defense against pathogens (Ng et al., 2011). In soybean, *Bradyrhizodium* and *Gammaproteobacteria* (*Proteobacteria* phylum) were dominant and associated with crop productivity during abiotic stresses (Chang et al., 2017). Similarly, *Actinobacteria*, *Chloroflexi*, *Proteobacteria*, *Ascomycota*, *Basidiomycota*, and *Mortierellomycota* phyla were significantly dominant in the soybean that was grown in different soil textures (Trépanier, 2019).

Little is known about the potential function of the single microbial family playing a dominant role during flooding stress. However, *Klebsiella variicola* and *Azospirillum* sp. were isolated and improved plant growth during flooding by forming adventitious roots in soybean plants (Kim et al., 2017; Tiwari et al., 2020). Due to flooding, there are more chances that the soil O_2 levels are quickly depleted by aerobic microbes, reaching anoxia even in the uppermost bulk soil layers within hours of a flooding event. This change in O_2 availability can then result in a progressive shift in the microbial community from aerobic organisms to facultative anaerobes and finally to strict anaerobes (Shabala et al., 2014). This shift toward anaerobic bacteria was hypothesized to be one possible explanation behind the increase in the relative abundance of *Aquaspirillum* in flooded poplar rhizosphere and root samples, as the genus contains a few known anaerobic species (Graff and Conrad, 2005). They hypothesized that shifts in the denitrifying bacterial community resulted from the combined effects of O_2 and N stress on the plant, which can reduce root C exudation. Although some evidence supports altered exudation of total organic carbon in plants exposed to flooding, changes in root exudates from flooded non-wetland species and consequent effects on root microbial communities remain relatively unexplored (Tiwari et al., 2020).

Under flooding and elevated levels of CO_2 stress, a higher amount of ROS is produced in various components of plant cells, disrupting normal plant metabolism (Jabeen et al., 2020; Lubna et al., 2022). Typically, ROS are formed when the electrons (one, two, or three) are transferred to molecular oxygen (O_2^-), which results in hydroxyl (OH), hydrogen peroxide (H_2O_2), or superoxide

(O_2^-) radicals (Bhattacharjee, 2010). To survive, plants activate the antioxidant defense system to mitigate oxidative damage (Hasanuzzaman et al., 2021). To alleviate flooding and eCO_2 stress-generated ROS, plants accelerate the production of antioxidant defense systems like SOD, POD, and other non-enzymatic antioxidants (Hasanuzzaman et al., 2021). SOD mediates the detoxification of superoxide radicals and prevents stress-induced cellular damage (Hasanuzzaman et al., 2021). Flooding stress induces interesting changes in gene expression, which coordinate morphological and metabolic adaptations to stress. The *SOD*, *APX*, and *POD* genes showed elevated expression in flooding and eCO_2 stresses alone and combined, as reported previously in *Luffa aegyptiaca* under flooding stress (Chiang et al., 2014) and durum wheat under eCO_2 stress (Medina et al., 2016). This indicates that elevated CO_2 in the presence of flooding might change the biochemical pathways soybeans use to cope with stress. This is also supported by the fact that catalase antioxidant was significantly downregulated in the stem compared with only flooding treatment. Although the change was not statistically significant, there was a slight decrease in *PPO* antioxidants, mostly in both the root and leaf, when elevated CO_2 was in conjunction with flooding treatments. Furthermore, the *Sub1* gene family regulates submergence tolerance in flooding stress. We presume that eCO_2 can be consumed by several classes of microbes and plant roots as a carbon source. This may lead to a reduction in oxidative stress in the root region. Alternatively, the weak carbonic acid and related radicals can react with flooding-induced radicals to develop a cascade of reactants and products to reduce oxidative stress in the rhizosphere. These genes enhance tolerance by minimizing the ethylene-promoted GA responsiveness by enhancing the accumulation of the GA signaling. In the current study, the *Sub1* gene was upregulated compared with control plants, and the expression patterns increased due to flooding stress (Fukao et al., 2019).

Conclusion

It has been noted that increasing our mechanistic understanding and real-world understanding of microbiome–plant interactions under flooding stress offers enormous potential for increasing the resilience of plants in such conditions (Van Der Heijden and Hartmann, 2016; De Vries et al., 2020). This topic is becoming essential as climatic risk events such as flooding and drought influence agricultural productivity. While we have studied flooding and its effect on plants, minimal studies consider the rising levels of CO_2 in our atmosphere. This is relevant because flooding creates hypoxic conditions that elevated CO_2 has the potential to worsen. Hence, we show that flooding and eCO_2 drastically impact plant growth physiology, gene expression profiling, and phenotype. Our findings show that these biochemical changes must be investigated further to understand the effect of flooding and eCO_2 on the holobiont. Elevated CO_2 levels reduce microbial activity in the soil, and the role it plays in the plant's biosynthetic pathways is not clear, opening up opportunities for future research investigating this. These environmental stressors, either alone or in combination, significantly impact the diversity and abundance of bacterial and fungal communities. Current fungal databases are missing an important fungal phylum identification that has the potential to play a critical role in soybean health and disease. Our findings highlight potential knowledge gaps in microbiome–plant relationships.

Materials and methods

Plant material, growth conditions, and treatment

Glycine max L. (Fiskeby III soybean) obtained from the US Department of Agriculture was selected due to its ability to show resistance against abiotic stresses. The soybean seeds were germinated in a soil mixture of peat moss (Miracle-Grow, USA), organic topsoil, and Ferti-lome perlite in 40:30:10 ratios, respectively. The soil mixture was thoroughly mixed and autoclaved to induce the development of the native microbiome in sterile conditions. A recent study has shown that soil disruption via autoclaving increased the colonization on the rhizosphere of potentially beneficial bacterial genera by reducing the number of microbial pathogens present and that these bacteria are shown to be crop-specific potentially (Dilegge et al., 2022). While this method may not present accurate agricultural settings, it will allow us to screen for genera specific to our plant of interest. This will allow us to find soybean-specific potential growth-promoting bacteria or fungi. It is customary to include bulk soil analysis; unfortunately, we could not collect samples and use the control as a reference for changes in this study. The plants were grown till the V3 stage in a growth chamber (Biora, MineARC Sys Inc., USA; relative humidity 60%–70% and light intensity of 800 μE m^{-2} s^{-1} from sunlight Z4NW; day/night cycle of 14 h at 28°C and 10 h at 25°C). The pots were watered with autoclaved DI water (ADW). After stage V3, the plants were arranged in a fully factorial experimental design with two factors: i) flooding and ii) eCO_2 treatments. Thus, it was comprised of i) control, ii) flooding, iii) eCO_2, and iv) flooding + CO_2. The control plants received only DW to maintain a natural soil moisture level of 50%. The flooding stress was induced by exposing plants to submergence for 7 days at 7 inches above the soil surface (partial submergence). An eCO_2 stream was applied every 12 h to maintain an eCO_2 level of 680 ± 80 ppm for 7 days with or without flooding stress. The eCO_2 levels were monitored using a sensor (Vaisala, Helsinki, Finland). Each treatment comprised 21 plants which were all harvested 7 days after the beginning of treatments. After 7 days of treatments, the different plant growth parameters (plant length, biomass, and chlorophyll contents) were taken. The plant and soil samples were harvested with gloves being used, and the plants were removed from pots. The soil surrounding the roots was shaken into an ethanol-cleaned bin and collected for soil samples. The roots were then rinsed in water to remove the remaining soil particles. The plant biomass was weighted and the roots and stems were cut to be ground in liquid nitrogen separately. After grinding the samples with liquid nitrogen, they were kept at −80°C until further analysis.

Plant growth and oxidative stress analysis

Plant growth attributes, including shoot and root length and biomass, were recorded. Chlorophyll content, total nitrogen, and leaf surface humidity were measured using a chlorophyll meter (Minolta, Japan). For a detailed analysis, chlorophyll (*a*, *b*, and total), carotenoids, and flavonoids were analyzed via spectrophotometry (Imran et al., 2021). Oxidative stress enzymes (superoxide anions and H_2O_2) were also analyzed for all the treatments. Leaf and root samples were ground to a fine powder, and a 0.2-g subsample was used for each extraction. Superoxide anions were extracted with 5 ml of buffer [25 ml of 10 mM phosphate buffer pH 7.8 + 15 ml of 0.05% nitroblue tetrazolium chloride (NBT) + 10 ml of 10 mM NaN_3]. The samples were incubated for 30 min at room temperature with shaking and were then incubated in a water bath at 70°C for 15 min. After cooling to room temperature, the samples were centrifuged at 10,000 rpm for 15 min. The supernatant was taken at a volume of 250 µl and added to 96-well plates to be read at 580 nm absorbance (Khan et al., 2020a). To determine the hydrogen peroxide (H_2O_2) level, 10% trichloroacetic acid (TCA) was added to the samples. The samples were vortexed and then centrifuged at 4,000 rpm for 10 min. The supernatant was collected, and 50 µl was added to a 96-well plate. Then, 100 µl of 1 M potassium iodide and 50 µl of 10 mM phosphate buffer were added to all the wells. The absorbance was read at 390 nm. TCA was added to the samples for reduced glutathione 5 ml of 10%. The samples were vortexed and then centrifuged at 4,000 rpm for 10 min. The collected supernatant reacted with Ellman's reagent in the presence of a phosphate buffer (pH 6.8; 100 mM). The plate was read at 420 nm absorbance. Similarly, catalase, polyphenol oxidase, peroxidase, and superoxide dismutase were analyzed using an extraction buffer (30 mM Tris–HCl + 6 mM $MgCl_2$ + 1 mM EDTA + 3.5 PVP). Samples were vortexed and centrifuged (4,000 rpm for 10 min at 4°C). The supernatant was used for catalase reaction (50 µl of supernatant, 150 µl of 10 mM phosphate buffer (pH 6.8) + 50 µl of 0.2 M H_2O_2) that was read on a spectrophotometer (Tecan 10M; at 240 nm, 255 nm, and 280 nm; Aebi, 1984). For polyphenol oxidase, the supernatant (50 µl) was mixed with 50 µl of pyrogallol (50 µM) and 100 µl of phosphate (pH 6.8; 100 mM). The plate was read at 420 nm absorbance. For peroxidase, the 50-µl supernatant was mixed with 50 µl of pyrogallol (50 µM), 25 µl of H_2O_2 (50 µM), and 100 µl of phosphate buffer (100 mM, pH 6.8) and read using a spectrophotometer (Tecan 10M; at 420 nm). All the experiments were performed in triplicates (Khan et al., 2020a).

Extracellular enzyme analysis

A solution of MUB (4-methylumbelliferone, 1 µM) in sodium acetate (pH 5.2) buffer was used as the fluorescent substrate. Testing was performed according to previous protocols from both Marx et al and Jian et al (Marx et al., 2005; Jian et al., 2016). Exozymes used in the study were β-D-cellubiosidase (BDC), α-glucosidase (AG), β-glucosidase (BG), N-acetyl-β-glucosaminidase (NAG), and phosphatase (Phos). Each exozyme used was quantified on the fluorescence spectrophotometer (Shimadzu, Tokyo, Japan). The rhizospheric soil samples from all treatments were incubated in sodium acetate buffer (pH 5.2) for 24 h on shaking (150 rpm). The samples were centrifuged (4°C, 12,000 rpm for 20 min), and resulting supernatants were collected. If turbidity was present a 0.22 mm filtered syringe was used. Five replicates for each substrate were taken per enzyme analysis. The samples were run on the same machine following exozyme quantification. Readings were taken at absorbance 360 nm and 460 nm for excitation and emission respectively at times 0 and 30 minutes. The concentrations were calculated in µmol h^{-1} L^{-1} (Stroud et al., 2022).

Microbiome DNA extraction and analysis

The samples (rhizospheric soil, root, and shoot) were harvested from four treatments—i) control, ii) flooding, iii) eCO_2, and iv) flooding + eCO_2—after the stress conditions. The plant tissues were processed according to Mcpherson et al. (2018). The leaves and roots were ground into a fine powder using a mortar and pestle using liquid nitrogen. The MagMAX™ Plant DNA Kit (Thermo Scientific, Massachusetts, USA) was used to extract DNA from plant leaves and roots. The manufacturer's instructions were used with a few modifications to extract high molecular weight DNA. A modified method (Verma et al., 2017) was used to extract soil DNA. Briefly, soil (0.2 g) samples were suspended in 1.4 ml of extraction buffer [100 mM of Tris/HCl (pH 8.0), 100 mM of EDTA (pH 8.0), 100 mM of sodium phosphate buffer (pH 8.0), 1.5 M of sodium chloride, 1% (w/v) CTAB, 100 mM of calcium chloride, 100 mg of lysozyme/ml]. The soil slurry was incubated at 37°C for 1 h with shaking at 200 rpm. Following incubation, 0.3 ml of SDS (20%) was added and incubated at 65°C for 1 h in a water bath with shaking every 10 min. The samples were centrifuged at 7,000*g* for 20 min at 4°C, and the supernatant was collected. Equal volumes of chloroform:isoamyl alcohol (24:1) were added to the supernatant and then centrifuged at 14,000*g* for 20 min at 4°C. The samples were kept on ice after the addition of chloroform:isoamyl alcohol. The top aqueous phase was collected, and 0.1 volume of 3 M sodium acetate and 0.4 volume of 30% PEG-8000, w/v, were added and then incubated at −20°C for 45 min. The samples were again centrifuged at 14,000*g* for 15 min at 4°C. The supernatant was discarded, and the pellet was dissolved in 70% ethanol. After dissolving in ethanol, the samples were centrifuged at 14,000*g* for 15 min at 4°C with the supernatant discarded. After drying, the pellets were resuspended in 60 µl of nuclease-free water. The quality and purity of all DNA samples were checked using a Thermo Scientific NanoDrop Lite Spectrophotometer (Massachusetts, USA) and an Invitrogen™ Qubit™ 4.0 Fluorometer (California, USA).

Microbiome sequencing

The DNA was processed for amplicon sequencing. PCR-free libraries of each DNA sample were generated by amplifying the internal transcribed spacers (ITS2 and ITS4) and 16S rRNA (V3–

V4) for fungal and bacterial communities, respectively. For 16S rRNA, peptide nucleic acid (PNA) clamps were used to reduce mitochondrial and chloroplast contamination. A paired-end sequencing approach of 300 bp was conducted on an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA) operating with v2 chemistry (User Guide Part # 15,027,617 Rev. L). All quality reads related to the study are available at NCBI under BioProject (PRJNA875044), BioSample (SAMN30594393), and accession number (SRR23345057–SRR23345080).

Bioinformatics analysis

The sequencing reads were analyzed with QIIME2.0 (Bolyen et al., 2019). The read quality was assessed with fast QC. We used the Mothur and DADA2 algorithms for denoising and generating the amplicon sequence variants (ASVs) (Callahan et al., 2016). In the denoising, sequences were filtered by overall quality and trimmed in low-quality regions, and chimeric sequences were removed (Callahan et al., 2017a). The 16S rRNA gene reads were trained on the SILVA database for the taxonomic classification (Quast et al., 2012), while the UNITE database was used to classify the ITS sequences (Nilsson et al., 2019). Sequences classified as mitochondria and chloroplast were removed from the 16S rRNA gene ASV table. For beta-diversity analyses, the Bray–Curtis distance and unweighted UniFrac PCoA matrix were generated for the sequence dataset and exported to RStudio software for statistical analysis. The Shannon diversity index and the observed ASV richness were calculated for alpha-diversity analyses. Permutative multivariate analysis of variance (PERMANOVA, 999 permutations) was used to test for significant effects of the factors (plant compartment, flooding, and eCO_2) and their interaction on bacterial and fungal community composition using the "adonis function." ANCOM-BC2 (Lin and Peddada, 2020) and analysis of similarity (ANOSIM) were also used to test the effects of the factors on the fungal and bacterial communities using RStudio. Differences in species diversity (Shannon index) and richness (observed ASVs) for the same factors were assessed using the Kruskal–Wallis test in QIIME 2.0 (Bolyen et al., 2019). The DESeq2 package was used to implement a negative binomial generalized model to test the effect of eCO_2 and flooding on the ASV abundances.

Molecular gene expression analysis

High molecular weight RNA was extracted from the aerial (shoot/leaf) samples using the MagMAX™ Plant RNA Isolation Kit (Thermo Fisher Scientific, Massachusetts, USA). The extracted RNA was analyzed for quantity and integrity through Qubit 4.0 (Qubit RNA IQ Assay and RNA HS Assay kits; Thermo Fisher Scientific, Massachusetts, USA). The cDNA synthesis was performed using the standard protocol of the kit (High Capacity cDNA Reverse Transcription; Applied Biosystems, California, USA). RNA (10 µl and 100 ng/µl) was added to the master mix, and cDNA was synthesized through polymerase chain reaction (PCR) in a thermocycler under specific conditions (25°C for 10 min, 37°C for 2 h, and 85°C for 5 min). The synthesized cDNA was stored at −80°C until further use. The synthesized cDNA was normalized and used for gene amplification. Power up "SYBR" green Master Mix has been used for the thermocycler (QuantStudio 7 Pro Flex, Applied Biosystems, California, USA) PCR reaction. Primers ordered from Azenta (forward and reverse) were used at 10 pM for all reactions (Supplementary Table 12). The qPCR reaction conditions were 94°C for 10 min, followed by 35 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for min. Gene expression results were analyzed using delta CT calculation methods, and the experiment was repeated three times. Fold changes in gene expression were calculated using the formula described previously (Khan et al., 2021).

Statistical analysis

At least three replicates per treatment were analyzed during this study. The data for the enzyme study are presented as the mean ± standard error (SEM). The significant differences were determined using a two-way analysis of variance (ANOVA). The two factors, flooding and eCO_2, were considered and computed across treatments to know the significance level. The mean values were considered significant at $p< 0.05$ and were calculated by GraphPad Prism Version 9.01 (GraphPad Software, San Diego, CA, USA).

Data availability statement

The original contributions presented in the study are included in BioProject (PRJNA875044), BioSample (SAMN30594393), accession number (SRR23345057–SRR23345080) and in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

Author contributions

LC: Formal analysis, Methodology, Software, Visualization, Writing – original draft. HM: Methodology, Writing – review & editing. YA: Investigation, Methodology, Writing – review & editing. RM: Investigation, Methodology, Writing – review & editing. WA: Software, Validation, Visualization, Writing – review & editing. KC: Resources, Writing – review & editing. AK: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing, Investigation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The corresponding author wishes to thank the financial support of the University of Houston-National University Research Funds (NURF-R0507404) and United State Department of Agriculture (USDA; 2023-77040-41264).

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1295674/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 04 March 2024
DOI 10.3389/fpls.2024.1364807

OPEN ACCESS

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RECEIVED 03 January 2024
ACCEPTED 09 February 2024
PUBLISHED 04 March 2024

CITATION
Sharma P, Pandey R and Chauhan NS (2024) Biofertilizer and biocontrol properties of *Stenotrophomonas maltophilia* BCM emphasize its potential application for sustainable agriculture.
Front. Plant Sci. 15:1364807.
doi: 10.3389/fpls.2024.1364807

Biofertilizer and biocontrol properties of *Stenotrophomonas maltophilia* BCM emphasize its potential application for sustainable agriculture

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Introduction: Microbial biofertilizers or biocontrol agents are potential sustainable approaches to overcome the limitations of conventional agricultural practice. However, the limited catalog of microbial candidates for diversified crops creates hurdles in successfully implementing sustainable agriculture for increasing global/local populations. The present study aimed to explore the wheat rhizosphere microbiota for microbial strains with a biofertilizer and biocontrol potential.

Methods: Using a microbial culturing-based approach, 12 unique microbial isolates were identified and screened for biofertilizer/biocontrol potential using genomics and physiological experimentations.

Results and discussion: Molecular, physiological, and phylogenetic characterization identified *Stenotrophomonas maltophilia* BCM as a potential microbial candidate for sustainable agriculture. *Stenotrophomonas maltophilia* BCM was identified as a coccus-shaped gram-negative microbe having optimal growth at 37°C in a partially alkaline environment (pH 8.0) with a proliferation time of ~67 minutes. The stress response physiology of *Stenotrophomonas maltophilia* BCM indicates its successful survival in dynamic environmental conditions. It significantly increased ($P < 0.05$) the wheat seed germination percentage in the presence of phytopathogens and saline conditions. Genomic characterization decoded the presence of genes involved in plant growth promotion, nutrient assimilation, and antimicrobial activity. Experimental evidence also correlates with genomic insights to explain the potential of *Stenotrophomonas maltophilia* BCM as a potential biofertilizer and biocontrol agent. With these properties, *Stenotrophomonas maltophilia* BCM could sustainably promote wheat production to ensure food security for the increasing population, especially in native wheat-consuming areas.

KEYWORDS

biofertilizer, biocontrol agent, wheat rhizosphere, plant growth promotion, genome characterization, sustainable agriculture, comparative genomics

Introduction

Wheat is a principal source of calories and nutritional sustenance for most of the world's population. The escalating global population poses a formidable challenge to food production systems, necessitating a heightened focus on augmenting wheat production by at least 1.5% per year by 2050 to meet burgeoning dietary demands. We have easily achieved it using fertilizers, hybrid seeds, and pesticides. However, their continuous use has adversely affected soil quality, which limits us from using another chemical-based green revolution. The disruption of soil ecology highlights the urgency for sustainable and integrated interventions to meet requirements without affecting soil ecology. The development of biofertilizers and their integration into agricultural practices pave the way toward achieving growth targets sustainability. Biofertilizer microbial strains are pivotal in enhancing plant growth and productivity through their intricate interactions with the rhizosphere. These strains, often comprising beneficial bacteria or mycorrhizal fungi, contribute to sustainable agriculture by promoting nutrient availability, facilitating nutrient uptake, and inducing plant systemic resistance. Symbiotic microbes like *Rhizobium*, *Sinorhizobium*, *Azoarcus*, *Mesorhizobium*, *Frankia*, *Allorhizobium*, *Bradyrhizobium*, *Burkholderia*, *Azorhizobium*, and *Achromobacter* strains (Nosheen et al., 2021) and free-living microbes like *Azospirillum*, *Azotobacter*, *Azoarcus*, *Gluconacetobacter*, and *Herbaspirillum* (Steenhoudt and Vanderleyden, 2000) have proven their potential to meet plant nitrogen requirements (Shah et al., 2021). The phosphate solubilization potential of *Agrobacterium* sp., *Azotobacter* sp., *Bacillus* sp., *Burkholderia* sp., *Enterobacter* sp., *Erwinia* sp., *Pseudomonas* sp., etc. (Shah et al., 2021) can be employed to meet our plant phosphate requirement. The potassium-solubilizing microbe like *Bacillus edaphicus* (Sheng and He, 2006), *Bacillus megaterium*, *Arthrobacter* sp (Keshavarz Zarjani et al., 2013), and *Paenibacillus glucanolyticus* (Sangeeth et al., 2012) enhances plant production. Various microbes were characterized for their plant growth-promoting potential by plant hormone secretion (Kumar et al., 2023), siderophores generation (Kumar et al., 2023), nutrient assimilation (Fatima and Senthil-Kumar, 2015), and biotic and abiotic stress resistance (Tsukanova et al., 2017). Employment of these microbial biofertilizers could fulfill the plant growth requirements for increased crop production. The wheat yield enhancement also requires employing biocontrol agents to overcome phytopathogen infestation. Wheat phytopathogens *Rhizoctonia solani* and *Fusarium oxysporum* severely affect seed germination and seedling growth (Harris and Moen, 1985; El Chami et al., 2023). It ultimately results in bare patches in crop areas up to 20% (Anees et al., 2010). These pathogens have significantly reduced the production of wheat. Australia's southern and western cropping regions have documented annual losses of $59 million and $166 million, respectively (Murray and Brennan, 2009). Also, this pathogen has a wide host range (Cook et al., 2002), and thus, it is much more difficult to control. Crop rotation strategy implementation may reduce the fungal infestation to some extent. However, even this strategy reduced grain production (Volsi et al., 2022). Various biocontrol agents like *Trichoderma* sp., Mycorrhizal fungi, and *Pseudomonas fluorescens* demonstrated antagonism toward *Rhizoctonia solani* and *Fusarium oxysporum*. The applicability of these control agents in wheat cultivation lies in their ability to enhance plant defense mechanisms, induce systemic resistance, and compete for resources with pathogenic fungi. Biocontrol agents primarily excel in mitigating phytopathogens; however, their role in directly promoting plant growth in wheat may be comparatively less pronounced.

A limited number of strains are known to exert the dual effect of biocontrol and biofertilization. *Bacillus subtilis* and *Bacillus amyloliquefaciens* represent a group with dual functionality, producing antimicrobial compounds such as polyketides, ribosomal peptides, and bacteriocins for biocontrol and contributing to plant growth promotion through the production of siderophores (Caulier et al., 2019). *Pseudomonas fluorescens* is recognized for biocontrol against various pathogens such as soil-borne *Fusarium solani* and *Sclerotinea rolscii* (Ganeshan and Manoj Kumar, 2005) and is known to stimulate plant growth by producing growth-promoting substances such as Indole-3-acetic acid and siderophores (Sah et al., 2021). However, their poor survivability in dynamic soil ecosystems, host specificity, etc. (Shah et al., 2021) requires enriching a catalog of plant growth-promoting strains. Therefore, identifying dual players from the host native niches will bestow the advantage of natural colonization and prepare a stage for its utilization up to its full potential. Hereby, the present study was designed to explore/elucidate the wheat rhizosphere microbial world to identify potential biocontrol agents with plant growth-promoting potential to increase wheat crop yield.

Methods

Isolation of wheat rhizosphere microbes

Rhizospheric soil was collected from wheat plants grown in an experimental field at Maharshi Dayanand University Rohtak (28° 52' 44" NL and 76° 37' 19" EL), Haryana, India. A measurement of 5.0g of soil was suspended in 20 ml ultrapure sterile water to perform physiochemical analysis. Furthermore, soil suspension was serially diluted up to 10^{-8}. A measurement of 0.1ml of each dilution was spread evenly on self-devised minimal media A (pH 7.2) [Urea (200mg), calcium phosphate (250mg), Ferrous sulfate (20mg), Synthetic Sea salt (200mg), Pectin (50mg), Inulin (50mg), Starch (50mg), Sorbitol (50mg), Carboxyl methyl cellulose (50mg), and Ammonium sulfate (50mg) dissolved in 100ml distilled water]. Culture plates were incubated at 16°C, 25°C, and 37°C to isolate diverse microbes. The bacterial growth was observed for 48 hours, and morphologically diverse microbial colonies were sub-cultured at 37°C to achieve their pure cultures.

Screening of rhizosphere isolates for antifungal potential

Isolated bacterial cultures were screened to assess their antifungal activity using a disc diffusion assay (Balouiri et al., 2016). Wheat rhizosphere microbes, *Rhizoctonia solani*, and

Fusarium oxysporum were grown in LB and YEPD broth, respectively, with continuous shaking at 200 rpm for 24 hours at 28°C. A measurement of 0.1 ml of 1.0 OD_{600nm} overnight-grown fungal culture was spread evenly on PDA plates in sterile conditions, discs were placed at the center of the plates, and 50µl of the overnight-grown bacterial culture (A600nm: 1.0) was applied to the disc, followed by incubation at 28°C for 48 hours. The fungal growth inhibition was checked by observing the presence of the growth inhibition zone (Balouiri et al., 2016).

Molecular, physiological, and biochemical characterization of microbial isolate BCM

Gram staining of biocontrol microbe was performed with a gram staining kit (K001-1KT, Himedia). Growth of biocontrol microbe was observed at different pHs (3, 4, 5, 7, 8, 9, 10, 11, and 12) and temperatures (10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, and 60°C) to identify its optimal growth conditions. Its growth pattern was observed in LB broth for 48 hrs at 37°C with constant shaking at 200rpm to check its doubling time (Wang et al., 2015). Substrate utilization preference of the identified microbe was assessed with a Hi-carbo kit (Himedia, KB009A-1KT, KB009B-1KT, and KB009C-1KT). Biochemical properties of the identified strain were performed using assays for amylase (Swain et al., 2006), catalase (Iwase et al., 2013), pectinase (Oumer and Abate, 2018), cellulase (Kasana et al., 2008), esterase (Ramnath et al., 2017), and protease (Vijayaraghavan et al., 2017). The antibiotic susceptibility of the microbial isolate was assessed using a Combi IV kit (Himedia, OD023) and G-VI-plus (Himedia, OD034). The stress response physiology of the identified strain was assessed by performing assays for salt stress tolerance, metal stress tolerance, and oxidative stress (Yadav et al., 2023). DNA was extracted from the microbial isolate using the alkali lysis method (Chauhan et al., 2009). The qualitative and quantitative analysis of the DNA was performed with agarose gel electrophoresis and Qubit HS DNA estimation kits (Invitrogen, USA), respectively. The 16S rRNA gene was amplified and sequenced to decode its taxonomic affiliation using a standardized methodology (Yadav et al., 2023).

Genome characterization and comparative genomics

Stenotrophomonas maltophilia BCM was sequenced using Illumina MiSeq using Nextera XT DNA Library Prep kit. Raw reads were quality checked using FASTQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and fastQ Validator v0.1.1 (https://github.com/statgen/fastQValidator). Contaminated reads were removed to get the corrected reads. The SPAdes v3.15.1 assembler was used for the *de-novo* assembly. Further, BUSCO v5.0.0 assessment tools were used with the latest bacterial orthologue catalog (bacteria_odb10) for analyzing the completeness of a set of predicted genes in bacterial genome assemblies. Assembled contigs were used for functional annotation via PROKKA. SSU rRNA gene was extracted, and the BLASTn was performed to identify the taxonomic affiliation of BCM. Database homologs with more than 97% 16S rRNA gene similarity were chosen for comparative analysis. Genomes were downloaded from the NCBI web server and annotated via PROKKA (Seemann, 2014). J-species software (http://jspecies.ribohost.com/jspeciesws/) assessed the genome level similarity using average nucleotide identity and tetra-correlation values. CRISPR/Casin genome was identified using the CRISPR identifier. Antibiotic resistance genes using CARD identifier were assembled and contigs were used to draw a circular genomic map via the Proksee tool (https://proksee.ca/). The antibiotic resistance, metal/metalloid resistance, and oxidative stress resistance protein features were identified using rapid annotation using a subsystem technology (RAST) server (https://rast.nmpdr.org/rast.cgi?page=Jobs). The genome was checked for pathogenesis with the Island Viewer 4 with the default parameters. Phylogenomic characterization of *Stenotrophomonas maltophilia* BCM and shortlisted strains were plotted using roary_plots.py v0.1.0 (https://github.com/sanger-pathogens/Roary/blob/master/contrib/roary_plots/roary_plots.py). The core multiple sequence alignments were used to infer the phylogenomic tree using FastTree v2.1.10 (Price et al., 2010).

Assessment of antifungal and antibiofilm activity of *Stenotrophomonas maltophilia* BCM

The biocontrol potential of *Stenotrophomonas maltophilia* BCM was assessed against *Rhizoctonia solani* and *Fusarium oxysporum* in terms of their effect on seed germination efficiency, root and shoot length of wheat plantlets (Moraes et al., 2014), and alpha-amylase activity (Singh and Kayastha, 2014). Biofilm inhibition activity of *Stenotrophomonas maltophilia* BCM was checked against *Chromobacterium violaceum*. Anti-biofilm activity was calculated by estimating the amount of violacein production by *Chromobacterium violaceum* in the presence of *Stenotrophomonas maltophilia* BCM (Adeyemo et al., 2022).

Role of *Stenotrophomonas maltophilia* BCM on seed germination under salt stress conditions

Wheat seed germination assay was performed in the presence of *Stenotrophomonas maltophilia* BCM. Seeds were initially soaked in overnight-grown microbial culture corresponding to 10^{11} cells/ml containing different concentrations of NaCl ranging from 0.0 M to 1.0 M for 16 hours at 37°C, while control seeds were soaked directly at different concentrations of NaCl ranging from 0 to 1M for 16 hours at 37°C. Seeds were finally wrapped in germination sheets, inserted in 50 ml culture tubes containing 5 ml Hoagland solution, and incubated for 7 days in the dark at room temperature. Seed germination percentage, alpha-amylase activity, and root and shoot length were measured after the incubation (Singh and Kayastha, 2014).

Bio-fertilizer potential of *Stenotrophomonas maltophilia* BCM

Stenotrophomonas maltophilia BCM were screened for nitrate reductase activity (Kim and Seo, 2018), auxin production (Ehmann, 1977), ammonia production (Bhattacharyya et al., 2020), and siderophore biosynthesis (Himpsl and Mobley, 2019) for the assessment of their bio-fertilization potential.

Results

Isolation and screening of microbes with biocontrol potential

The wheat rhizospheric soil from where the microbe was isolated has been collected and identified to have 7.3 pH, 22.6°C temperature, and 11.5 ± 1.10% moisture content. Twelve morphologically diverse microbes were purified from the wheat rhizosphere, of which three showed antifungal activity against at least one fungal strain. Only the BCM strain was found to show activity against both fungal strains and was used for further study. Antifungal activity assay showed that the microbial isolate BCM led to a growth inhibition zone of 17 ± 0.57 mm and 15 ± 0.57735 mm against *Rhizoctonia solani* and *Fusarium oxysporum*, respectively. These results indicate that microbial isolate BCM harbors the potential to develop an efficient biocontrol agent.

Taxonomic, physiological, and biochemical characterization of microbial isolate BCM

The 16S rRNA gene of the microbial isolate BCM shared 99.48% homology with *Stenotrophomonas maltophilia* LMG 25348 in the NCBI 16S rRNA gene database, indicating it as a species of *Stenotrophomonas maltophilia*. The16S rRNA gene-based phylogenetic analysis also confirms similar observations (Figure 1). Based on taxonomic and phylogenetic observations, microbial isolate BCM was labeled *Stenotrophomonas maltophilia* BCM for downstream analysis. Microscopic investigation indicated *Stenotrophomonas maltophilia* BCM as a gram-negative, rod-shaped, and motile bacterium. *Stenotrophomonas maltophilia* BCM showed optimum growth at pH 7.0 and 35°C (Figure 2). Growth pattern analysis indicates that it attains a log phase of growth after 15 hours and has a doubling time of around 67.8 minutes (Supplementary Figure SF1). *Stenotrophomonas maltophilia* BCM showed growth of 0.493 O.D. at 600nm when grown anaerobically for 24 hrs at 37°C, indicating its facultative anaerobic nature. *Stenotrophomonas maltophilia* BCM was positive for amylase, esterase, lipase, protease, and catalase activity. Substrate utilization assay of *Stenotrophomonas maltophilia* BCM indicates that its substrate utilization profile is similar to other *Stenotrophomonas maltophilia* species (Supplementary Table S1). Antibiotic susceptibility assay suggests it is resistant toward bacitracin, cephalothin, erythromycin, novobiocin, oxytetracycline, ceftazidime, cefotaxime, and ofloxacin antibiotics while showing sensitivity

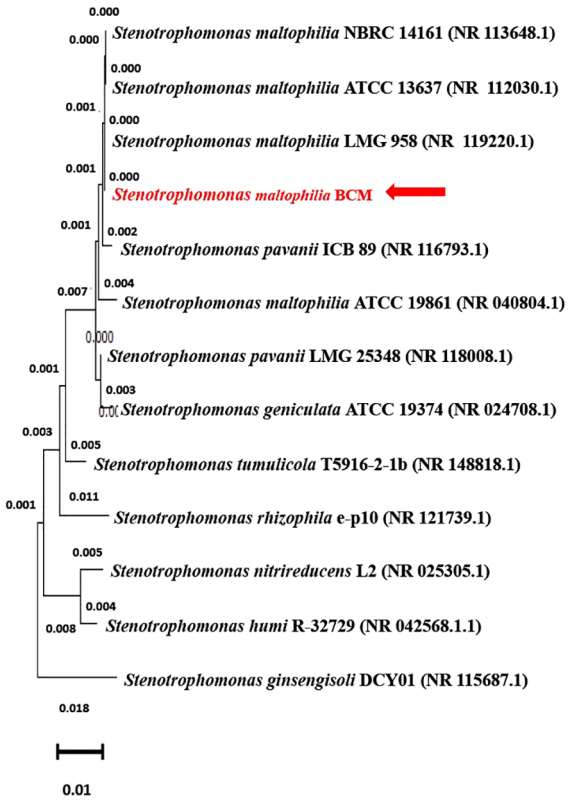

FIGURE 1
Phylogenetic affiliation of *Stenotrophomonas maltophilia* BCM with the other *Stenotrophomonas* species. The phylogenetic tree was constructed with the Neighbor-joining method of phylogenetics with 1000 bootstrap replications using MEGA-X software. The 16S rRNA gene sequence of *Stenotrophomonas ginsengisoli* DCY01 was represented as out-group.

toward nation, lincomycin, claxon, and amikacin. The antibiotic resistance profile of *Stenotrophomonas maltophilia* BCM was similar to other *Stenotrophomonas maltophilia* species while overlapping with *Stenotrophomonas maltophilia* smyn44 (Supplementary Table S2). Similar biochemical, substrate utilization, and antibiotic resistance profiles of *Stenotrophomonas maltophilia* BCM to other *Stenotrophomonas maltophilia* species strengthen the 16S rRNA gene-based taxonomic observations. Stress response physiology assays indicate that it can successfully grow in the presence of salts [up to 5.85% NaCl (w/v), 8.9% KCl (w/v), and 4.2% LiCl (w/v)] (Figure 3), metals [up to 0.1% Na_3AsO_4 (w/v), 0.12% $NaAsO_2$ (w/v), and 0.54% $CdCl_2$(w/v)] (Figure 4), oxidizing agents [up to 5.96% (v/v) H_2O_2] (Figure 5), as observed for other *Stenotrophomonas* sp. (Supplementary Table S3).

Genomic characterization of *Stenotrophomonas maltophilia* BCM

Genome sequencing of *Stenotrophomonas maltophilia* BCM resulted in the generation of 551495 paired-end raw reads. *Stenotrophomonas maltophilia* BCM genome was assembled into 447 contigs, accounting for a total 4519592 bp size, with 66.5% GC

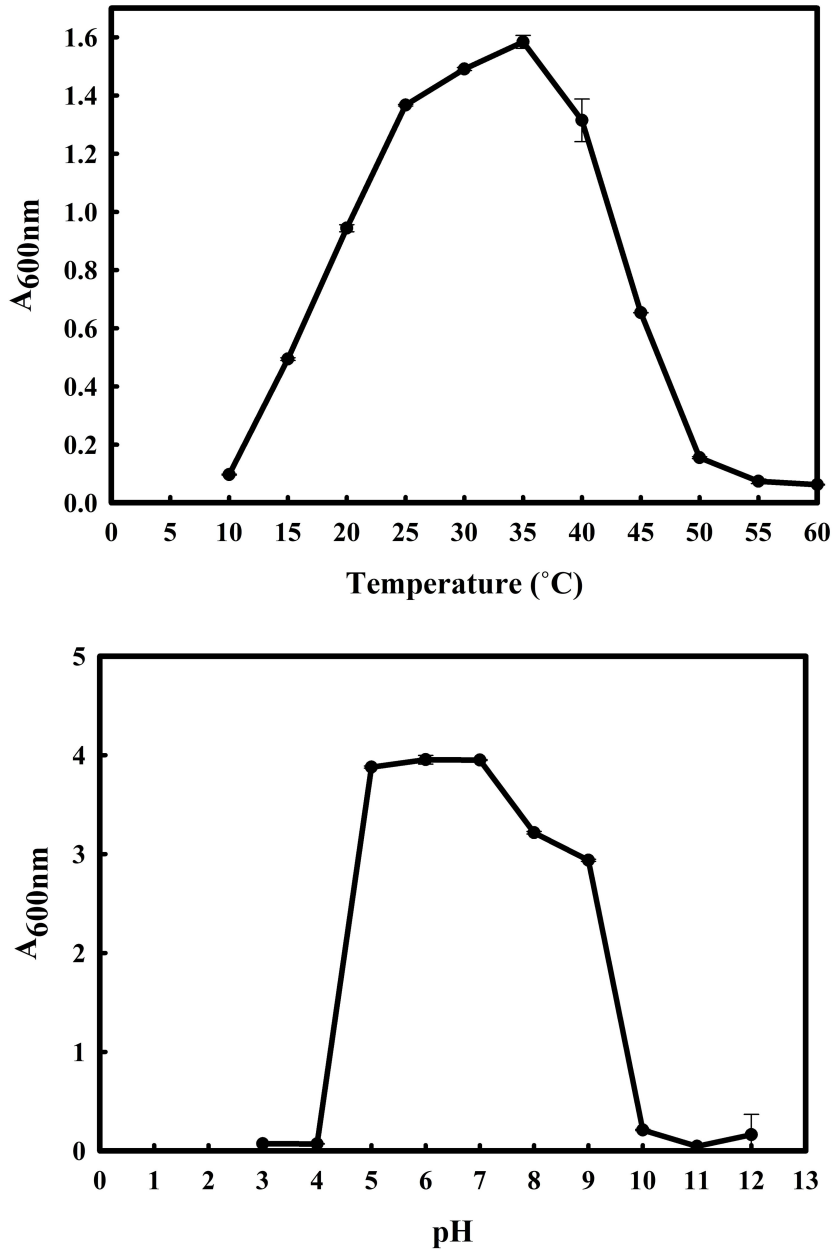

FIGURE 2
Growth profile of *Stenotrophomonas metophilia BCM* under diverse temperatures (10°C to 60°C with an interval of 5°C) and pH (from 3 to 12 with an interval of one pH unit) conditions. *Stenotrophomonas maltophilia BCM* growth was observed in LB broth after 16 hrs of uninterrupted growth at respective temperature and pH conditions with constant shaking at 200 rotations per minute (rpm). The experiments were carried out in triplicates, and growth was observed by reading the absorbance of the culture at 600nm. Plotted values are the mean of triplicates along with the observed standard deviation.

content and N50 length of 139100bp (Supplementary Table S4). Functional annotation of the genome identified 3949 protein-coding, 7 rRNA, 74 tRNA, and 1 tmRNA gene (Figure 6A). Average nucleotide identity (ANI) was performed to check the relationship of microbe at the genomic level. The average ANI among different species of *Stenotrophomonas* was 77-99% toward the lower end of the 77-100% spectrum, suggesting significant interspecific genomic variations. Furthermore, the ANI score of *Stenotrophomonas maltophilia* BCM with *Stenotrophomonas maltophilia* smyn44 was 99.57, while it was comparatively higher compared with other species members (Supplementary Table S5). The affiliation of *Stenotrophomonas maltophilia* BCM as a member of *Stenotrophomonas maltophilia* species was further confirmed using terra correlation. *Stenotrophomonas maltophilia* BCM has been awarded a 0.9996 z-score against *Stenotrophomonas maltophilia* AU12-09 during terra-correlation, confirming its similarity with *Stenotrophomonas maltophilia*. Other *Stenotrophomonas* species exhibited good similarity (z-score ~ 0.93-0.99) (Supplementary Table S6). The matrix generated using the Roary tool showed the comprehensive nature of the genome in which the microbial isolate showed the highest similarity with *Stenotrophomonas maltophilia* smyn44 (Figure 6B). The genomic

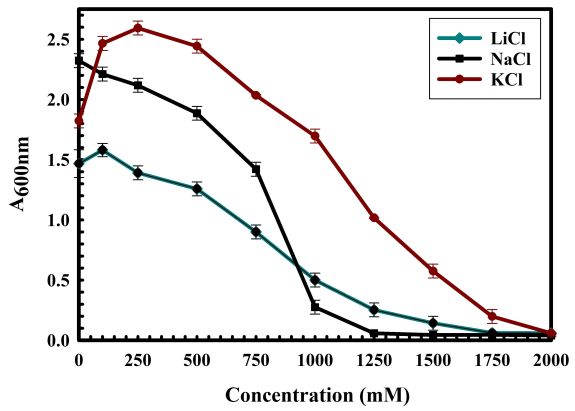

FIGURE 3
Growth profile of *Stenotrophomonas maltophilia BCM* in the presence of NaCl, KCl, and LiCl. *Stenotrophomonas maltophilia BCM* growth was observed in salt-supplemented LB broth after 16 hrs of uninterrupted growth at 37°C with constant shaking at 200 rpm. The experiments were carried out in triplicates, and growth was observed by reading the absorbance of the culture at 600nm. Values plotted here are the triplicates' mean and the observed standard deviation.

matrix also revealed that all *Stenotrophomonas* genomes share only a few numbers of genes as their core genome. Shell and cloud genome collectively forms the central part of the genomes. *Stenotrophomonas maltophilia* BCM genome has neither a pathogenic gene/island nor any virulence-related genes, indicating its non-pathogenic nature. In addition to the genes for antimicrobial activity (Table 1), *Stenotrophomonas maltophilia* BCM genome harbors genes encoding proteins for plant growth promotion activities like auxin biosynthesis, nitrogen assimilation, siderophore biosynthesis, and phosphate solubilization (Table 2). *Stenotrophomonas maltophilia* BCM genome also harbors genes responsible for arsenic resistance, oxidative stress tolerance, metal stress tolerance, and salt tolerance (Supplementary Table S7), explaining its stress response physiology. The 24 CAZymes clusters within its genome also justify its diverse carbohydrate utilization profile (Supplementary Table S8). Additionally, its genome encodes various hydrolases, some of which might extend anti-pathogenic behavior to the host (Supplementary Table S9).

Several proteins were identified as essential for effective colonization in plant rhizosphere (Kumar et al., 2023). An in-depth analysis of *the Stenotrophomonas maltophilia* BCM genome identifies *the* presence of genes encoding proteins for the synthesis of Type 1 and IV pili, exopolysaccharide (Table 3) essential for plant surface adhesion, auto-aggregation, and early biofilm formation (Kumar et al., 2023).

Assessment of the antifungal potential of *Stenotrophomonas maltophilia* BCM

The protective effect of *Stenotrophomonas maltophilia* BCM on wheat seed germination was assessed. A 10% ± 1 and 5% ± 0.57735 seed germination was observed in the presence of *Rhizoctonia solani*

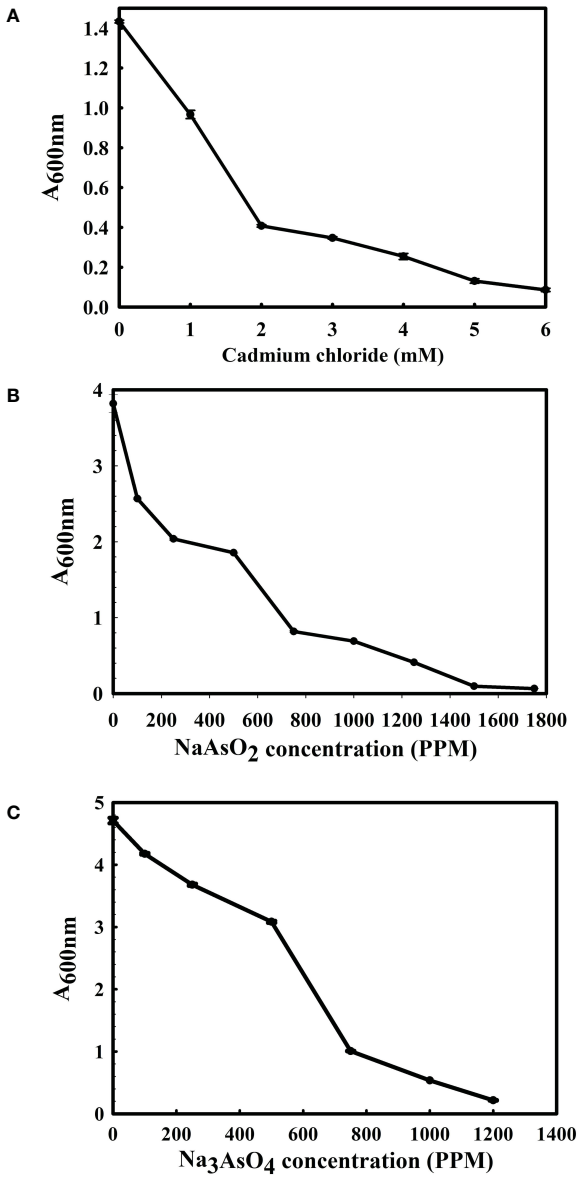

FIGURE 4
Growth profile of *Stenotrophomonas maltophilia* in the presence of cadmium chloride **(A)**, sodium arsenite **(B)**, and sodium arsenate **(C)**. *Stenotrophomonas maltophilia BCM* growth was observed in metal/metalloid-supplemented LB broth after 16 hrs of uninterrupted growth at 37°C with constant shaking at 200 rpm. The experiments were carried out in triplicates, and growth was observed by reading the absorbance of the culture at 600nm. Values plotted here are the triplicates' mean and the observed standard deviation.

and *Fusarium oxysporum*, respectively. Seeds' pre-treatment with *Stenotrophomonas maltophilia* BCM showed a germination efficiency of 75.33 ± 0.57735% and 87.66 ± 0.57705% in the presence of *Rhizoctonia solani* and *Fusarium oxysporum*, respectively (Figure 7). *Stenotrophomonas maltophilia* BCM was observed to increase ~750 and ~1753-fold seed germination in the presence of *Rhizoctonia solani* and *Fusarium oxysporum*, respectively. These results strongly indicate the potential biocontrol behavior of *Stenotrophomonas maltophilia* BCM. *Stenotrophomonas maltophilia* BCM enhanced seed germination

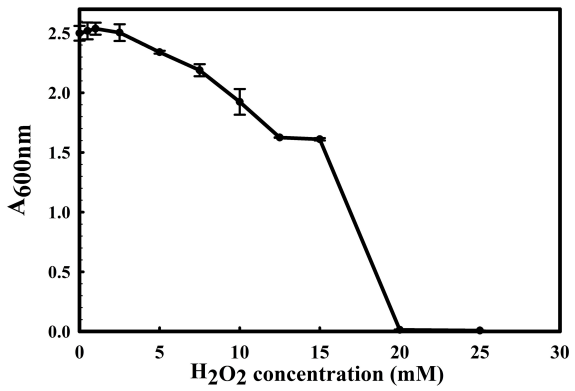

FIGURE 5
Growth profile of *Stenotrophomonas maltophilia* BCM in the presence of NaCl , KCl, and LiCl. *Stenotrophomonas maltophilia* BCM growth was observed in salt-supplemented LB broth after 16 hrs of uninterrupted growth at 37°C with constant shaking at 200 rpm. The experiments were carried out in triplicates, and growth was observed by reading the absorbance of the culture at 600nm. Values plotted here are the triplicates' mean and the observed standard deviation.

in the presence of phytopathogens and significantly improved seed germination in reference to the control (P=0.024) (Figure 7). *Rhizoctonia solani* and *Fusarium oxysporum* were also found to significantly reduce (P=0.0001 and P=0.0018) alpha-amylase activity in wheat seeds to 0.42 IU and 0.22 IU, respectively. Seeds' pre-treatment with *Stenotrophomonas maltophilia* BCM significantly increased alpha-amylase activity (P=0.000136 and P=0.0000262) in the presence of *Rhizoctonia solani* and *Fusarium oxysporum*, respectively (Figure 8). The significant improvement of alpha-amylase activity in wheat seeds after pre-treatment with *Stenotrophomonas maltophilia* BCM could be a possible reason for enhanced seed germination.

Pre-treatment of seeds with *Stenotrophomonas maltophilia* BCM not only enhanced seed germination but also improved the growth of wheat plantlets. Wheat seeds pre-treated with *Stenotrophomonas maltophilia* BCM showed a significantly enhanced shoot length (P=0.027) and root length (P=0.010) compared to the untreated seeds (Figure 9). *Stenotrophomonas maltophilia* BCM was also found to significantly improve the root (P=0.017 and 0.039) and shoot length (P=0.020 and 0.136) of wheat plantlets infected with *Rhizoctonia solani* and *Fusarium oxysporum*, respectively. The average shoot and root length of wheat plantlets treated with *Stenotrophomonas maltophilia* BCM were significantly higher (P=0.0297 and 0.0023) compared to the control even after *Rhizoctonia solani* and *Fusarium oxysporum* exposure, indicating its biocontrol behavior. *Stenotrophomonas maltophilia* BCM also interacted with *Chromobacterium violaceum* and significantly reduced violacein production (P=0.001). This indicates the potential of *Stenotrophomonas maltophilia* BCM as a quorum-sensing inhibitor and antibiofilm activity, which is good for a bio-controlling agent.

Stenotrophomonas maltophilia BCM's role in wheat seed germination in abiotic stress like high salinity (a key bottleneck in wheat germination and crop production) was also assessed. Increased salt concentration significantly reduced seed germination (Figure 10A). Seeds' pre-treatment with *Stenotrophomonas maltophilia* BCM showed enhanced seed germination at high salinity conditions (Figure 10B). Pre-treatment of seeds with *Stenotrophomonas maltophilia* BCM not only enhanced seed germination but also improved the growth of wheat plantlets. Wheat seeds pre-treated with *Stenotrophomonas maltophilia* BCM showed a significantly enhanced shoot length (P=0.001) and root length (P=00.0018) compared to the untreated in high salinity conditions.

Plant growth potential of *Stenotrophomonas maltophilia* BCM

Stenotrophomonas maltophilia BCM genome harbors key genes responsible for plant growth promotion activities like auxin biosynthesis, nitrogen assimilation, siderophore biosynthesis, and phosphate solubilization. *Stenotrophomonas maltophilia* BCM showed nitrate reductase activity (14IU), extracellular alkaline (0.22IU), and acid phosphatase activity (0.1 IU). It was also found to produce and secrete plant growth-promoting hormones in the surrounding environment. The presence of genes responsible for plant growth promotion and their bioactivity indicate biocontrol behavior. Stenotrophomonas maltophilia BCM also harbors plant growth-promotion activity.

Discussion

A boost in crop production of cereals is required to ensure food security for the expanding global population (Tilman et al., 2011). In the past, applying agrochemicals has boosted agricultural yield and helped to fulfill food requirements. Continuous applications of agrochemicals negatively impact the environment and human health. These agrochemicals severely affect soil fertility, creating hurdles in enhancing crop production to ensure food security (Gamage et al., 2023). In addition, the evolution of plant pathogens for pesticide resistance, higher infectivity, and broad host range are significant challenges (Newman and Derbyshire, 2020). Sustainable agricultural practices offer solutions to ensure healthy soil ecology, limit infections, and enhance crop production (Mehmet Tuğrul, 2020). Plants harbor several microbial companions on underground and above-ground surfaces (Van Dijk et al., 2022). These micro-residents improve the host plant's growth by enhancing nutrient assimilations, cell division, and plant reproduction and preventing the invasion of pathogens (Koza et al., 2022). Sustainable agricultural practices advocate the employment of such micro-residents to improve crop production. Identification and characterization of such candidate microbes became a quest of researchers around the globe.

Wheat is one of the prime food sources for fulfilling the hunger of most of the global population. Increases in soil salinity, reduced soil fertility, emergence of phytopathogens, and climate change threaten wheat production (Srinivas et al., 2019). There is an emergent need to identify potential microbial agents that can

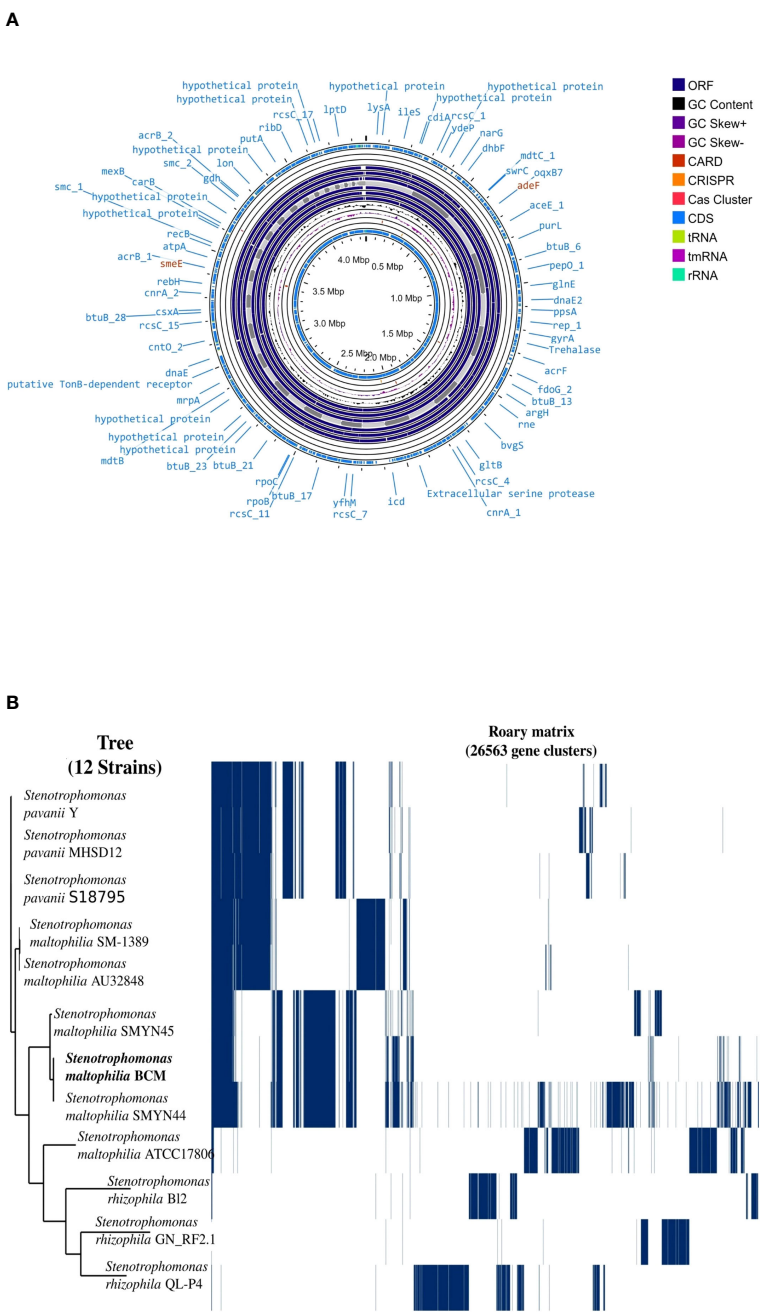

FIGURE 6
Genome map **(A)** and phylogenomic profile **(B)** of the *Stenotrophomonas maltophilia* BCM. The circular genome map **(A)** was drawn using the Proksee online tool (https://proksee.ca. The phylogenomic tree **(B)** was constructed with the FastTree v2.1.10 tool via Roary.

improve resistance toward both biotic (phytopathogens) and abiotic (salinity) stress in wheat for better crop production. In the present study, an effort was made to explore the wheat rhizosphere microbiota to identify potential microbial candidates to enhance plant growth by inhabiting phytopathogens infection, improving nutrient assimilation, and extending resistance toward various stressors.

Culture-dependent exploration of wheat rhizosphere microbiota identified 12 morphologically different bacterial isolates. Antifungal assays indicate that the isolate BCM showed effective growth inhibition of *Rhizoctonia solani* and *Fusarium oxysporum* phytopathogens. The 16S rRNA gene-based phylogenetic characterization of BCM isolate reveals suitable homology with *Stenotrophomonas maltophilia*, accordingly labeled as *Stenotrophomonas maltophilia* BCM. *Stenotrophomonas maltophilia* is a group of diazotrophic bacteria isolated from diverse habitats, predominantly from plant-associated ecosystems (Pinski et al., 2020). Rhizospheric *Stenotrophomonas maltophilia* species have the potential as biofertilizers, biocontrol agents, pesticide remediation (Kumar et al., 2023), and stress resistance (Singh and

TABLE 1 Genetic features involved in *Stenotrophomonas maltophilia* BCM genome involved in biocontrolling activity.

CDS start Position in the genome	CDS stop position in the genome	Strand	Function
A. Phenazine biosynthesis and resistance protein			
11738	10860	–	Phenazine biosynthesis protein PhzF
12685	11810	–	Phenazine biosynthesis protein PhzF
18001	16053	–	Phenazine antibiotic resistance protein EhpR
B. Bacteriocin resistance protein			
37151	36447	–	Bacteriocin resistance protein
C. Chitinase			
181286	180096	–	Chitinase
61552	63651	+	Chitinase

Jha, 2017). These studies strengthen the candidature of *Stenotrophomonas maltophilia* BCM as a biofertilizer and biocontrol agent. Most of this information was drawn based on experimentation with diverse plant species (Kumar et al., 2023). As a result, it is essential to understand its physiological, genomic, and plant-associated properties to understand its efficiency.

The morphological, physiological, and chemotaxonomic profile of BCM was similar to other *Stenotrophomonas maltophilia* strains, confirming 16S rRNA gene-based phylogenetic observations. Likewise, other *Stenotrophomonas* sp., *Stenotrophomonas maltophilia* BCM, was observed to successfully thrive under diverse physicochemical (pH and temperature) and stress conditions (saline, exposure to metal/metalloid, and antibiotic). Survival in diverse environments extended the ubiquitous nature of *Stenotrophomonas* sp (Kumar et al., 2023). *Stenotrophomonas maltophilia* BCM harbors a G+C-rich genome of 4519592bps encoding a diverse array of proteins, extending metabolic robustness to the host. Average Nucleotide Identity and phylogenomic observations further validate its taxonomic affiliation. *Stenotrophomonas maltophilia* BCM encodes a diverse array of hydrolytic enzymes (CAzymes, proteases, chitinase, glucanases, and lipases), phytohormones production (IAA), nutrients (Phosphate) solubilization, and phenazine production. The presence of protein-encoding features for phosphate solubilization, siderophore production, nitrogen fixation, and phytohormone production could significantly boost plant growth (Souza et al., 2015) to act as a biofertilizer. Various *Stenotrophomonas* sp. were already characterized for the presence of these features and were placed under the category of PGPRs (Majeed et al., 2015). Chitinolytic and proteolytic enzymes could effectively hydrolyze fungal cell walls and inhibit fungal growth (Hamid et al., 2013). Phenazine is another potent antifungal compound, and

TABLE 2 Genetic features identified within *Stenotrophomonas maltophilia* BCM genome encoding various proteins involved in nutrient assimilation and solubilization.

CDS start position in the genome	CDS stop position in the genome	Strand	Function
A. Nitrogen transport and regulation			
69574	69969	+	Nitrogen regulatory protein P-II, GlnK
94361	95422	+	Nitrogen regulation protein NtrB (EC 2.7.13.3)
95415	96863	+	Nitrogen regulation protein NR(I), GlnG (=NtrC)
26694	25342	–	nitrogen regulation protein NtrY, putative
348	10	–	Nitrogen regulatory protein P-II, GlnK
36588	37013	+	PTS IIA-like nitrogen-regulatory protein PtsN
145605	147023	+	Nitrate/nitrite transporter NarK/U
147096	150839	+	Respiratory nitrate reductase alpha chain (EC 1.7.99.4)
150839	152383	+	Respiratory nitrate reductase beta chain (EC 1.7.99.4)
152383	153063	+	Respiratory nitrate reductase delta chain (EC 1.7.99.4)
153060	153767	+	Respiratory nitrate reductase gamma chain (EC 1.7.99.4)
B. IAA biosynthesis			
36312	35470	–	Indole-3-glycerol phosphate synthase (EC 4.1.1.48)
C. Phosphate regulation, transport, and solubilization			
189720	188125	–	Alkaline phosphatase
23081	23770	+	Phosphate regulon transcriptional regulatory protein PhoB (SphR)
23877	25208	+	Phosphate regulon sensor protein PhoR (SphS) (EC 2.7.13.3)
39099	38392	–	Phosphate transport system regulatory protein PhoU
40006	39176	–	Phosphate ABC transporter, ATP-binding protein PstB (TC 3.A.1.7.1)
40889	40026	–	Phosphate ABC transporter, permease protein PstA (TC 3.A.1.7.1)
41857	40889	–	Phosphate ABC transporter, permease protein PstC (TC 3.A.1.7.1)

(Continued)

TABLE 2 Continued

CDS start position in the genome	CDS stop position in the genome	Strand	Function
C. Phosphate regulation, transport, and solubilization			
43028	41940	–	Phosphate ABC transporter, substrate-binding protein PstS (TC 3.A.1.7.1)
44453	43437	–	Phosphate ABC transporter, substrate-binding protein PstS (TC 3.A.1.7.1)
45887	44676	–	Phosphate/pyrophosphate-specific outer membrane porinOprP/OprO
D. Siderophore Biosynthesis			
83949	81742	–	Putative OMR family iron-siderophore receptor precursor
1	546	+	TonB-dependent siderophore receptor
77730	78392	+	Ferric siderophore transport system, biopolymer transport protein ExbB
104477	105670	+	Isochorismate synthase (EC 5.4.4.2) @ Isochorismate synthase (EC 5.4.4.2) of siderophore biosynthesis
105667	107319	+	2,3-dihydroxybenzoate-AMP ligase (EC 2.7.7.58) of siderophore biosynthesis
107319	107951	+	Isochorismatase (EC 3.3.2.1) of siderophore biosynthesis
108205	112095	+	Siderophore biosynthesis non-ribosomal peptide synthetase modules
112086	112844	+	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase (EC 1.3.1.28) of siderophore biosynthesis
202780	203736	+	Iron siderophore sensor protein
203920	207042	+	Iron siderophore receptor protein

TABLE 3 Genetic features within the genome encoding proteins for *Stenotrophomonas maltophilia* BCM colonization in wheat rhizosphere.

CDS start position in the genome	CDS stop position in the genome	Strand	Function
A. Pili formation Protein			
36034	35504	–	Type IV pili signal transduction protein PilI
61031	61546	+	Type IV fimbrial biogenesis protein FimT
61543	62040	+	Type IV fimbrial biogenesis protein PilV
62049	63206	+	Type IV fimbrial biogenesis protein PilW
63212	63733	+	Type IV fimbrial biogenesis protein PilX
63747	67514	+	Type IV fimbrial biogenesis protein PilY1
67538	67945	+	Type IV pilus biogenesis protein PilE
B. Mannose-6-phosphate isomerase			
67945	68009	+	Mannose-6-phosphate isomerase
C. EPS biosynthesis			
19212	19874	+	Exopolysaccharide synthesis ExoD

phenazine-producing microbes can effectively protect plants against fungal phytopathogens (Karmegham et al., 2020). *Stenotrophomonas maltophilia* BCM genome harbors genes encoding chitinase, protease, and proteins involved in phenazine production, indicating its potential as a biocontrol agent. Type I and IV pili encoding genes are essential for adhesion, autoaggregation, and biofilm formation (Elvers et al., 2001); these proteins allow the PGPR *Stenotrophomonas* sp. to associate with plant host in the rhizosphere (Kumar et al., 2023). The presence of these genes in the *Stenotrophomonas maltophilia* BCM genome further confirms its strong interaction with wheat rhizosphere. Despite the enormous potential of *Stenotrophomonas* sp., their application for crop improvement is challenged by the pathogenic nature of *Stenotrophomonas maltophilia* (Ryan et al., 2009). Surprisingly, the *Stenotrophomonas maltophilia* BCM genome lacks gene clusters for inducing pathogenicity in animals and plants, confirming its safe application. *Stenotrophomonas maltophilia* BCM genome indicates its application as a biocontrol agent and biofertilizer; however, these properties must be validated experimentally.

In-vitro experiments confirm phosphate solubilization, nitrate reduction, and auxin synthesis properties of *Stenotrophomonas maltophilia* BCM. These experimental observations confirm genomic observations based on the biofertilizer potential of *Stenotrophomonas maltophilia* BCM. Additionally, *in-vitro* antifungal experiments confirm its potential as a biocontrol agent. PGPR and biocontrol behavior of *Stenotrophomonas maltophilia* BCM were further confirmed in *in-vivo* studies. Wheat seed germination experiments in the presence of phytopathogens, *Fusarium oxysporum*, and *Rhizoctonia solani* indicated that *Stenotrophomona smaltophilia* BCM effectively protects the host seedling from fungal infection. These observations support the

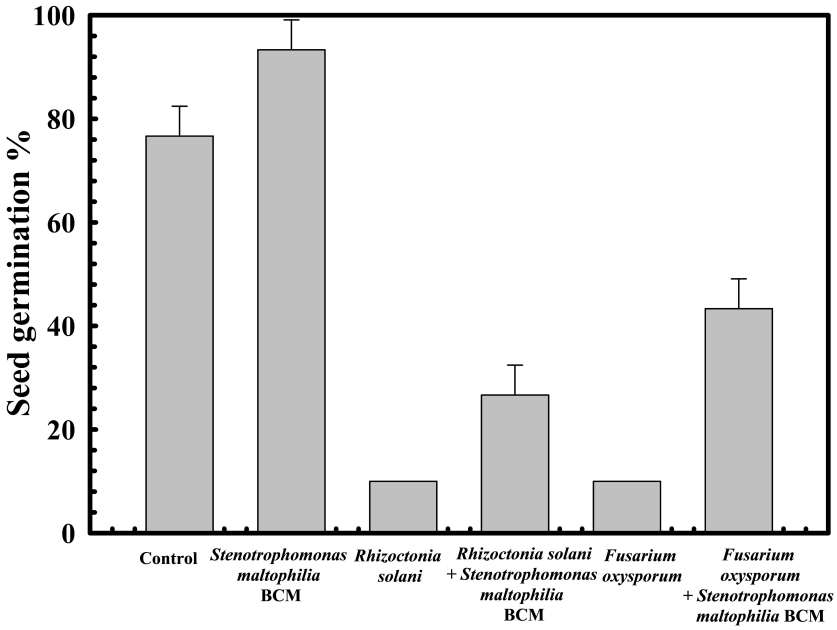

FIGURE 7
Impact of *Stenotrophomonas maltophilia* BCM on the wheat seed germination percentage under biotic stress. Seeds were pre-inoculated with 2× 10^8 CFU/ml of test organisms (*S. maltophilia* BCM and phytopathogenic fungal strains as per experimental conditions) for 16 hours before seed germination experiments. All assays were carried out in triplicates.

genome-based observations indicting *Stenotrophomonas maltophilia* BCM as a biocontrol agent. *Stenotrophomonas maltophilia* BCM not only protects the wheat seedlings from fungal infection but also significantly improves seed germination percentage and plant growth in the presence and absence of phytopathogens. *Stenotrophomonas maltophilia* BCM also improved wheat seed germination percentage and seedling growth under saline conditions, indicating its potential to overcome salty conditions. Conclusively, wheat rhizosphere isolates *Stenotrophomonas maltophilia* BCM showed good PGPR

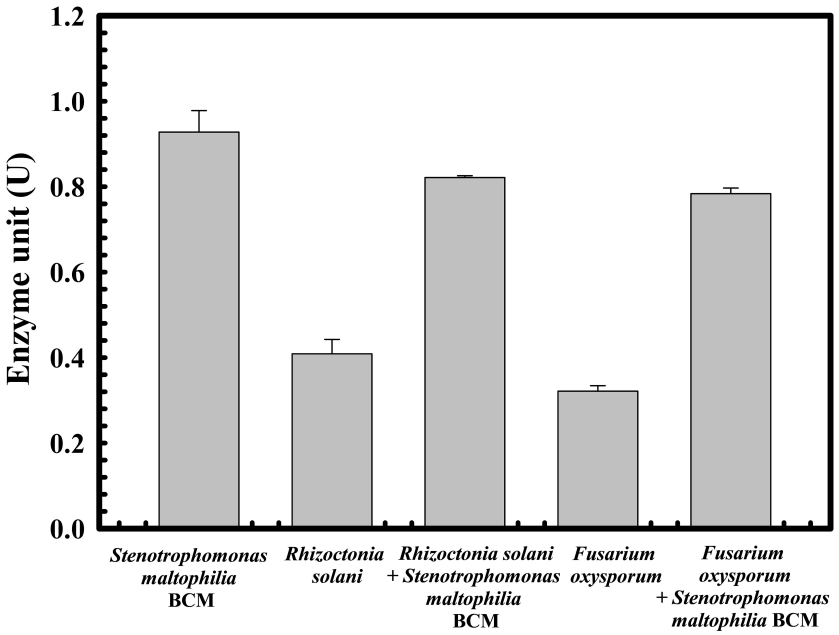

FIGURE 8
Impact of *Stenotrophomonas maltophilia* BCM on the alpha-amylase activity in wheat seeds during germination under biotic stress. Seeds were pre-inoculated with 2× 10^8 CFU/ml of test organisms (*S. maltophilia* BCM and phytopathogenic fungal strains as per experimental conditions) for 16 hours before alpha-amylase activity assays. Plotted values are the mean of triplicates along with the observed standard deviation.

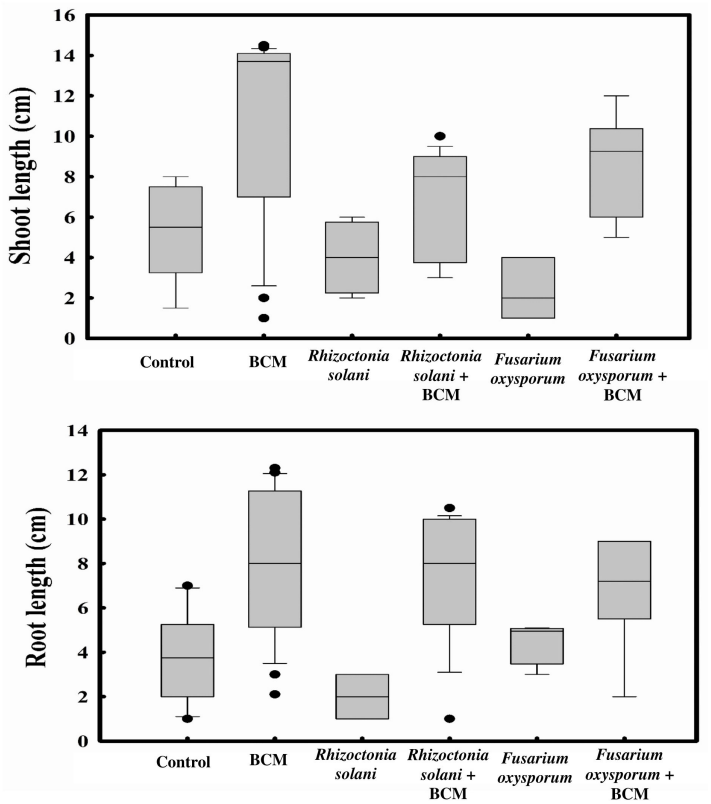

FIGURE 9
Impact of *Stenotrophomonas maltophilia* BCM on root and shoot length of WC-306 plantlets under biotic stress. Seeds were pre-inoculated with 2× 10^8 CFU/ml of test organisms (*S. maltophilia* BCM and phytopathogenic fungal strains as per experimental conditions) for 16 hours before seedling growth experiments. Plotted values are the mean of triplicates along with the observed standard deviation.

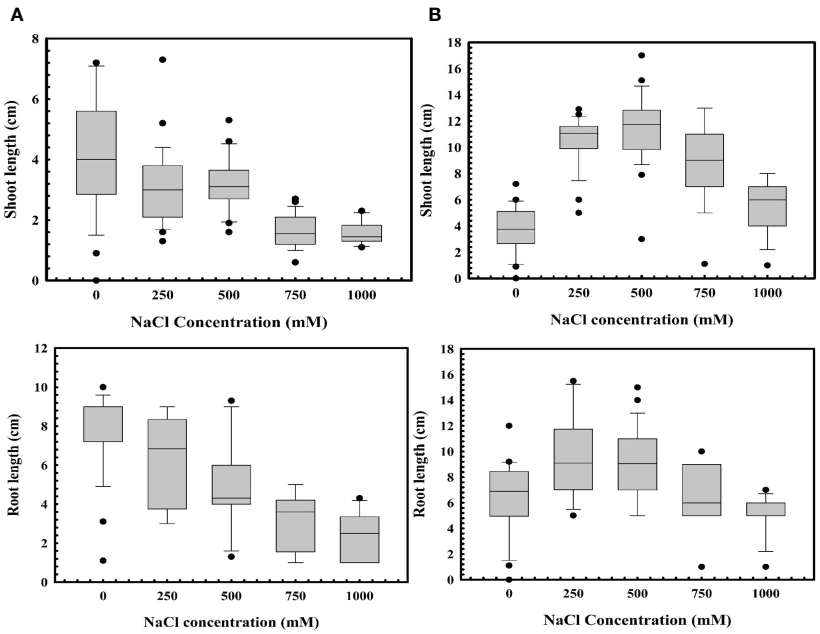

FIGURE 10
Impact of *Stenotrophomonas maltophilia* BCM on root and shoot length of WC-306 plantlets under saline stress (NaCl **(A)** and KCl **(B)**). Seeds were pre-inoculated with 2× 10^8 CFU/ml of test organisms (*S. maltophilia* BCM and phytopathogenic fungal strains as per experimental conditions) for 16 hours before seedling growth experiments. Plotted values are the mean of triplicates along with the observed standard deviation.

and biocontrol potential under a diverse range of stresses in the study, projecting its potential application for sustainable agriculture. However, long-term analysis under field conditions is required to validate the outcomes. These analyses are essential for implanting the isolate in agricultural practices.

Data availability statement

The whole genome sequence of *Stenotrophomonas maltophilia* BCM has been uploaded to the NCBI server with an SRA accession number SRX23009035 and Bio project accession ID PRJNA1056133.

Author contributions

PS: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. RP: Writing – original draft, Writing – review & editing. NC: Data curation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors acknowledge CSIR-Institute of Genomics and Integrative Biology, New Delhi, India, for DNA sequencing facility.

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/fpls.2024.1364807/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 12 March 2024
DOI 10.3389/fpls.2024.1332840

OPEN ACCESS

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RECEIVED 03 November 2023
ACCEPTED 14 February 2024
PUBLISHED 12 March 2024

CITATION
Adamo I, Acin-Albiac M, Röttjers S, de Prado DR, Benito BM, Zamora J, Godara R, García-Jiménez B, Jiang-Rempel P, Cline LC and Acedo A (2024) Short impact on soil microbiome of a *Bacillus amyloliquefaciens* QST713 based product that correlates with higher potato yield across USA. *Front. Plant Sci.* 15:1332840. doi: 10.3389/fpls.2024.1332840

Short impact on soil microbiome of a *Bacillus amyloliquefaciens* QST713 based product that correlates with higher potato yield across USA

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Potato (*Solanum tuberosum L.*) is considered one of the most widely consumed crops worldwide, due to its high yield and nutritional profile, climate change-related environmental threats and increasing food demand. This scenario highlights the need of sustainable agricultural practices to enhance potato productivity, while preserving and maintaining soil health. Plant growth-promoting bacteria (PGPB) stimulate crop production through biofertilization mechanisms with low environmental impact. For instance, PGPB promote biological nitrogen fixation, phosphate solubilization, production of phytohormones, and biocontrol processes. Hence, these microbes provide a promising solution for more productive and sustainable agriculture. In this study, the effects of *Bacillus amyloliquefaciens* QST713 based-product (MINUET™, Bayer) were assessed in terms of yield, soil microbiome, potato peel and petiole nutrient profile as a promising PGPB in a wide range of potato cultivars across the United States of America. Depending on the location, potato yield and boron petiole content increased after biostimulant inoculation to maximum of 24% and 14%, respectively. Similarly, nutrient profile in potato peel was greatly improved depending on the location with a maximum of 73%, 62% and 36% for manganese, zinc and phosphorus. Notably, fungal composition was shifted in the treated group. Yield showed strong associations with specific microbial taxa, such as *Pseudoarthrobacter, Ammoniphilus, Ideonella, Candidatus Berkiella, Dongia*. Moreover, local networks strongly associated with yield, highlighting the important role of the native soil microbiome structure in

indirectly maintaining soil health. Our results showed that treatment with *B. amyloliquefaciens* based product correlated with enhanced yield, with minor impacts on the soil microbiome diversity. Further studies are suggested to disentangle the underlying mechanisms of identified patterns and associations.

KEYWORDS

biostimulant, soil microbiome, potato yield, potato leaf petiole, potato peel, network properties

1 Introduction

Potato (*Solanum tuberosum L.*) is one of the most widely consumed crops worldwide. Because of its high yield and nutritive values, it is considered one of the most important agricultural crops (Beals, 2019; Kanter and Elkin, 2019), whose production reached a total 376 million tonnes in 2021 as per Unicef organization (FAO, 2022). However, current environmental threats to the agricultural sector related to climate change, biodiversity loss, land degradation and agricultural practices raised interest in more sustainable agricultural practices to preserve and maintain soil health (Muller et al., 2017). In addition, the total global food demand is expected to increase by 35% - 56% between 2010 and 2050 (Van Dijk et al., 2021). Therefore, an intensified effort to enhance food production is needed to overcome these global future challenges, while reducing the environmental impact on the ecosystems (Rouphael and Colla, 2020). Plant growth-promoting (PGP) microbes are known for their capacity to stimulate crop production through biofertilization mechanisms with low environmental impacts. For instance, PGP microbes promote biological nitrogen fixation, phosphate solubilization, production of phytohormones, and biocontrol processes (Vacheron et al., 2013; Bashan et al., 2014; Shahrajabian et al., 2023). More in particular, plant growth-promoting rhizobacteria (PGPR) suggest a promising solution for more productive and sustainable agriculture practices (Chaudhary et al., 2023). The use of biological products in agriculture has increased substantially in the last decade. In 2022, the plant-growth promoters and biostimulants market was valued 2.9 billion and ca. USD 3.5 billion, respectively (Markets and Markets, 2022).

Several studies have already assessed the positive direct effects of PGPR on potato yield (Sarwar et al., 2018; Ekin, 2019; Imam et al., 2021). Therefore, microbial biostimulants are an innovative and promising group of agricultural inputs. Nevertheless, more effort is needed to explore their use and the possible positive impacts on productivity and soil health in potato cultivars. This may provide inputs to give recommendations to farmers on the use of more sustainable practices (Shahrajabian et al., 2023). Because of the importance of soil microbial communities in maintaining soil quality and global nutrient cycling, the effects of plant growth-promoting bacteria soil inoculation should be further assessed (Bharti et al., 2016; Imam et al., 2021; Chaudhary et al., 2023).

Moreover, studies at global and local levels observed that soil microbial communities are affected by changes in soil physio chemistry and climate (Tedersoo et al., 2014; Glassman et al., 2017; Plassart et al., 2019; Zeng et al., 2019). However, associations between soil physio chemistry and soil microbiome before and after product inoculation are still poorly understood. In addition, potato petiole analysis can give indirect information on the effectiveness of products by monitoring nutrient levels in potato cultivars (Collins et al., 2016; Kong et al., 2022). For instance, phosphorus, boron, nitrogen, zinc and manganese concentrations increased upon rhizobacteria inoculation in strawberry and wheat crops (Ipek et al., 2014; Kumar et al., 2014). Nevertheless, still little is known about to what extent potato petiole nutrients are important to assess the efficacy of biological products and how it can relate to potato yield and soil microbiome.

Similarly, a previous study analyzed the microbial composition and structure of bulk and rhizosphere soils to assess the effects of *B. amyloliquefaciens* QST713-based product (Imam et al., 2021). Imam et al. (2021) investigated the effects of this biological product in three different geographical regions in the United States. Their results showed that the product positively promoted potato yield in two geographic locations. Moreover, microbial taxa abundances and community structure changed after inoculation, but long-lasting effects on soil microbial alpha and beta-diversity were not observed. Finally, yield prediction model including all three locations was built, which incorporated product use and soil microbiome information, such as microbiome network properties. These properties define how microorganisms tend to co-occur or co-exclude and measure how the microbial network tends to cluster together in a specific ecological niche. Imam et al. (2021) concluded that soil fungal network properties were the most important predictors of yield.

In our study, the effects of *B. amyloliquefaciens* QST713 application were addressed in a wide range potato cultivars across the United States of America, in 21 geographic locations. This product has already demonstrated a broad fungicide and bactericide activity on potato crops (US Environmental Protection Agency, 2006). Moreover, its antibiotic production and *in vitro* suppression of pathogens were examined from root surfaces through HPLC and MS quantification (Kinsella et al., 2009). In this study, previous work of Imam et al. (2021) was extended by exploring the possible

effects of *B. amyloliquefaciens* based product on a substantially broader geographic distribution. Here, product effects on soil microbiome composition and structure were determined, comparing both control and treated samples at growth stage 2 (around 30 days after emergence) with control before planting. Moreover, crop yield, soil physicochemical properties, potato peel and leaf nutrients, along with environmental data, were integrated to explore the effetcs of *B. amyloliquefaciens* QST713-based product on soil microbiome associated with crop performance in different locations. Indeed, this study demonstrated the impacts of this biological product on soil microbiome and potato productivity. Finally, the results provided insights on the functioning of soil ecosystems and its associations with crop performance. This may serve to provide better recommendations to growers on the use of more sustainable products in the current global change context.

2 Materials and methods

2.1 Agronomic trial design and sample collection

Potatoes (Varieties: Russet Ranger, Russet Burbank, French Fingerling, Russet Norkotah, FL1867, FL2137, Red Norland) were planted between April and May 2020 in 21 different geographical regions in the United States (Supplementary Figure S1; Supplementary Table S1). All trials were managed by Bayer Crop Science. Treatment consisted of a biological product containing minimum of 2.7 x 10^{10} colony forming units (CFU) of *B. amyloliquefaciens* strain QST713 (NCBI accession number: CP025079; MINUET™, Bayer Crop Science). All trials comprised three replicated sub-plots per treatment condition for a total of 6 sub-plots per location. Harvest was conducted by corresponding Bayer trial cooperators located in Idaho, Washington, Texas, Michigan, Maine, New York, North Dakota, Wisconsin, Colorado and Nebraska. Potato tubers were sampled at harvest and yields were evaluated and recorded in pounds and hundredweight per acre (cwt/ac). Soil samples were collected at two different time points: before planting (T0) and at growth stage 2, around 30 days after emergence (T1) of each variety. In addition, each variety had different time frame duration from time points T0 and T1, depending on their growth speed. Therefore, potato variety influence was mitigated. Detailed sample collection dates can be found in Supplementary Table S1. To encompass the variability of field, bulk soil core samples were collected from sub-plots to form a well-blended composite soil sample, using 1 inch diameter soil probe (Hartz, 2007). For each time point, samples were collected from both control and treated samples to isolate the effect of the treatment. For T0, a total of 132 samples were collected (66 control 66 treated), while for T1 a total of 130 samples (65 control 65 treated).

2.2 Weather measurements

Weather variables were downloaded from several public databases such as Bioclim (Karger et al., 2017), Long-term Moderate-Resolution Imaging Spectroradiometer (MODIS) - Land Surface Temperature (LST) day-time and night-time temperatures, sd and differences at 1 km based on the 2000–2017 time series (Hengl et al., 2017). Moreover, soil variables were downloaded from Copernicus Global Land services (https://land.copernicus.eu/global/index.html) and International Soil Reference and Information Centre (ISRIC) World Soil Information (Hengl et al., 2017). Then, a correlogram was constructed to check and reduce for collinearity between variables. Feature selection was performed based on Principal Component Analysis (PCA). Then, the number of dimensions that explained a percentage of variance between 90-95% and the variables that most contributed to each selected dimension were selected: Monthly median soil temperature during day time based on data from the MODIS sensor; average normalized difference vegetation index (NDVI) of the first third of April (m04) between 1999 and 2019; mean temperature of wettest quarter, temperature seasonality (standard deviation ×100); precipitation of the coldest quarter of the year, soil sand percent in the first 30 cm of soil, soil clay percent in the first 30 cm of soil and average NDVI of the first third of July (m07) between 1999 and 2019 were used. Full description of selected environmental features can be found in Supplementary Table S2.

2.3 Soil physicochemical, leaf petiole and potato peel quantification

A total of 120 observations were taken for soil physicochemical properties at time point T0, while a total of 112 observations were taken for petiole at time point T1, and 98 observations for yield at harvest. Mean and standard deviation of each soil property in each location can be found in Supplementary Table S3. Leaf petioles were collected from the last mature leaf of potato plants, all leaf tissues were removed. Potato peel consisted of 216 observations taken at harvest. Soil and leaf petiole chemistry were analyzed by Ward Laboratories Inc (Nebraska, United States) with common analytical methods (https://www.wardlab.com/services/plant-analysis/). Potato peel metabolomics were analyzed by Bayer AG. Briefly, potatoes were washed and peeled off for weighing. Then, potato peels were put into liquid nitrogen for metabolomics. Lastly, metabolomics quantification was done using mass spectrometry. Soil physicochemical properties in parts per million (ppm) included nitrate, potassium, sulfur, zinc, manganese, copper, calcium, phosphorus. Soil physicochemical properties in weight percentage: organic matter (LOI), sand, silt and clay. Petiole nutrients in weight percentage included nitrogen, phosphorus, potassium, magnesium and calcium, while nutrients in ppm included zinc, iron, copper and boron. Finally, potato peel nutrients in unit percent included total nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, sodium, while nutrients in ppm included zinc, iron, copper and boron.

2.4 DNA extraction and library preparation

After collection, soil samples were immediately sent for molecular analysis to the Biome Makers laboratory in

Sacramento, CA. DNA extraction was performed with the DNeasy PowerLyzer PowerSoil kit from Qiagen. To characterize both bacterial and fungal microbial communities associated with bulk soils, BeCrop® custom primers were used for PCR amplification, specifically targeting the 16S rRNA V4 region and the ITS1 region (Becares and Fernandez, 2017). Next, amplicons were purified using the KAPA Pure Beads (Roche) kit, while correct 16S and ITS amplification was assessed through agarose gel. Purified PCR products were then subjected to library preparation, following a two-step PCR Illumina protocol (Gobbi et al., 2019; Liao et al., 2019). Next, DNA was quantified using a Qubit fluorometer with Qubit HS Assay Kit 500 (Thermo Fisher Scientific). Finally, libraries were sequenced on an Illumina MiSeq instrument (Illumina, San Diego, CA, USA) using 2×251 paired-end reads.

2.5 Bioinformatic processing

Primers were removed from paired end reads using Cutadapt (Martin, 2011). Then, the trimmed reads were merged with a minimum overlapping of 100 nucleotides. Next, the sequences were quality filtered by Expected Error with a maximum value of 1.0 (Edgar et al., 2011). After quality pre-processing, reads having single nucleotide differences were iteratively clustered together to form ASVs (Amplicon Sequencing Variants) using Swarm (Mahé et al., 2022). *De novo* chimeras and remaining singletons were subsequently removed (Edgar et al., 2011). Finally, taxonomy was assigned from ASVs using a global alignment with 97% identity, against a curated reference database from SILVA 138.1 for 16S sequences, and UNITE 8.3 for ITS sequences (Glöckner et al., 2017; Nilsson et al., 2019).

2.6 Computation of local network properties

Local network properties were determined following the procedure described by (Ortiz-Álvarez et al., 2021). Briefly, microbial community networks were built for 16S and ITS samples independently following the methodology described by Veech (2013). Presence-absence metanetwork with all samples was built using rarefied counts, and the ASV pairs occurring significantly more or fewer than expected by chance were preserved to create the co-occurrence or co-exclusion network, respectively. Local network properties were retrieved from subsetting ASV pairs from the corresponding metanetwork present in the individual sample. The following network properties were computed for both co-occurrence and co-exclusion in 16S and ITS: modularity, transitivity and average path length. Modularity defines microorganisms that tend to co-occur or co-exclude frequently in specific ecological niches (clusters). Next, transitivity (clustering coefficient) measures the degree to which nodes in a network tend to cluster together. Finally, average path length quantifies the degree of connectivity to go from one side of the network to another.

2.7 Computation of global networks integrating yield and leaf physiochemical properties

The ASV data were aggregated to genus-level and rarefied to 10.000 reads for 16S, 18.750 for ITS. Five samples had insufficient reads and were removed. Here, both treated and untreated samples available were used. Then, treatment was introduced as a metadata variable to assess associations to MINUET™ biological fungicide or control treatment. A prevalence filter of 20% was then applied to the genera. Throughout all steps, the total reads per sample were preserved, with the final data set containing 63 genera across 39 samples for the 16S data and 197 genera across 44 samples for the ITS data. Next, a permutation test was performed to identify taxa with higher or lower degree than expected given their prevalence in the network. Yield, petiole and environmental data were centered and scaled and used in network inference, while location and treatment were used as one-hot encoded variables. Nodes that represent these metadata variables were referred to as MVs. FlashWeave v0.18.1 was run to assess direct associations in Julia version 1.7.1, with heterogeneous set to false and sensitive to true (Tackmann et al., 2019).

2.8 Statistical analysis

Statistical analyses of microbiome data were done mainly using phyloseq, microbiome and vegan R packages (McMurdie and Holmes, 2013; Oksanen, 2013; Shetty and Lahti, 2019). First, the generation of rarefaction curves allowed estimating sample intradiversity in terms of bacterial and fungal richness and Shannon index at the same sequencing depth. Then, pairwise comparison of treated vs control samples for microbiome indexes (biodiversity and network properties), yield, petiole and potato quality were performed through Wilcox-test using Z-score transformation, standardized by location. Moreover, correlations between microbiome indexes (network properties and biodiversity), yield, soil physico-chemical, petiole nutrients, potato peel nutrients and environmental variables at time T0 and T1 were performed through Spearman correlation. Resulting p-values were further corrected by False Discovery Rate (FDR). Then, significant correlations were visualized as a network graph. Here, Louvain clustering was performed to identify clusters of positive associations among variables. Microbiome composition was assessed through beta diversity analysis, using Principal Coordinate Analysis (PCoA) ordination and Bray-Curtis distance matrix. Then, explained variance of resulting ordination by treatment, time and location and their interaction was determined through PERMANOVA. In addition, soil, petiole, potato quality physico-chemical properties and environmental properties were correlated to bacterial and fungal ordination, through the envfit. In addition, constrained distance-based Redundancy Analyses (RDAs) were generated using log-transformed data of microbial counts. Next, microbiome data was centered based on the location and fitted to

a linear model containing both time and treatment, along with potato peel, petiole and soil physiochemical properties and weather variables. Significance of independent variables was determined through ANOVA. Only significant variables were visualized in the ordinations. Then, yield, petiole and environmental data were centered and scaled and used in the global network inference, while location and treatment were used as one-hot encoded variables. Networks were visualized in Cytoscape 3.9.0. Prevalence of conserved soil prokaryotic and fungal genera was assessed in control and treated samples at T0 and treated samples at T1. This was visualized as heatmaps at varying detection thresholds. Then, shared taxa at ASV and genus level among control and treated samples at time T0 and time T1 was visualized through Venn diagrams with constrained intersections by location. Finally, differential abundant (DA) taxa due to product application were determined through negative binomial regression at various taxonomic levels (Love et al., 2014).

3 Results

3.1 Application of the *B. amyloliquefaciens* based product increased yield, leaf petiole boron, potato peel manganese, phosphorus and zinc content.

Yield significantly increased in treated sample, when standardizing by location (Wilcoxon-test: p-value = 0.003, Figure 1A). For individual locations, a similar trend was observed, with a percentage of increment between 24 and 2% in almost all locations (Supplementary Figure S2). However, no statistical significant differences in treated vs control were seen per location. Conversely, yield showed significant differences across locations (Kruskal-Wallis: p-value < 0.001); with, Loc12 and Loc 15 resulting in the highest and lowest yield, respectively (Supplementary Figure S3).

Leaf petiole boron was also significantly enhanced in treated samples, when standardised by location (increment across locations: 14 - 2%, Wilcoxon-test: p-value = 0.01, (Figure 1A). Similarly, *B. amyloliquefaciens* significantly enhanced potato peel manganese, phosphorus and zinc content (increment across locations: 73 - 2%, 36 - 2%, 62 - 2%, respectively, Wilcoxon-test: p-value = 0.007, p-value = 0.002, p-value = 0.001, Figure 1B).

Bacterial and fungal richness and evenness significantly differed across locations independently of time point, as shown by Chao1 and Shannon indexes, respectively (Supplementary Figure S4). Metadata together with computed alpha diversity per sample are found in Supplementary Table S1. No significant differences were detected for either bacterial or fungal biodiversity indexes when comparing net changes from T0 to T1 between control and after *B. amyloliquefaciens* based product application (Figure 1C). Finally, no significant changes were observed in network properties due to treatment application (data not shown).

3.2 Yield associated with microbiome network properties

The global correlation network across soil, leaf petiole and peel physico-chemical properties, microbiome indexes and yield separated in four main clusters containing variables that significantly correlated (p-value threshold < 0.05) (Supplementary Table S4). Here, both treated and untreated samples were used to provide a global overview of the relations between microbiome,

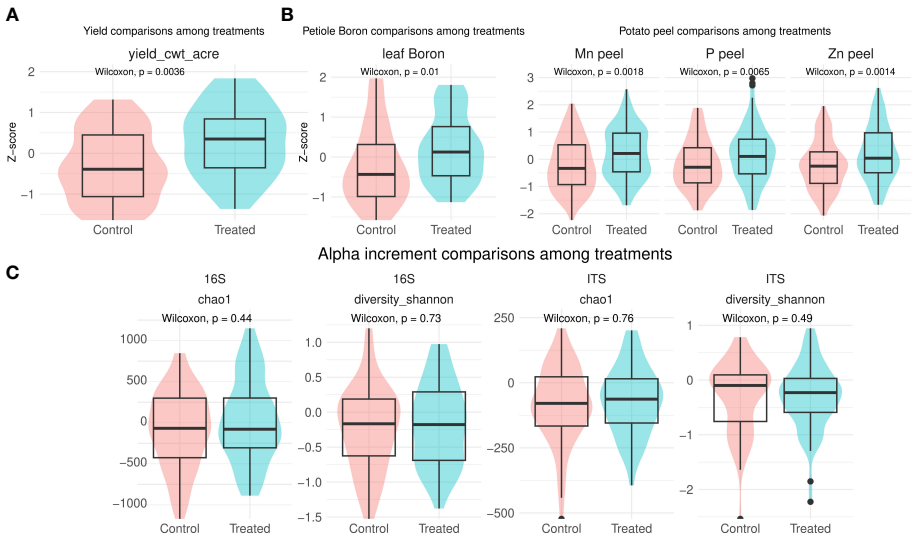

FIGURE 1
Yield **(A)**, leaf boron and and potato peel **(B)** comparisons between treatment and control normalized per location. Only significant comparisons are shown. Net change in biodiversity from T0 to T1 comparison between treatment and control **(C)**. Complete comparisons of leaf petiole and potato peel nutrients can be found in Supplementary Figure S1.

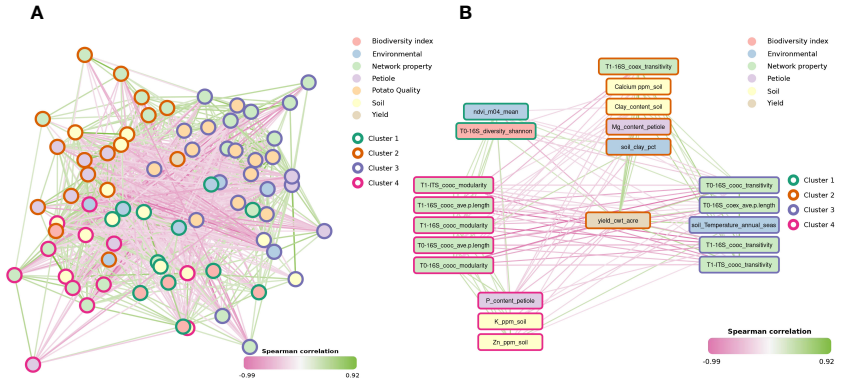

FIGURE 2
Spearman correlation network of microbiome indexes, environmental and physicochemical data from soil, leaf petiole and potato peel **(A)**. Only significant correlations are shown (p-value < 0.05). Each edge represents a Spearman correlation between two variables, while nodes represent the different variables. Nodes are coloured by variable type and borders represent the clusters assigned using Louvain clustering on the positive-edge only network. Subgraph showing only the direct neighborhood of yield **(B)**.

yield and physico-chemical properties. Cluster 1 mainly comprised biodiversity related variables. Cluster 2 comprised yield, specific network and leaf petiole properties, such as T0 16S co-exclusion transitivity, T0 ITS co-exclusion average path length, T0 ITS co-exclusion modularity, T0 ITS co-exclusion transitivity, calcium, nitrogen, sulfur content, and iron (Figure 2A; Supplementary Table S4). Cluster 3 included mainly potato peel and specific network properties, such as T0 16S co-exclusion modularity, T0 16S co-occurrence transitivity and T0 16S co-exclusion average path length, T1 16S co-exclusion modularity, and T1 16S co-exclusion transitivity (Supplementary Table S4). Finally, cluster 4 included network properties and soil properties such as T0 16S co-occurrence average path length, T1 16S co-occurrence average path length, T1 ITS co-occurrence modularity, sulfur, zinc, boron (Supplementary Table S4).

Notably, yield positively correlated with soil calcium, soil clay, soil clay percent from selected environmental features (see Supplementary Table S2) and leaf petiole magnesium content (pairwise Spearman correlation: R = 0.44, p-value < 0.001, R = 0.51, p-value < 0.001, R = 0.28, p-value = 0.034, R = 0.41, p-value = 0.001, respectively). Similarly, yield positively correlated with T0 16S diversity Shannon and T0 16S co-occurrence transitivity (pairwise Spearman correlation: R = 0.27, p-value = 0.046; R = 0.41, p-value = 0.01). Moreover, both bacterial and fungal co-occurrence transitivity positively correlated with yield, both in T1 (pairwise Spearman correlation: R = 0.42, p-value = 0.001, R = 0.44, p-value < 0.001) (Figure 2B). On the other hand, yield negatively correlated with bacterial co-occurrence modularity in T0 (pairwise Spearman correlation: R = -0.54, p-value < 0.001). Similarly, yield negatively correlated with both bacterial and fungal co-occurrence modularity in T1 (pairwise Spearman correlation: R = -0.48, p-value <0.001, R = -0.40, p-value = 0.002, respectively). Likewise, yield negatively correlated with bacterial co-occurrence average path length in T0 (pairwise Spearman correlation: R = -0.43, p-value < 0.001), petiole phosphorus content (pairwise Spearman correlation: R = -0.45, p-value < 0.001), NDVI of the first third of April (m04), soil potassium and zinc content (pairwise Spearman correlation: R = -0.53, p-value < 0.001, R = -0.39, p-value = 0.003, R = -0.35, p-value = 0.008).

Yield and potato quality were not correlated with each other (Figure 2B). Potato peel manganese positively correlated with leaf petiole manganese (pairwise Spearman correlation: R = 0.31, p-value = 0.007). Similarly, potato peel manganese positively correlated with precipitation of the wettest quarter (pairwise Spearman correlation: R = 0.28, p-value = 0.011) and NDVI m07 which is a proxy of yield (pairwise Spearman correlation: R = 0.27, p-value = 0.009) (Supplementary Figure S5). In addition, potato peel manganese showed associations with fungal direct neighbor nodes (Supplementary Figure S5). More specifically, peel manganese positively correlated with both T0 and bacterial Shannon diversity (pairwise Spearman correlation: R = 0.23, p-value = 0.04) and T1 bacterial Shannon diversity and Chao1 (pairwise Spearman correlation: R = 0.25, p-value = 0.03, R = 0.46, p-value < 0.001). On the other hand, peel manganese concentration in potato peel negatively correlated with T0 fungal Shannon diversity.

Moreover, positive correlation through pairwise Spearman correlations (p-value threshold < 0.05) were found between potato peel zinc and abiotic and biotic soil characteristic. For instance, it positively correlated with soil sand content, soil temperature of the wettest quarter, T1 ITS co-occurrence transitivity. Moreover, it positively correlated with main potato peel properties such as calcium, iron, copper and manganese (Supplementary Figure S6). Finally, by exploring individual correlations between microbiome indexes and metadata, some correlations were detected to possibly be location driven (Supplementary Figure S7). For instance, the correlation between bacterial and fungal Shannon diversity and Chao1 with soil phosphorus, organic matter and leaf petiole zinc seemed to be driven by Loc16. Similarly, the correlation between bacterial and fungal Shannon diversity and Chao1 and soil calcium seemed to be driven by Loc1 and Loc20 (Supplementary Figure S7A). The correlation with phosphorus content was driven by Loc9 and

Loc11 (Supplementary Figure S7B), this tendency was not observed for potato quality properties (Supplementary Figure S7C).

3.3 Application of the *B. amyloliquefaciens* based product significantly impacts fungal microbiome composition

Beta diversity analysis showed that location played a major role in determining soil microbiome composition (Permanova: 16S $F_{[20]}$ = 21.76, R = 45.46%; ITS $F_{[20]}$ = 13.59, R = 45.15%, p-value <0.001) followed by its interaction with treatment (16S $F_{[1,20]}$ = 1.63, R = 3.71%; ITS $F_{[1,20]}$ = 1.39, R = 4.61%, p-value <0.001 (location: treatment) (Figure 3A; Table 1). Therefore, the interaction location: treatment:time significantly explained microbiome composition variation depending on the location, especially for fungal community ($F_{[1,20]}$ = 1.52, R = 3.47%, p-value <0.001 Bacteria, $F_{[1,20]}$ = 1.39, R = 4.61%, p-value <0.001 fungi (Figure 3A; Table 1). Notably, silt content and soil manganese correlated with bacterial microbiome ordination (R = 45.55%, p-value = 0.01; R = 39.93%, p-value=0.01), while soil zinc and sand contents correlated with fungal ordination (R = 38.27%, p-value = 0.01; R = 33.38%, p-value = 0.01) (Supplementary Table S5). Moreover, soil temperature of the wettest quarter correlated with both bacterial and fungal microbiome ordination (R = 51.14%, p-value = 0.01; R = 40.37%, p-value = 0.01), while precipitation only with bacterial microbiome ordination (R = 40.23%, p-value = 0.01) (Supplementary Table S5). Correlation

TABLE 1 PERMANOVA analysis on microbiome ordination for time, treatment and location factors.

Marker	Term	Variance explained	p-value
16S	Location	49.45%	0.0001
	Time	2.48%	0.0001
	Location : Treatment	3.71%	0.0001
	Location : Time	20.38%	0.0001
	Location : Treatment:Time	3.47%	0.0002
ITS	Location	45.15%	0.0001
	Time	1.60%	0.0001
	Location : Treatment	4.61%	0.0001
	Location : Time	14.11%	0.0001
	Location : Treatment:Time	4.61%	0.0001

Significant terms (p-value < 0.05) and their variance explained by marker.

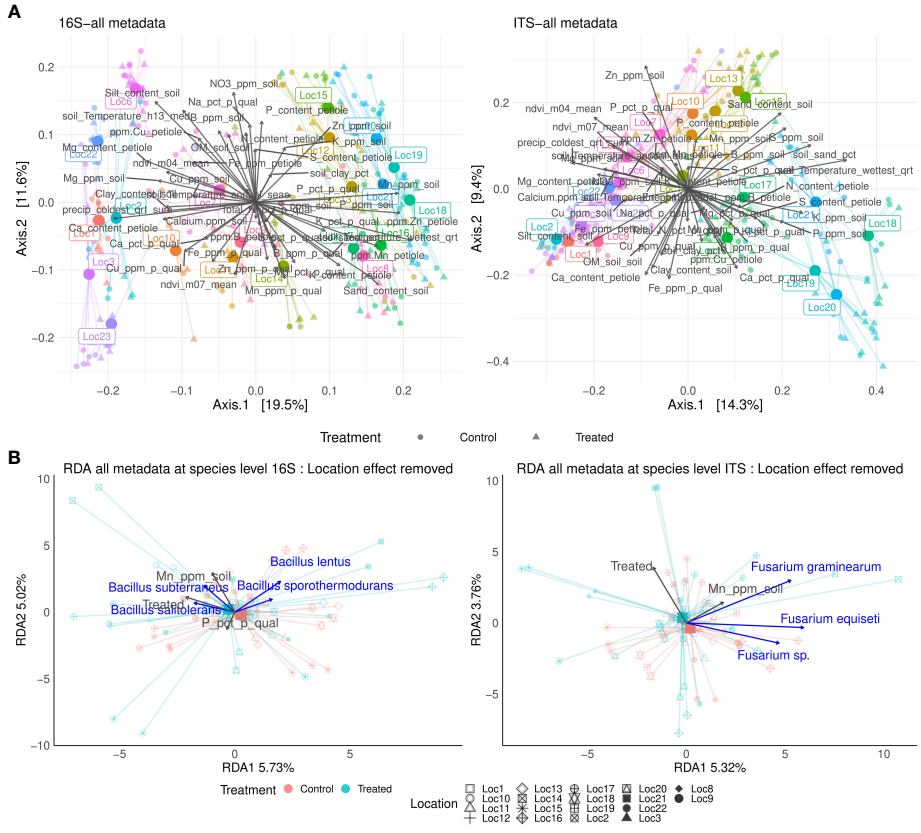

FIGURE 3
Principal coordinate analysis (PCoA) of the microbial community based on Bray-Curtis distances for 16S and ITS markers at global scale. Correlation with soil, leaf petiole, potato peel nutrients to microbiome ordination are shown for each marker **(A)**. Constrained ordination for 16S and ITS community composition fitting treatment, soil, leaf petiole, potato peel and environmental features. Location effect was removed. Only significant terms (p-value < 0.05) are reported **(B)**.

of all soil, leaf petiole nutrients, potato quality and environmental variables can be found in Supplementary Table S6.

Next, constrained microbiome ordination by location using the above-mentioned variables was performed to remove the location effect. Application of the *B. amyloliquefaciens* explained the significant changes in the fungal composition, (Table 2, Figure 3B), but not in bacterial composition. In fact, potential plant associated pathogens, such as *Fusarium* species (e.g. *Fusarium* sp., *F. graminearum* and *F. equiseti*) tended to have positive association with control samples. (Figure 3B). Regarding nutrients, phosphorus content in potato peel significantly modulated bacterial composition variation. Conversely, soil manganese was the only meaningful physico-chemical factor to explain fungal composition variation (Table 2).

3.4 Yield directly associated with specific microbial taxa

Yield was highly and positively associated with several bacterial taxa, such as *Pseudoarthrobacter* (FlashWeave association weight: 0.34) and, *Ammoniphilus* (+) (FlashWeave association weight: 0.34), but negatively with *Ideonella, Candidatus Berkiella, Dongia* (-) (FlashWeave association weight: -0.38 -0.30, -0.36, respectively) (Figures 4A–C). Moreover, yield associated with fungal taxa such as *Psathyrella and Naganishia* (FlashWeave association weight: 0.36, -0.35, respectively) (Figure 4D). *B. amyloliquefaciens* based product had no significant associations at taxa level, but slighter effects might be blurred if those effects can be explained by other nodes in the network (Figure 4A). On the other hand, NDVI of the month of April (m04) was negatively associated with yield (FlashWeave association weight: -0.35), as shown by its central position in the network (Figure 4C).

In the bacterial network, the phyla Proteobacteria and Actinobacteriota were responsible for most associations; the total degree (connectance of each taxa) for these groups was 467 and 293 respectively, compared to 148 for Firmicutes, the third most-connected phylum (Figure 4A). Additionally, the degree and betweenness centrality was strongly correlated for both 16S and ITS networks ($p < 0.001$, and respectively). However, the total degree for both Proteobacteria and Actinobacteria was lower than expected. On the other hand, Acidobacteriota, Firmicutes and Planctomycetota had a total degree at least 10 greater than the expected total degree.

Finally, for the fungal global network, Ascomycota played a larger role in the fungi community structure, followed by Basidiomycota (green nodes) (Figures 4B, C, green nodes). Moreover, soil temperature, precipitation and sand percentage occupied central positions, but NDVI was relatively more connected in the ITS network (Figures 4B, C). Ascomycota had a higher total degree than expected, while Basidiomycota had a lower total degree than expected. Here, the classes Sordariomycetes, Tremellomycetes and Eurotiomycetes had a higher total degree relative to the expected degree (Figure 4B).

TABLE 2 RDA analysis significant model terms (p-value< 0.05) and their explained variance.

Marker	Term	Variance explained	p-value
16S	P pct	2.07%	0.046
ITS	Treatment	2.05%	0.01
	Mn ppm	2.21%	0.007

3.5 *B. amyloliquefaciens* based product impacts preserved and accessory microbiome fraction

In order to identify core microbiome members, only taxa found in all locations with a detection threshold of 0.01 and a prevalence of 25% were considered for the analysis. The preserved microbial fraction tended to be 25% higher, when comparing the preserved and accessory microbiome fraction of bacterial and fungal communities in T0 and in untreated T1, vs samples treated with *B. amyloliquefaciens* in T1. A more conserved fraction of bacterial genera was detected compared to fungal genera (Figure 5A). *Candidatus*, *Nitrosocosmicus* and *Sphingomonas* core members were higher in samples treated with the *B. amyloliquefaciens* based product, in comparison to the control at T1 (Figure 5A). No major differences were detected in the bacterial core microbiome between control and treated samples. Regarding fungal microbiome, *Mortierella* was the only fungal genus with a global prevalence higher than 80% (Figure 5A). Notably, *Fusarium* and *Trichoderma* were present in the control core microbiome at higher prevalence (Figure 5A). In order to determine core microbiome size, genera found in all locations were considered. Core size number of taxa decreased in all samples at T1 when compared to their T0, for both communities (Figure 5B). Moreover, the shared number of genera between control and treated samples was lower in T1 compared to T0 (Figure 5B). None of the bacterial taxa were differentially abundant (Wald test: pval > 0.05) due to application of the *B. amyloliquefaciens* based product. However, application of *B. amyloliquefaciens* based product impacted the abundance of several fungal taxa. For instance, *Mortierella* increased in relative abundance after treatment with the *B. amyloliquefaciens* based product, while *Stemphylium* and *F. proliferatum* increased after treatment with *B. amyloliquefaciens* (Figure 5C; Supplementary Figure S9).

4 Discussion

In this work, the effects of *B. amyloliquefaciens* based product on soil microbiome were further explored across 21 locations from the United States integrating yield, soil, leaf petiole nutrients, potato peel quality and environmental data.

Yield and leaf petiole boron showed a significant increase after treatment with *B. amyloliquefaciens* based product. Our results on yield are in agreement with the previous study applying *B. amyloliquefaciens* QST713 in the United States (Imam et al., 2021). Imam et al. (2021) observed a significant yield increase

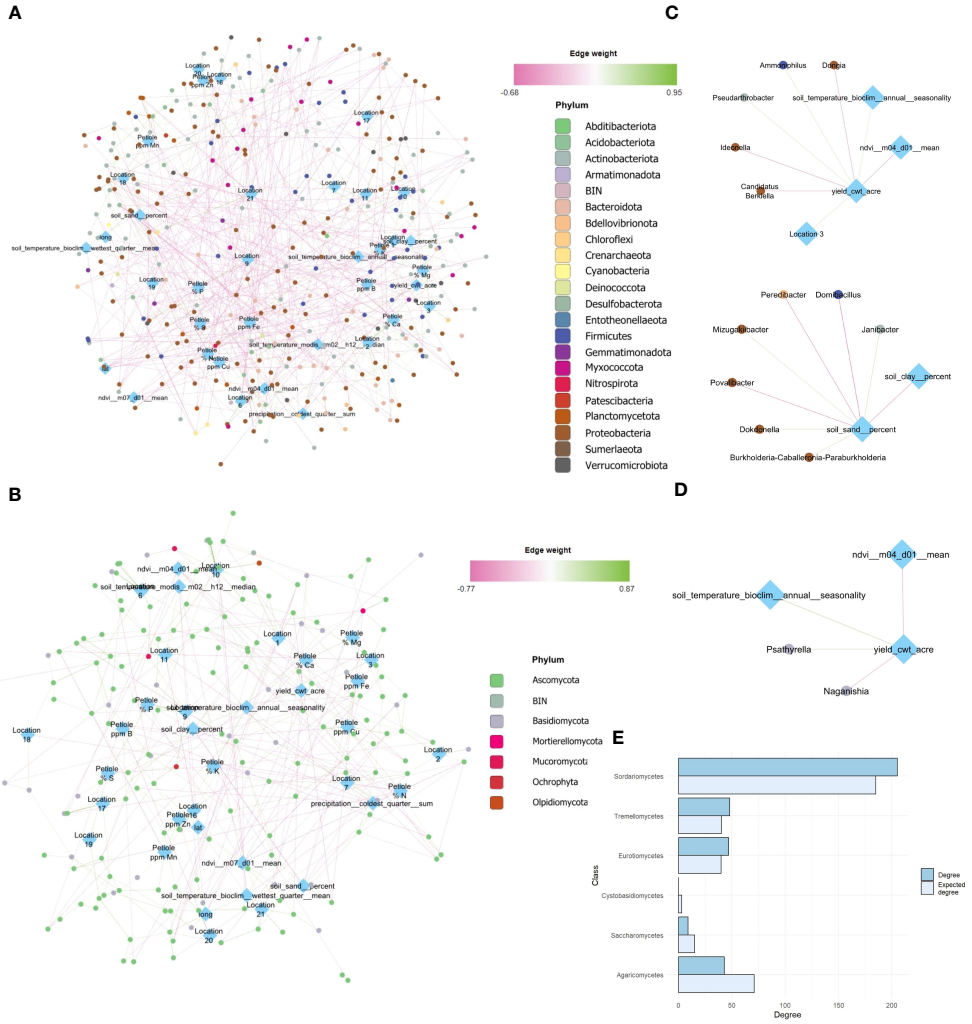

FIGURE 4
Global network analysis at genus taxa level after MINUET^{TM} application, introducing metadata variables (yield, climate and petiole, peel physicochemical properties). Metadata variables are annotated in blue rhomboid nodes, while taxa is annotated in circular nodes colored by the Phylum they belong to. Connections among nodes are represented by positive or negative edges, ranging from green to pink, respectively. Global network for bacterial community **(A)**. Global network for fungal community **(B)**. Direct neighbors of relevant metadata variables, such as yield for bacterial **(C)** and fungal network **(D)**. Difference between observed and expected degree for the six fungal classes with the largest positive or negative difference in degree in the ITS network. Classes with a higher degree than expected are shown above, and those with a lower degree than expected are below **(E)**.

after inoculation of *B. amyloliquefaciens* QST713 in two geographical locations, now expanded to more USA locations. In addition, previous studies already assessed the importance of boron for plant growth (Tombuloglu et al., 2017; Pereira et al., 2021). Boron is involved in cell elongation, nucleic acid synthesis, hormone responses and membrane function (Pereira et al., 2021) and its plant absorption capacity increases with higher soil clay content (Padbhushan and Kumar, 2017). Our results showed a significant positive association with soil clay content. This is well known to be an important factor for potato productivity, since its fine texture prevents nutrient leaching and enhances water availability (Zebarth et al., 2021). Therefore, the enhancement of yield and boron after product inoculation in these soil conditions highlights the indirect role that *B. amyloliquefaciens* may have in nutrient mobilization and yield improvement (Rana et al., 2012).

Moreover, potato peel manganese, phosphorus and zinc content significantly increased after *B. amyloliquefaciens* inoculation. Our results are in line with previous findings, where increased concentrations of the above nutrients improved after rhizobacteria inoculation (Ipek et al., 2014). The increased concentration of peel phosphorus after inoculation may be due to the bacterial solubilization of P, increasing its availability in the soil for the plant (Rouphael and Colla, 2020).

Focusing on different geographic regions, yield, bacterial and fungal richness and evenness significantly changed across locations independently of time. These results confirm that geographical location is one of the main drivers of potato yields and soil microbiome, due to edaphic and climate variation of the different regions (Rasche et al., 2006; Weinert et al., 2010). In addition, bacterial and fungal communities were mainly explained by

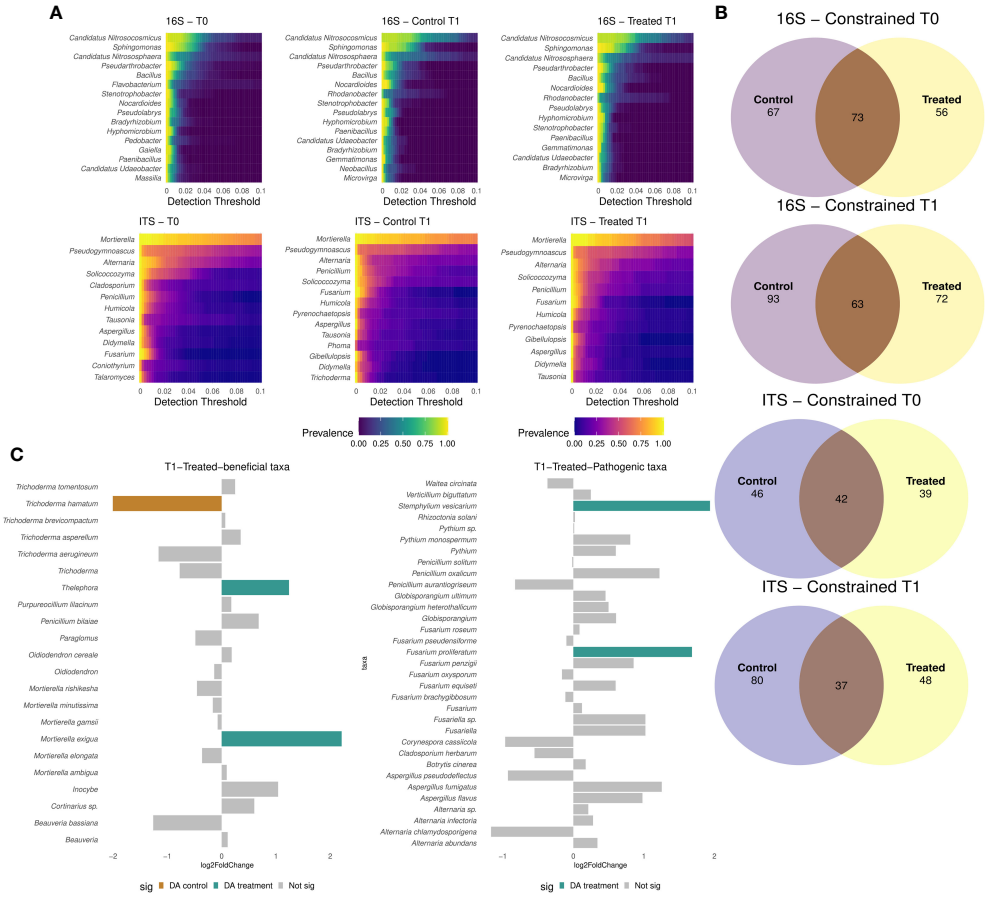

FIGURE 5
Heatmaps showing the genus prevalence proportion across different detection thresholds for 16S and ITS markers **(A)** in combined treated and control samples at T0 and control and treated samples in T1 **(A)**. Constrained shared and exclusive members of bacterial and fungal microbiomes at Genus level, for 16S and ITS, respectively **(B)**. Barplot showing potential beneficial differential abundant taxa **(C)** and potential pathogenic taxa **(C)** in treated samples at T1 when compared to the control reporting their log fold change.

location, followed by its interaction with treatment. The impact of geographical location in the bacterial communities was slightly higher than for the fungal communities. This was also evidenced when correlating geographical distances with beta diversity distance matrix (Supplementary Figure S8). Moreover, our results showed that treatment with *B. amyloliquefaciens* based product biological fungicide impacted soil microbiome communities depending on the location, especially for the fungal community (see Table 1). This indicates that the treatment may impact soil microbiome differently due to the climatic and edaphic conditions, which might potentially further explain the different nutrient mobilization effect in different geographical locations. Previous studies already assessed biogeographical patterns as the main drivers of soil microbiome (Hanson et al., 2012; Bahram et al., 2013; Tedersoo et al., 2014; Plassart et al., 2019).

The biological product had no significant impact on either bacterial or fungal alpha-diversity. Similarly, Imam et al. (2021) detected no changes in either bacterial or fungal alpha diversity in any location, even if they observed shifts in soil microbial structure (Imam et al., 2021). However, the application of a plant growth promoting bacteria did not affect the soil bacterial community structure in maize (Kari et al., 2021). These observations were explained by possible resilience of the ecosystem, driven by the interactions between plant and soil microbes. Nevertheless, yield had a significant positive associations with both bacterial and fungal co-occurrence transitivity, while negative associations with modularity. This indicates that communities that are more interconnected (increased transitivity) may lead to higher yields. On the other hand, a highly modular microbiome (increased modularity), where taxa form specific niches, may lead to lower yields. Therefore, both bacterial and fungal local network properties may be good drivers of yields due to their strong correlation profile, as similarly shown in previous studies (Imam et al., 2021). In conclusion, preserving microbial community structure where communities are more interconnected and cooperative, may be an important target to achieve higher crop performance.

Notably, yield was positively correlated with soil calcium. These results are in agreement with previous studies that assessed the important role of calcium for potato productivity (Gondwe et al., 2019; Koch et al., 2020). Calcium is a fundamental micronutrient that enhances plant growth and is a signaling molecule mediating plant response to environmental stresses and hormones (Thor, 2019). However, the negative correlation with the NDVI during the month of April indicates that lower NDVI after planting season

may not be predictive of yields. In fact, higher yields at the harvesting season were associated with lower NDVI in the month of April.

Moreover, treatment with *B. amyloliquefaciens* based product had a significant effect on fungal community composition at T1 but not on bacterial community. These results are in agreement with previous studies, where the inoculation of *B. amyloliquefaciens* BNM122 in soybean did not significantly change the bacterial community of the rhizosphere (Correa et al., 2009). Similarly, in potato, the inoculation of *B. subtilis* did not significantly affect the bacterial or fungal community, while improving potato yield (Song et al., 2021). Conversely, the inoculation of *Stenotrophomonas rhizophila* indirectly promoted plant growth by shaping the soil fungal community in tomato and sweet peppers (Schmidt et al., 2012). In addition, Fusarium species such as *Fusarium* sp. and *F. equiseti* tended to be associated with control samples. Therefore, potential plant pathogens may have reduced presence when *B. amyloliquefaciens* is applied.

The efficacy of *B. subtilis* against *Fusarium* was already observed in potato cultivars (Gachango et al., 2012; Stefańczyk et al., 2016; Tiwari et al., 2020). In particular, the inoculation of *B. subtillis* in potato crops effectively reduced *Fusarium* spp. abundance, including *F. graminareum* (Khedher et al., 2021). Similarly, a previous study in apple showed that *B. amyloliquefaciens* QSB-6 inhibited several *Fusarium* species, while significantly improving seedling growth (Duan et al., 2021). Nevertheless, further studies for different potato genotypes are needed to assess the pathogenicity of *Fusarium* spp. and possible biocontrol action of *B. amyloliquefaciens* against it. Conversely, *B. amyloliquefaciens* based product promoted the potential pathogen *F. proliferatum* (Gachango et al., 2012). Therefore, treatment with the *B. amyloliquefaciens* based product may have a greater impact on specific *Fusarium* taxa. Notably, soil sand content significantly explained fungal community composition variation.

At taxa level, the *B. amyloliquefaciens* based biological fungicide had no direct association with bacteria and fungi network when taxa were included in the global network integrating yield and petiole nutrients. However, weaker or indirect treatment effects on community structure may not be detected if those effects can be explained by other nodes in the network. Nevertheless, yield had strong associations with several bacterial taxa, such as positive associations with *Pseudoarthrobacter* (FlashWeave association weight: 0.34) and *Ammoniphilus* (+) (FlashWeave association weight: 0.34), and negative with *Ideonella, Candidatus Berkiella, Dongia* (-) (FlashWeave association weight: -0.38 -0.30, -0.36, respectively). A recent study assessed the effects of the inoculation of chlorophenolicus in *Geum aleppicum*, which showed a significant increase in root development and plant growth (Ham et al., 2022). Therefore, *Pseudarthrobacter* may promote potato yield, through the stimulation of root development. However, further studies should be carried out on rhizosphere targeting potato crop and *Pseudarthrobacter*. In addition, several uncultured taxa are involved in nitrogen fixation, which could promote N availability to the plant (Mus et al., 2016). Regarding relevant taxa, Proteobacteria and Actinobacteriota were responsible for most associations. Notably, these taxa have a fundamental role in Nitrogen cycling (Kielak et al., 2016; Mosley et al., 2022). and may promote nitrogen availability. In addition, Firmicutes and Planctomycetota had a total degree of prevalence in the network at least 10 units higher than the expected total degree. This indicates that Firmicutes and Planctomycetota occupy more central positions in the network, highlighting their importance on community structure. *Bacillus* a is well-known beneficial taxon for soil health (Saxena et al., 2020). Hence, favoring *B. amyloliquefaciens* presence through inoculation may lead to better connected networks due to Firmicutes high degree and central role. For the ITS network, Ascomycota had a higher total degree than expected compared to Basidiomycota, suggesting this phylum played a larger role in the community structure. In particular, the class Sordariomycetes includes many potential saprotrophic taxa fundamental for crop litter decomposition (Wang et al., 2021). Moreover, species belonging to the Sordariomycetes may be able to grow in fecal material and they can be an indicator of differences in fertilization application between locations (Guo et al., 2022).

Lastly, *B. amyloliquefaciens* biological fungicide slightly modulated the soil core microbiome from T0 to T1. In particular, a higher conserved fraction of *Sphingomonas sp* was observed. A recent study on maize showed that *Sphingomonas* sp. Hbc-6 increased microbiome rhizosphere diversity and could help plant growth promotion by recruiting beneficial bacteria in inoculated soils (Wang et al., 2022). Conversely, genus *Fusarium* in treated condition showed a lower detection threshold in T1, when compared to control at T1. In addition, no changes in the detection threshold were seen for the genus Mortierella between conditions. *Mortierella* is a widely spread genus which is known to be beneficial in soils (Ozimek and Hanaka, 2020). Therefore, treatment with *B. amyloliquefaciens* may modulate the presence of potential pathogens such as *Fusarium*, while indirectly promoting beneficial taxa through indirect effect (Cao et al., 2011; Khedher et al., 2021). Moreover, species like *Stemphylium* and *F. proliferatum* were differentially abundant after treatment with *B. amyloliquefaciens*. Therefore, these species may be more resistant to the product and a better competitor than other taxa. Hence, these taxa may increase after treatment application while other taxa decrease. However, further analyses should be performed to decipher the effects that treatment with *B. amyloliquefaciens* can have on different *Fusarium* species.

Finally, our results showed that soil inoculation with a sustainable *Bacillus*-based product correlated with higher yields. In addition, nutrient solubilization and soil health were promoted, without major disruption of the soil microbiome. Indeed, these findings contribute to disentangle sustainable long term solutions in view of the future global climate change, increasing global food demand.

5 Conclusions

Our results showed that treatment with *B. amyloliquefaciens* based product correlated with enhanced yield. In addition, the

treatment was associated with leaf petiole boron and improved nutrient uptake in potato peel. Therefore, *B. amyloliquefaciens* based product may indirectly promote nutrient solubilization with minor impacts on the soil microbiome diversity 30 days after inoculation. Moreover, yield was strongly correlated to specific local network properties, which are associated with cohesive and cooperative microbial community. This highlights the importance of the native soil microbiome with a complex and interconnected structure, indirectly promoting healthier soils. In addition, treatment with *B. amyloliquefaciens* had an impact on fungal community composition, reducing *Fusarium* presence without major impacts on the fungal community structure. Moreover, the *B. amyloliquefaciens* based product modulated the soil core microbiome after 30 days by modulating the presence of *Fusarium*, while indirectly promoting beneficial taxa like *Sphingomonas* and *Mortierella*. However, further studies should focus on the long term effects of *B. amyloliquefaciens* based product on soil bacterial and fungal communities. Lastly, our results showed that the use of a sustainable biostimulant product correlated with higher yield and indirectly promoted soil health. These evidences are crucial to further enhance agriculture sustainability, increasing food production while reducing's environmental footprint.

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/, PRJNA981118.

Author contributions

IA: Data curation, Formal analysis, Investigation, Resources, Software, Visualization, Writing – original draft. MA-A: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. SR: Data curation, Investigation, Software, Validation, Writing – review & editing. DP: Data curation, Investigation, Software, Validation, Writing – review & editing. BB: Data curation, Writing – review & editing. JZ: Data curation, Writing – review & editing. RG: Conceptualization, Methodology, Writing – review & editing. BG-J: Conceptualization, Data curation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. PJ-R: Conceptualization, Data curation, Methodology, Resources, Supervision, Writing – review & editing. LC: Conceptualization, Data curation, Methodology, Project administration, Resources, Writing – review & editing. AA: Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The authors received funding from Bayer U.S. for this project.

Acknowledgments

We thank all the members from the BiomeMakers team involved in this project, mainly to the Data Science and IT departments people.

Conflict of interest

The biostimulant used in this article is commercialized by Bayer under the name MINUET™.

AA is a cofounder and IA, MA-A, SR, DP, BMB, JZ, and BG-J are current employees of Biome Makers, Inc. PJ-R, LCC, and RG are current employees of the Bayer Germany and U.S.

The authors declare that this study received funding from Bayer U.S. The funder had the following involvement in the study: study design, sample collection, writing of the manuscript, and the decision to submit it for publication.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1332840/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 10 May 2024
DOI 10.3389/fpls.2024.1396754

OPEN ACCESS

EDITED BY
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RECEIVED 06 March 2024
ACCEPTED 22 April 2024
PUBLISHED 10 May 2024

CITATION
Ma Y, Zheng C, Bo Y, Song C and Zhu F (2024) Improving crop salt tolerance through soil legacy effects.
Front. Plant Sci. 15:1396754.
doi: 10.3389/fpls.2024.1396754

Improving crop salt tolerance through soil legacy effects

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Soil salinization poses a critical problem, adversely affecting plant development and sustainable agriculture. Plants can produce soil legacy effects through interactions with the soil environments. Salt tolerance of plants in saline soils is not only determined by their own stress tolerance but is also closely related to soil legacy effects. Creating positive soil legacy effects for crops, thereby alleviating crop salt stress, presents a new perspective for improving soil conditions and increasing productivity in saline farmlands. Firstly, the formation and role of soil legacy effects in natural ecosystems are summarized. Then, the processes by which plants and soil microbial assistance respond to salt stress are outlined, as well as the potential soil legacy effects they may produce. Using this as a foundation, proposed the application of salt tolerance mechanisms related to soil legacy effects in natural ecosystems to saline farmlands production. One aspect involves leveraging the soil legacy effects created by plants to cope with salt stress, including the direct use of halophytes and salt-tolerant crops and the design of cropping patterns with the specific crop functional groups. Another aspect focuses on the utilization of soil legacy effects created synergistically by soil microorganisms. This includes the inoculation of specific strains, functional microbiota, entire soil which legacy with beneficial microorganisms and tolerant substances, as well as the application of novel technologies such as direct use of rhizosphere secretions or microbial transmission mechanisms. These approaches capitalize on the characteristics of beneficial microorganisms to help crops against salinity. Consequently, we concluded that by the screening suitable salt-tolerant crops, the development rational cropping patterns, and the inoculation of safe functional soils, positive soil legacy effects could be created to enhance crop salt tolerance. It could also improve the practical significance of soil legacy effects in the application of saline farmlands.

KEYWORDS

saline soil, halophytes, salt-tolerant crops, beneficial microorganism, salt tolerance

1 Introduction

In recent years, land degradation caused by climate change has posed a huge challenge to agricultural production. In the absence of major technological breakthroughs in agriculture, existing arable land resources are hardly sufficient to support global food security (German et al., 2017; Hartmann and Six, 2023). Saline farmland is an important reserve resource of arable land with great potential for ensuring food security and sustainable agricultural development (Negacz et al., 2022). Therefore, finding solutions to increase the productivity of saline farmland and improve crop tolerance to saline stress has become an important research topic currently (Munns and Tester, 2008).

Soil salinization is a global environmental problem, with more than 833 million hectares of soil and more than 10% of farmland affected by salinization (FAO, 2021), causing at least 25% of crops to suffer from varying degrees of yield loss due to persistent salt stress, with a serious impact on food security (Farooq et al., 2017; Kumar et al., 2022). Soil salinization leads to reduced crop yield because the significant negative impacts on seed germination by disrupting the membrane permeability of the seed embryo and increasing the osmotic stress on seeds (Deng et al., 2014). For salt-sensitive crops, seed germination rate, germination time, and the length of the plumule are all affected by salt stress (Abbas et al., 2012). Persistent salt stress during the crop growth phase leads to crop water loss and ion toxicity due to increased cellular osmotic pressure and disruption of cell membranes (Läuchli and Grattan, 2007). Salt stress also reduces nutrient uptake by inhibiting crop root growth (Burssens et al., 2000; West et al., 2004), inhibits photosynthesis by decreasing the crop's leaf area (Hu et al., 2022), and ultimately affects crop yield and quality.

Moreover, the survival of microorganisms is directly associated with plant and soil environments (Pulleman et al., 2012). Salt stress can reduce the abundance and activity of soil microbial communities (Rietz and Haynes, 2003), affecting the composition of functional soil microbes (Zhang et al., 2019), and disrupting the stability of microbial networks (Li et al., 2023a). This disruption affects nutrient cycling (Bai et al., 2012) and material utilization (Elmajdoub and Marschner, 2013) ultimately affecting the ecological functions of soil microbial communities (Zhang et al., 2023). Weakened ecological functions of microbial community, in turn, affect plant-microbe interactions (Etesami and Beattie, 2017), as manifested by reduced microbial colonization (Li et al., 2023a) and impaired plant growth (Jansson et al., 2023).

Both plants and soil microorganisms have developed specific abilities and mutualistic associations to cope with various stresses (Zhao et al., 2020; Liu et al., 2022). Halophytes and salt-tolerant plants, as the dominant vegetation in saline environments, are better adapted to saline stresses and have formed unique strategies improving their adaptability through such pathways as salt gland excretion (Yuan F. et al., 2016), ionic and osmotic regulation (Zhu, 2016), antioxidant defenses (Apse and Blumwald, 2002) and root structural modifications (Yu et al., 2022). Soil microorganisms also have various salt-tolerance strategies, such as salt accumulation and synthesis of organic osmotic material to adapt to high-salt environments (Gunde-Cimerman et al., 2018). Meanwhile, beneficial microorganisms can influence performance of their host plants under harsh conditions (Wang and Song, 2022). For example, arbuscular mycorrhizal fungi can help host plants to cope with abiotic stresses like drought, salt, etc., by improving plant water utilization, regulating photosynthesis and maintaining osmotic balance (Borde et al., 2017).

In addition, soil legacy effects are microbiological and functional substance traits retained in the soil by the plants, which influence the growth of succeeding plants (Van der Putten et al., 2013). The formation of soil legacy effects is the process of plant-microbe interactions in which plants respond to stressful stimuli and mobilize the required metabolites and functional microorganisms, thus promoting the growth of their own and succeeding plants as well as increasing their tolerance (Bakker et al., 2018). So, the application of soil legacy effects may also help to refine the way we cultivate and manage crops for agricultural production (Mariotte et al., 2018; Carrión et al., 2019; Cordovez et al., 2019). Therefore, based on the theoretical foundation of soil legacy effects in natural ecosystems, it is important to further explore the mechanism of crop-soil-microbe interactions in saline farmlands, which has profound implication for mitigating crop salt stress, increasing crop productivity and improving the environment of saline farmlands (Vukicevich et al., 2016).

2 Formation and role of soil legacy effects in natural ecosystems

In natural ecosystems, plants and soil organisms have various effects to soil legacy (Wardle et al., 2004; Faucon et al., 2017). Plant species with different root structures, growth habits and ways of interacting with soil organisms have important impacts on soil legacy effects (Oliver et al., 2021), while plant species composition and diversity also significantly modify such effects at the community level (Kowalchuk et al., 2002; Lange et al., 2015). Soil organisms, playing important roles in soil ecosystems, influence soil legacy effects by affecting soil organic matter decomposition, nutrient cycling and soil structure (Bardgett and Wardle, 2010).

Therefore, natural ecosystems have become a 'database' for exploring the mechanisms of soil legacy effects in the context of a highly diversified plants, microorganisms and soil environmental factors. An increasing number of studies have been carried out on the growth characteristics, resource utilization and survival strategies of plants and microorganisms that contribute to a better understanding about the soil legacy effects (Bezemer et al., 2006; Cortois et al., 2016; Bezemer et al., 2018; Heinen et al., 2020). The diversity of plant species, plant functional traits and soil microorganisms in natural ecosystems contributes to extensive research on species interactions and stress adaptations. The intricate interactions between plants and soil microorganisms play a crucial role in promoting the stabilization of soil ecosystems (Grayston et al., 1998; Berg and Smalla, 2009; Berendsen et al., 2012). Above- and below-ground interactions of plants have long-term legacy effects on biotic stresses in natural

ecosystems and can improve plant performance and resistance by manipulating soil microbial communities (Wurst and Ohgushi, 2015; Pineda et al., 2017). For abiotic stresses, soil microorganisms are able to implement a variety of mechanisms to fight against them and keep soil fertility as well as plant development in good condition (Abdul Rahman et al., 2021). For example, drought stress-induced dominance of fungal communities can influence succeeding plant drought adaptation by maintaining higher rates of litter decomposition and soil respiration (Mariotte et al., 2015). Inoculation of drought-conditioned phyllosphere and soil microbial communities can make plants capable of coping with repeated drought stress (Li et al., 2022).

Plant functional group is a common concept in the study of soil legacy effects, which refers to a group of plants that respond similarly to ecological processes and environmental changes, such as the grasses, forbs and legumes that are frequently mentioned in the literature (Kulmatiski et al., 2008; Cortois et al., 2016). Different plant functional groups can create positive or negative soil legacy effects by accumulating soil pathogens, recruiting beneficial microorganisms and regulating interactions with insects, etc (Petermann et al., 2008; Latz et al., 2012; Heinen et al., 2019). Such soil legacy effects, mediated by aboveground plant functional groups and soil microorganisms, play a role for succeeding plant growth in terms of soil physical properties, soil nutrient availability, soil microbial community structure, stress tolerance and competitive coexistence relationships (Byun et al., 2013; Strecker et al., 2015; Fischer et al., 2018; Mackie et al., 2018; Adomako and Yu, 2023).

Different plant functional groups play distinct roles in shaping soil legacy effects. For instance, grasses may improve soil physical structure and water retention through dense root systems (Hanamant et al., 2022), while legumes retain soil nutrients through nitrogen fixation (Spehn et al., 2002). The soil legacy effects resulting from these changes in the soil environment create more favorable conditions for succeeding plant growth. Simultaneously, the interaction between various plant functional groups and soil microorganisms yields diverse soil legacy effects. Grasses and forbs secrete different carbon compounds into the soil, recruiting different soil microorganisms (Philippot et al., 2013). For example, the presence of the grasses *Lolium perenne* not only increased the density of active bacteria in the soil but also elevated the expression of biocontrol genes associated with these bacteria, thereby contributing to the productivity of succeeding plant communities (Latz et al., 2015). Moreover, grasses positively influence other plant functional groups by altering soil microbial communities and soil nutrients (Cortois et al., 2016). Forbs, however, with more decomposers and higher concentrations of chemicals in their litter, may negatively impact succeeding plants (Bonanomi et al., 2006).

To foster positive soil legacy effects, it is essential to manage specific plant functional groups, regulate appropriate levels of beneficial microorganisms, decomposers and pathogenic microorganisms, and develop diverse plant-microbe community interactions (Carrión et al., 2019; De la Fuente Cantó et al., 2020; Xiong et al., 2020; Song et al., 2021). However, there is a current lack of studies exploring the application of the principle of soil legacy effects in understanding plant salt tolerance. Most studies have focused on the mechanism of plant's intrinsic salt tolerance and the utilization of specific microorganisms to enhance salt tolerance in laboratory and simulation experiments (Li et al., 2020a; Li et al., 2020b; Li et al., 2021a; Schmitz et al., 2022). Therefore, it is important to address how the rules of soil legacy effects can be developed and applied in saline farmlands.

3 Processes of plant response to salt stress

Plants have various strategies to cope with salt stress, involving refinement in their cellular physiology, phenotypic structures, osmoregulation, antioxidant production, and the regulation of signaling pathways (Van Zelm et al., 2020; Zhao et al., 2020). For instance, plants eliminate excess salt through a salt excretion mechanism to minimize salt-damage (Dassanayake and Larkin, 2017). Plants can also modify their root structure, such as developing deeper root systems to increase water uptake and mitigate the impact of salinity (Galvan-Ampudia and Testerink, 2011). In addition, plants respond to salt stress-induced damage by producing antioxidants, osmotic substances and protective enzymes (Hasegawa et al., 2000). ABA-dependent protein kinases are activated in response to salt stress, affecting cellulose distribution, controlling root tip cells, thus promoting salt avoidance in plant (Yu et al., 2022). Plant roots also secrete peptides that are transferred to the leaves to induce ABA accumulation, thereby driving stomatal closure to prevent leaf (Takahashi et al., 2018; Yu et al., 2020). Therefore, the combined application of these strategies enables plants to better adapt and survive in high-salt environments.

Besides plant innate responses, the complex microbial communities in rhizosphere soil play a critical role in host performance and tolerance to stresses (Durán et al., 2018; Carrión et al., 2019). These microbial communities help plants adapt to harsh conditions by forming mutualistic relationships, participating in nutrient uptake, producing beneficial compounds, and inducing immune responses that support plants against stress (Hou et al., 2021).

In terms of salinity tolerance, microorganisms establish mutually beneficial symbiotic relationships with plants through various mechanisms, assisting them in adapting to high salt environments. Rhizosphere microorganisms can secrete specific compounds, such as bacterial exopolysaccharides (EPS), which improve plant ion balance, promote soil aggregation, and thus maintain plant growth in high-salt (Morcillo and Manzanera, 2021). Arbuscular mycorrhizal fungi (AMF) enhance host plant salt tolerance by manipulating the osmotic balance through mycelium, improving access to water and nutrients (Hammer et al., 2011; Ruiz-Lozano et al., 2012). Moreover, rhizosphere microorganisms also play a role in physiological regulation and defense processes (Mishra et al., 2021). Plant growth promoting rhizobacteria (PGPR) can stimulate root development and enhance nutrient utilization under salt stress. For instance, the IAA-overproducing strain *Sinorhizobium meliloti* has been found to enhancive salt tolerance of alfalfa in saline soils by stimulating root proliferation (Bianco and Defez, 2009). Under salt stress conditions, the increase in the number and weight of root nodules in *Acacia gerrardii* inoculated with *Bacillus subtilis* contributed to the enhancement of nitrogen fixation by the roots, as well as uptake

and systemic translocation of phosphorus by the plant (Hashem et al., 2016a, Hashem et al., 2016b). AMF can activate an antioxidant protection system, maintaining cell membrane stability by decreasing permeability and malondialdehyde (MDA) content in plants (Yang et al., 2014).

These complex processes converting salinity tolerance cannot be separated from the dynamic interactions between plants and microorganisms (Liu et al., 2022). In the context of climate change-induced stress, introducing new microbial taxa had been shown to improve plant survival in stressful environments, and plant tolerance can be predicted by the climatic history of the microbial community (Allsup et al., 2023). Building on this, plant-soil-microbe interactions in salt-stressed environments may result in a history of stress response for soil microbes and the soil environment, generating soil legacy effects that aid succeeding plants in overcoming salt stress (Figure 1; Li et al., 2021a; Jing et al., 2022).

4 Creating soil legacy effects to improve crop salt tolerance

Farmlands vulnerable to saline stress often experience extreme environmental conditions and undergo specific agricultural management practices. These practices include high surface evapotranspiration, low precipitation, elevated ambient temperatures, and the application of chemicals, along with heavy irrigation during production (Arora et al., 2018; Enebe and Babalola, 2018). In contrast to natural ecosystems, the production function of farmland directly determines its monoculture structure, resulting in low plant diversity and nutrient use efficiency, imbalanced dynamics between above-ground crops and below-ground soil food webs, and altered crop defense mechanisms (Savary et al., 2019). Crops cultivated in farmlands tend to prioritize growth over defense compared to their wild counterparts of the same species. This preference, combined with the monoculture structure, increases the likelihood of negative soil legacy effects between previous and succeeding crops (Mariotte et al., 2018). The multiple stresses of saline farmlands challenge the growth of crops and soil microbes, and there is a need to rethink how to create soil legacy environments that are conducive to crop growth, while optimizing agricultural practices and fostering sustainable methods to enhance soil health and crop (Li et al., 2014).

4.1 The use of plants to create soil legacy effects

The productivity constraints of saline farmlands primarily result from the highly stressful environment directly impacting the growth of aboveground crops. Most staple crops in agricultural

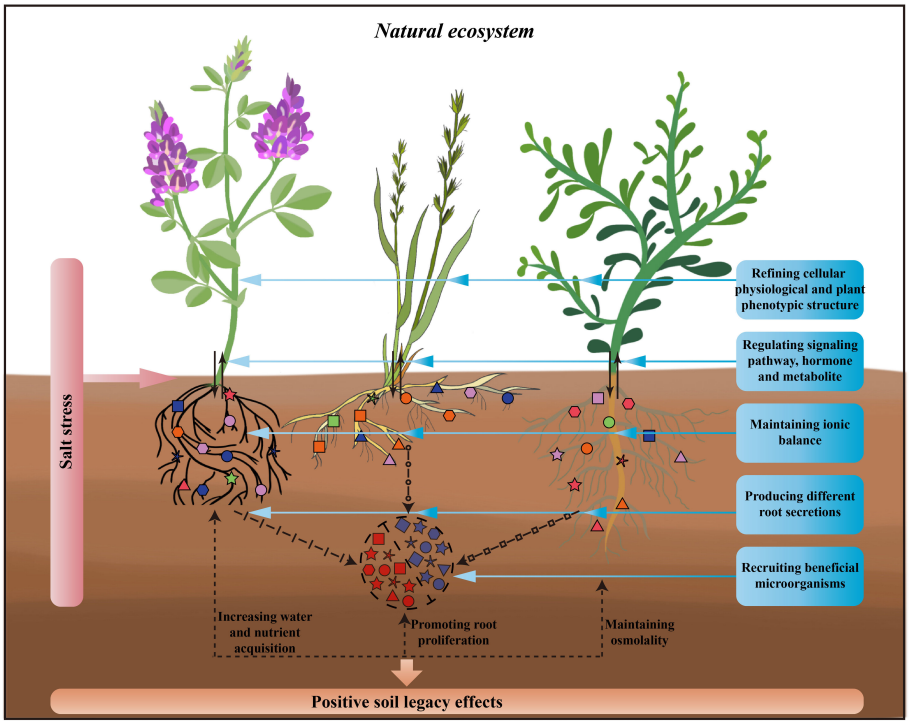

FIGURE 1
Processes of plant response to salt stress in natural ecosystems and possible soil legacy effects by plants. This figure shows, from left to right, three different plant functional groups, legume, grass, and forb, which respond simultaneously through above-ground and below-ground parts to salt stress. For above-ground parts of the plant, by refining cellular physiological and plant phenotypic structure, regulating signaling pathway, hormone and metabolite and thus responding to salt stress. For below-ground parts of the plant, by maintaining ionic balance, producing different root secretions, recruiting beneficial microorganisms and thus responding to salt stress. The response of above- and below-ground parts to salt stress simultaneously with increasing the plant's own acquisition of soil water and nutrients, promoting plant root proliferation, and maintaining the osmolality of the plant as well as the rhizosphere, thus creating the positive soil legacy effects through this favourable response processes.

production, such as maize, wheat, and rice, show high sensitivity to salinity stress. This sensitivity manifests itself in increased crop water loss, plant and fruit wilting, reduced crop photosynthesis, lowered carbon fixation, inhibited crop nutrient uptake, and slowed growth (Atta et al., 2023). To overcome the production bottlenecks in saline farmlands, it is necessary to harness biological resources with inherent salt tolerance found in natural environments. Additionally, establishing positive soil legacy effects through the introduction of specific plant species and plant functional groups is crucial (Figure 2).

4.1.1 The use of salt-tolerant biological resources

One feasible approach is to utilize the ability of halophytes and salt-tolerant plants in the natural environment. Firstly, some halophytes can absorb salt ions, and they are effective in reducing surface soil salinity while fighting against the increase in ion levels in tissue cells through leaf succulence (Song and Wang, 2015). Halophytes also dissolve calcium in the soil through root respiration, where calcium ions replace sodium ions in the cation-exchange complex, and ultimately improve soil physical properties in the plant's root zone (Qadir et al., 2005). Desalinated soils resulting from these processes contribute favorably to the subsequent growth of plants. Secondly, both halophytes and salt-tolerant plants boast robust root systems with strong penetration and water-holding capacity, thus enhancing soil structure (Silva et al., 2016). This improvement increases soil permeability and water retention post-planting, with the positive effects on soil structure persisting over an extended period (Liang and Shi, 2021). Finally, certain salt-tolerant plants, such as the forage crop sweet sorghum, can develop salt tolerance through hormonal signaling and secondary metabolites (Chen et al., 2022). Notably, stress-induced plant secondary metabolites have demonstrated legacy effects on succeeding plant growth by manipulating the composition of soil microbiome (Hu et al., 2018). Consequently, the utilization of halophytes and salt-tolerant plants presents opportunities to desalinate saline farmlands, improve soil conditions, or directly leverage the soil legacy effects created by the metabolites they produce to enhance crop resilience to salinity.

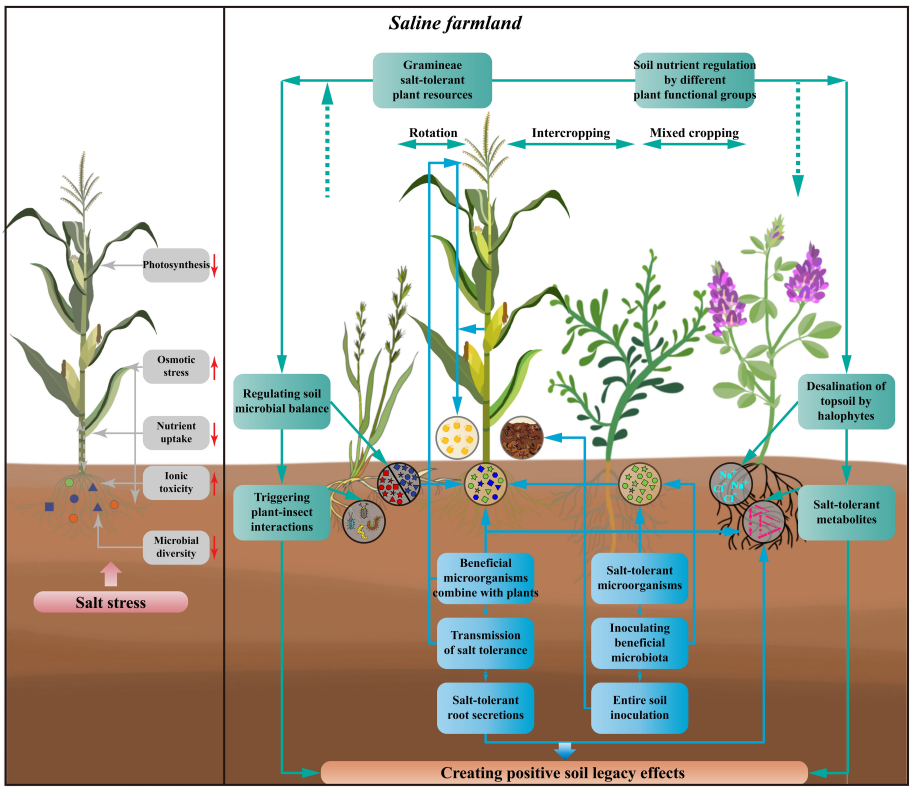

FIGURE 2
Effects of salt stress on crops and how to create soil legacy effects as well as improve crop salt tolerance in saline farmlands. The harmful effects of salt stress on crops include weakening crop photosynthesis, increasing osmotic stress, reducing crop nutrient uptake, adding ionic toxicity, and declining rhizosphere microbial diversity. By using halophytes, salt-tolerant plants, and plants of different functional groups, and developing the cropping patterns of rotating, intercropping, and mixed cropping with crops, the interactions between above- and below-ground parts of the plants can achieve the regulation of soil nutrients in saline farmlands, the desalination of surface soils, the secretion of salt-tolerant metabolite, and thus regulating the balance of soil microorganisms, as well as triggering the interactions between plants and insects. The improvement of salt tolerance in crops can also be achieved by screening for salt-tolerant microorganisms, inoculation with beneficial microbiota or entire soil inoculation. At the same time, new cultivation techniques could be used to combine the beneficial microorganisms directly with the plants and to transmit the tolerance. Crops with improved tolerance continue to produce salt-tolerant root secretions and to recruit beneficial microorganisms, thus creating an effective recycle of crop salt tolerance. All of these processes can create positive soil legacy effects through beneficial interactions between the above-ground and below-ground parts of the crop and influence succeeding crop.

4.1.2 Introducing plant functional groups into crop rotation systems

The soil legacy effects observed in natural ecosystems, facilitated by specific functional groups of plants, can significantly impact succeeding plants (Bezemer et al., 2006). This insight has inspired the development of effective cropping patterns for saline farmlands, especially considering that traditional monoculture patterns have contributed to soil resource depletion and decreased farmland productivity (Guo and Zhou, 2022). Grasses, have a solid research base in the field of ecology, known for carbon sequestration, nutrient cycling and improved soil stability (Franzluebbers, 2012; Hanamant et al., 2022). As the understanding of grassland ecosystem functioning continues to improve, forbs, representing a large proportion of species and functional richness, have also been recognized for their stress tolerance, indication of overgrazing, and maintenance of insect diversity (Siebert et al., 2021). Legumes, aside from being high-quality food and forage resources, are consistently recognized for sequestering nutrients and increasing diversity in cropping systems (Stagnari et al., 2017).

The crop rotation system of grasses and crops increased soil organic matter and earthworm numbers, resulting in improved soil structure compared to conventional crop rotations (Van Eekeren et al., 2008). This legacy effect of the grasses' influence on soil properties, then, increased the yield and seed nitrogen content of succeeding crops (Christensen et al., 2009). Legumes are even more beneficial to agricultural production by providing diverse services. One aspect is that the nitrogen-fixing capacity of legumes can continually increase the nitrogen yield of succeeding crops (Fox et al., 2020). Moreover, the growth process of legumes releases organic acids and other compounds, directly activate nutrients and indirectly promote the activity of soil microorganisms, thus increasing crop yields and soil fertility (Latati et al., 2016). Studies have shown that the deposition of rhizosphere nitrogen in legumes accounts for 70% of the total plant nitrogen (Fustec et al., 2010). These deposited nitrogens have mechanisms for transfer to other crops, affecting agricultural production potential. Although there are fewer practices on the involvement of forbs in crop rotation, studies have shown that forbs are rather less affected by changes in nutrient conditions than grasses due to their ability to store nutrients in their roots (Herz et al., 2017). Forbs are also important for maintaining the diversity of arthropods in the environment and some forb communities are more resistant to herbivores (Potts et al., 2010; Van Coller et al., 2018). Therefore, introducing these plant functional groups, such as grasses, forbs, and legumes, during crop rotation can strategically change soil nutrient levels or indirectly regulate the biotic and abiotic environment of saline farmlands.

Moreover, grasses and forbs exhibit different abiotic stress tolerance mechanisms and growth strategies. Due to obvious differences in growth, development and physiological structure between grasses and forbs, applying knowledge of forbs to improve salt tolerance in major cereal crops becomes challenging (Tester and Bacic, 2005). Meanwhile, the ability of grasses to accumulate salt ions in shoots and leaves may be weaker than that of forbs due to fewer salt glands (Semenova et al., 2010). So, although the planting of forbs like *Suaeda salsa* can effectively reduce soil salinity, it is difficult to apply the mechanism of salt ion accumulation and succulence in shoots of forbs to crops of grasses.

However, Poaceae, particularly within the functional group of grasses, has a unique history of salt tolerance, including major halophytic taxa identified as sources of halophytes (Flowers et al., 1986). Compared to the forbs, grasses usually maintain ion levels in aboveground tissues by limiting sodium uptake, having high potassium/sodium selectivity, and efficient potassium utilization, essential for survival under saline conditions (Flowers and Colmer, 2008). Many wild-type grasses are naturally tolerate to salt stress (Landi et al., 2017). For example, the study found that its close wild relatives *Tripsacum dactyloides* and *Zea perennis* both showed strong salt tolerance compared to maize (Li et al., 2023b). The leaf surface of wild rice, *Porteresia coarctata*, can excrete salts, maintaining intercellular ion concentrations and lower sodium to potassium ratios (Sengupta and Majumder, 2010). Grasses have been reported to produce positive soil legacy effects by altering soil microbial communities, influencing nutrient transfer, and even triggering interactions between above-ground plants and insects (Kos et al., 2015; Cortois et al., 2016; Schmid et al., 2021). Also, the ionic changes that occur in grasses during salt tolerance are closely related to their rhizosphere microorganisms (Hamdia et al., 2004; Paul and Lade, 2014). Thus, by introducing plant functional groups into the crop rotation system and combining their different ecological functions and salt-tolerate characteristics, positive soil legacy effects can be generated. This provides broader thinking for the improving the soil environment in saline farmland and enhancing of crop salt tolerance.

4.1.3 Introducing plant functional groups into crop intercropping system

The combination of plant functional groups within the same time and space can exert a significant influence on succeeding crops. One notable example is the legume and grass forage matching system, a typical forage mixing approach where the growth of grasses synergistically enhances both the symbiotic nitrogen fixation of legumes and the competitive nitrogen uptake of grasses (De Deyn et al., 2012; Suter et al., 2015). Beyond improving soil nutrient use efficiency, the extended growing period of mixed legumes and grasses also helps suppress topsoil salt accumulation, thereby enhancing soil quality (Li et al., 2021b).

While there are fewer studies on crop tillage systems and salt tolerance, similar to forage mixes, crop intercropping can weaken the negative impacts of saline farmland and may have legacy effects on succeeding crops. Firstly, intercropping systems increase the biodiversity of farmland ecosystems by direct introducing companion plants, such as differential crops or salt-tolerant plants, which provide services for saline farmland and the main crop (Yang et al., 2021). The introduction of different plants diversifies the rhizosphere environment, and the recruited microbial community can promote nutrient cycling, salt transformation, and degradation in the soil, thereby alleviating the damage of the saline environment to the crops. For example, the introduction of legumes can improve intercropping system resilience and resource use efficiency by enhancing crop growth and tolerance to abiotic stresses through

root distribution, vegetative cover, and nutrient activation (Chamkhi et al., 2022). Furthermore, the intercropping of the halophyte *Suaeda salsa* with maize significantly transferred more sodium ions to the rhizosphere of *Suaeda salsa*, thereby reducing the salt content of the maize rhizosphere (Wang S. et al., 2021). Regarding the rhizosphere enrichment by intercropping systems, it was shown that legume-grass crop intercropping (maize/faba bean) increased the abundance of rhizobia and reduced pathogens in the soil. The soil legacy effects it produced could be one of the reasons for the observed yield advantage in intercropping systems (Wang et al., 2020). Particularly under salt stress, the beneficial microorganisms recruited by the intercropping system (sorghum/peanut) achieved increased crop tolerance by altering the composition and content of metabolites (Shi et al., 2023). Therefore, the potential positive soil legacy effects of salt-tolerant forage mixtures and salt-tolerant crops of different functional groups can help to develop efficient intercropping systems for saline farmlands.

4.2 The use of soil microorganisms to synergistically create soil legacy effects

The presence of soil microorganisms in natural ecosystems depends on the soil environment, chemical signals provided by plants and nutrient resources (Bai et al., 2022). In response to the direct release of stress-responsive signals and compounds in plants, the associated soil microorganisms undergo specific changes (Hartman and Tringe, 2019). These changes are closely related to plants, especially alterations in rhizosphere microorganisms, and are critical to support the growth and recovery potential of plants under stress (Park et al., 2023). Salt-tolerant microorganisms capable of thriving and multiplying in high-salt environments, directly aiding plants in tolerating salt stress through their salt-tolerance mechanisms (Sharma et al., 2015; Wang R. et al., 2021). Plants in traditional environments, when confronted with salt stress, also respond by directly recruiting beneficial microorganisms through root secretions (Kumar et al., 2023). Furthermore, the mechanism by which soil microorganisms regulate plant salt tolerance also involves osmotic regulators, nutrients and soluble salts they provide to plants. These pathways can indirectly influence plant hormones and metabolism, stimulate plant growth and help plants overcome salt stress (Glick, 2012; Shrivastava and Kumar, 2015). These actions not only alleviate the negative effects of salinity but also establish soil legacy effects that confer tolerance to succeeding plants (see Figure 2; Zhalnina et al., 2018; Otlewska et al., 2020). Considering this, the question arises: How can we apply the direct and indirect effects of soil microorganisms on plant salt tolerance to saline farmland? What measures can be taken to sustain these positive effects in the farmland?

4.2.1 Direct utilization of soil microorganisms

Soil microorganisms play a crucial role in defending against saline stress, and saline soils serve as a significant source of salt-tolerant microorganisms (Zhang et al., 2023). Current research has successfully isolated several culturable salt-tolerant strains. For instance, 70% of the culturable strains of the root endophyte from the coastal perennial grass *Festuca rubra* exhibit salt tolerance (Pereira et al., 2019). The core microorganisms of the rhizosphere of *Suaeda salsa* have been found to harbor genes encoding salt stress adaptation and nutrient solubilization processes (Yuan Z. et al., 2016). Microbial inoculation is a direct method of utilizing these specialized salt-tolerant microbial resources, which can be applied to enhance plant salt stress adaptation and promote growth. Studies have demonstrated that inoculation with the salt-tolerant endophyte *Sphingomonas prati* significantly increases the salt tolerance of *Suaeda salsa* by improving the antioxidant enzyme system (Guo et al., 2021). *Curvularia* sp., isolated from *Suaeda salsa*, can establish a beneficial symbiotic relationship with poplar and promote its growth (Pan et al., 2018). Moreover, the inoculation of salt-tolerant microorganisms has been gradually extended to major crops, including soybean, maize, wheat, and peanut. Its positive effect in mitigating salt stress has been consistently verified in numerous indoor simulation experiments (Ramadoss et al., 2013; Goswami et al., 2014; Zerrouk et al., 2016; Khan et al., 2019; Shabaan et al., 2022).

In addition to the salt-tolerant microbial resources associated with saline soils and halophytes, salt stress is alleviated by the recruitment of beneficial microorganisms to the rhizosphere of plants when they face with salt stress in normal environments (Ilangumaran and Smith, 2017; Santoyo, 2021). For example, it has been shown that 1-aminocyclopropane-1-carboxylate (ACC), a stress-related amino acid in plants, can reshape the soil microbiome, enhancing plant tolerance to salinity stress (Liu et al., 2019). In addition, rice influences rhizosphere microorganisms by producing metabolites such as salicin and arbutin, enabling rhizosphere microorganisms associated salt stress tolerance (Lian et al., 2020). Moreover, beneficial rhizosphere microorganisms in plants can not only enhance salt-tolerant properties but also synergistically improve plant responses to salt stress by altering physiological growth processes, including seed germination, morphological structure, and biomass accumulation and partitioning (Pan et al., 2020). Regarding the inoculation of beneficial microbial strains to help crops tolerant salinity, studies have demonstrated that inoculation with *Pseudomonas flavescens* D5 strain effectively increased the biomass and antioxidant enzyme activities of barley, while reducing the adverse effects of salt stress on barley (Ignatova et al., 2022). Inoculation of candidate strains of *Azotobacter* has also been found to increase the potassium-sodium ratio, polyphenol and chlorophyll content, and decrease proline concentration in maize, thereby alleviating salt stress in maize by integrating multiple mechanisms (Rojas-Tapias et al., 2012).

Indeed, successful microbial inoculation often requires a combination of strains rather than a single strain to enhance the sustainability of its impact on (Verbruggen et al., 2012; Finkel et al., 2017). Notably, double inoculation with *Rhizobium* and *Pseudomonas* has been observed to elicit positive adaptive responses in alfalfa under salt stress (Younesi et al., 2013). Similarly, dual inoculation of plant growth-promoting bacteria with *Bradyrhizobium* strains has proven more effective in enhancing salt tolerance in soybean, reducing salt-induced ethylene production, and improving nutrient uptake (Win et al., 2023). Further studies have found that inoculation with species-specific microbiomes or whole-soil

inoculation can assist plants in coping with various biotic and abiotic stresses (De Vries et al., 2020; Ma et al., 2020; Trivedi et al., 2020). The introduction of microbiomes or the whole-soil achieves more complex ecological functions by coordinating microbial interactions (Pineda et al., 2019; Trivedi et al., 2021), and it avoids the potential issue of single strains struggling to survive inoculation into foreign soil (Mallon et al., 2018). However, it is crucial to acknowledge the possibility that introducing exotic microbial communities may reshape functions within the native microbial community (Amor et al., 2020). Recent evidence suggests that the beneficial effects of microbial inoculation on plant growth are best explained as changes in native microorganisms rather than direct effects on plants (Hu et al., 2021). This underscores the importance of understanding the intricate interactions occurring within the microbial community and their influence on plant health and resilience.

While practical examples of microorganism inoculation for saline farmland improvement are limited, the concept of soil legacy effects suggests that enhancing saline farmland and crops can be achieved through microbial-mediated processes. By inoculating salt-tolerant microbial strains and communities of beneficial microorganisms, and even inoculating the entire soil including most microorganisms, it becomes possible to modulate crop responses to salt stress and enhance salt tolerance. Concurrently, synergistic changes with the inoculated microorganisms involve stress response-related metabolites and alterations in the crop rhizosphere environments. These changes encompass crop rhizosphere secretions, microbial metabolites, and native microbial communities. Their persistent influence on succeeding crop growth in the form of soil legacy effects contributes to ongoing salt stress mitigation in saline farmland. Thus, the application of microbial interventions holds promise for sustainable improvements in saline farmland and crop resilience (Cuddington, 2011; Trivedi et al., 2020).

4.2.2 Indirect utilization of soil microorganisms

Alongside traditional plant- and microorganism-based methods for restoring saline farmlands, advanced modern agricultural techniques with their high efficiency and precision have also found application agricultural production (Varshney et al., 2011; Ahanger et al., 2017). Research has focused on integrating and applying the active components of rhizosphere exudates to soil microbial systems, revealing improvements in soil physicochemical environments and microbial communities associated with rhizosphere exudates. These improvements are speculated to have an impact on plant growth (Shi et al., 2011). Similar findings were observed in maize system, where a significant increase in bacterial density and altered metabolic potential in the maize rhizosphere after application of maize rhizosphere exudates (Baudoin et al., 2003). In terms of enhancing crop tolerance, research has shown that introducing the ability of releasing volatile organic compounds (VOCs) into maize varieties that do not release specific VOCs can reduce the threat of pests (Degenhardt et al., 2009). This suggests that the introduction of tolerant metabolites is not limited to rhizosphere exudates, and the application of below-ground volatiles, as well as other tolerant signals, offers additional possibilities for improving salt tolerance in crops on saline farmlands. The advances in agricultural technology have also inspired the exploration of beneficial root traits in wild relatives of crops, the introduction of which may solve the problems faced by saline farmlands (Preece and Peñuelas, 2020).

In the past decade, cultivation techniques have gradually emerged, pointing to the unique microbiome existing in plant seeds and how it spreads from generation to generation, aiding plants in adapting to their environment and increasing tolerance (Gopal and Gupta, 2016; Abdelfattah et al., 2023). In this context, delivering endophytes to the next generation of crops and ensuring the persistence of their tolerance has been achieved by combining relevant beneficial microorganisms with plants (Wei and Jousset, 2017). For example, a suspension of *Paraburkholderia phytofirmans* PsJN was sprayed in plots at the flowering stage of wheat in field experiment, and thus the maturation of its progeny plants was accelerated by the introduction of this endophytic bacteria into the flowers of the wheat parents (Mitter et al., 2017). The advantage of this approach lies in the ability of seed endophytes to avoid competition with native soil microorganisms, establishing closer interactions with the plant early on. While there is currently limited research related to this approach concerning salt tolerance in progeny plants, seed endophytes have long been shown to provide plants with tolerance against a wide range of stresses, participate in plant adaptation mechanisms, and enhance plant competitiveness (Samreen et al., 2021). Therefore, the use of these new bioculture techniques and the genetic mechanisms of plant microbes offer innovative avenues for improving saline farmland. These approaches are closely related to plant-microbe interactions and are centered around the concept of creating positive soil legacy effects.

Inspired by the mentioned approaches, microorganisms can be used indirectly, such as through the recruitment of microorganisms by plant rhizosphere exudates and intergenerational dissemination of beneficial microorganisms, to create positive soil legacy effects in saline farmland. However, it is acknowledged that microbial-related methods of creating soil legacy effects are imperfect, and their processes may introduce soil pathogens or other responsive substances, necessitating further in-depth research to explore safer methods of creating soil legacy effects (Jing et al., 2022).

5 Conclusion and future prospects

This paper provides a summary of the ways in which plants, in collaboration with soil microorganisms in natural ecosystems, jointly respond to salt stress. It suggests enhancing the salt tolerance of crops in saline farmlands through the perspective of soil legacy effects. The focus is on meeting the salt tolerance needs of crops by creating well-considered soil legacy effects. The paper explores both the direct use of plants and the synergistic use of soil microorganisms to establish positive soil legacy effects, offering innovative insights to boost production potential and improve the ecological environment of saline farmland. The emphasis lies on creating positive soil legacy effects through the selection of suitable salt-tolerant crops, the development of planting patterns with a rational match of crop functional groups, the inoculation of functional microorganisms, the inoculation of safe and efficacious soils, and the application of advanced agricultural technologies and bio-cultivation methods. This approach underscores the practical utility of crop-soil microorganism interactions

in agricultural production. In addition to plants and associated soil microorganisms, the role of soil animals in constructing soil food webs is acknowledged. These soil animals, through direct or indirect interactions with microorganisms and plants, contribute to the cycling of soil nutrient resources, influencing soil ecosystem function (Du et al., 2018). Multi-trophic interactions between mycorrhizal fungi, fungus-eating protozoa, and nematodes in the soil can enhance crop nutrient uptake, crop yield, and tolerance (Jiang et al., 2020). This suggests that future studies can more precisely and directly leverage soil legacy effects to trigger positive tolerant responses by regulating specific species or soil fauna in the soil food web of saline farmlands, or even by controlling certain trophic levels.

Author contributions

YM: Writing – original draft, Writing – review & editing. CZ: Funding acquisition, Writing – review & editing. YB: Funding acquisition, Writing – review & editing. CS: Funding acquisition, Writing – review & editing. FZ: Funding acquisition, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Young Scientists Project of National Key Research and Development Program of China (2021YFD1900200), the National Key Research and Development Program of China (2022YFF1302800, 2022YFD1900300), the Strategic Priority Research Program of Chinese Academy of Sciences (XDA26040103), the Special Key Grant Project of Technology Research and Development of Zhangjiakou City ("Take-and-lead" Program) (No.2022J001) and the "100 Talents Project" of Chinese Academy of Sciences.

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 Plant Science

TYPE Review
PUBLISHED 23 May 2024
DOI 10.3389/fpls.2024.1377793

OPEN ACCESS

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RECEIVED 28 January 2024
ACCEPTED 07 May 2024
PUBLISHED 23 May 2024

CITATION
Verma KK, Joshi A, Song X-P, Liang Q, Xu L, Huang H-r, Wu K-C, Seth CS, Arora J and Li Y-R (2024) Regulatory mechanisms of plant rhizobacteria on plants to the adaptation of adverse agroclimatic variables.
Front. Plant Sci. 15:1377793.
doi: 10.3389/fpls.2024.1377793

Regulatory mechanisms of plant rhizobacteria on plants to the adaptation of adverse agroclimatic variables

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The mutualistic plant rhizobacteria which improve plant development and productivity are known as plant growth-promoting rhizobacteria (PGPR). It is more significant due to their ability to help the plants in different ways. The main physiological responses, such as malondialdehyde, membrane stability index, relative leaf water content, photosynthetic leaf gas exchange, chlorophyll fluorescence efficiency of photosystem-II, and photosynthetic pigments are observed in plants during unfavorable environmental conditions. Plant rhizobacteria are one of the more crucial chemical messengers that mediate plant development in response to stressed conditions. The interaction of plant rhizobacteria with essential plant nutrition can enhance the agricultural sustainability of various plant genotypes or cultivars. Rhizobacterial inoculated plants induce biochemical variations resulting in increased stress resistance efficiency, defined as induced systemic resistance. Omic strategies revealed plant rhizobacteria inoculation caused the upregulation of stress-responsive genes—numerous recent approaches have been developed to protect plants from unfavorable environmental threats. The plant microbes and compounds they secrete constitute valuable biostimulants and play significant roles in regulating plant stress mechanisms. The present review summarized the recent developments in the functional characteristics and action mechanisms of plant rhizobacteria in sustaining the development and production of plants under unfavorable environmental conditions, with special attention on plant rhizobacteria-mediated physiological and molecular responses associated with stress-induced responses.

KEYWORDS

adverse agroclimatic conditions, physiological and omic aspects, plant responses, plant hormones, agricultural sustainability, rhizobacteria

Introduction

Plant rhizobacteria-mediated abiotic stress reduction occurs directly through hormone induction or indirectly via signaling in the host plant. The direct function in nitrogen fixation, phosphate solubilization, auxin, cytokinin, gibberellin, and abscisic acid production are all documented. It also makes it easier for necessary mineral elements to be absorbed from the rhizospheric soil along with the production of plant growth regulators. However, the indirect roles include the production of metabolites, siderophores, antibiotics, volatile HCN, etc. Some of the compounds that the microbes may produce include deaminase enzyme, microbiocidal enzyme, siderophores, plant hormones, and PO_4-solubilizing enzyme (Gujral et al., 2013; Ekinci et al., 2014; Saleem et al., 2015; Kumari and Khanna, 2016; Moustaine et al., 2017). Plants have unique microbiota, and the microbial structure in the rhizosphere is influenced by the bacteria and plants' production of signal molecules and the chemical composition of root exudates (Zhang et al., 2017; Jalmi and Sinha, 2022). Plant-growth regulators, phytohormones, and various secondary metabolites can be produced by PRs to stimulate plant development (Islam et al., 2014; Kaushal and Wani, 2016) (Figure 1).

The upregulated synthesis of metabolites, such as phytohormone, exopolysaccharides, siderophores, antioxidant enzymes, and volatile compounds, primarily minimizes plant resistance to environmental challenges. The production of phytohormones by rhizobacteria-inoculated plants, including cytokinins (CK), gibberellic acid (GA), indole-3-acetic acid (IAA), and abscisic acid (ABA) is employed during plant stressed conditions. 1-aminocyclopropane-1-carboxylate (ACC) deaminase plays a significant role in conferring stress resistance capacity to plants by downregulating the level of stress-induced ethylene level in plant roots system (Etesami and Maheshwari, 2018; Shahid et al., 2023). Plant-rhizobacteria downregulated the effects of abiotic stresses by modifying the expression of genes associated with the biosynthesis of hormones, i.e., *ACO* and *ACS* genes (ethylene biosynthesis), *MYC2* (Jasmonate), *PR1* (SA), genes encoding antioxidant enzymes, transcription factor *NAC1*, etc. (Tiwari et al., 2017) (Tables 1, 2). Extensive field trials are required to investigate the interaction between the functional activities of signaling networks and their association. The interaction between PRs and plants based on various factors, such as root composition, strains of bacteria, and exudation patterns from their roots (Kumar et al., 2019). Numerous secondary metabolites and root exudates depend as chemo-attractants in the rhizosphere, attracting beneficial soil bacteria and inhibiting phytopathogens, thereby stimulating a delicate network of signaling between microbes and plants (Ullah et al., 2021; Joshi et al., 2022; Mellidou and Karamanoli, 2022; Joshi et al., 2023). The physiological and molecular responses activated in plants in response to stress resistance are regulated by various key genes with metabolic and regulatory roles. Research demonstrations focusing on plant gene expression following plant-rhizobacteria inoculation may help understand which can be an effective environmentally friendly approach to alleviate the adverse environmental variables (Ferrante

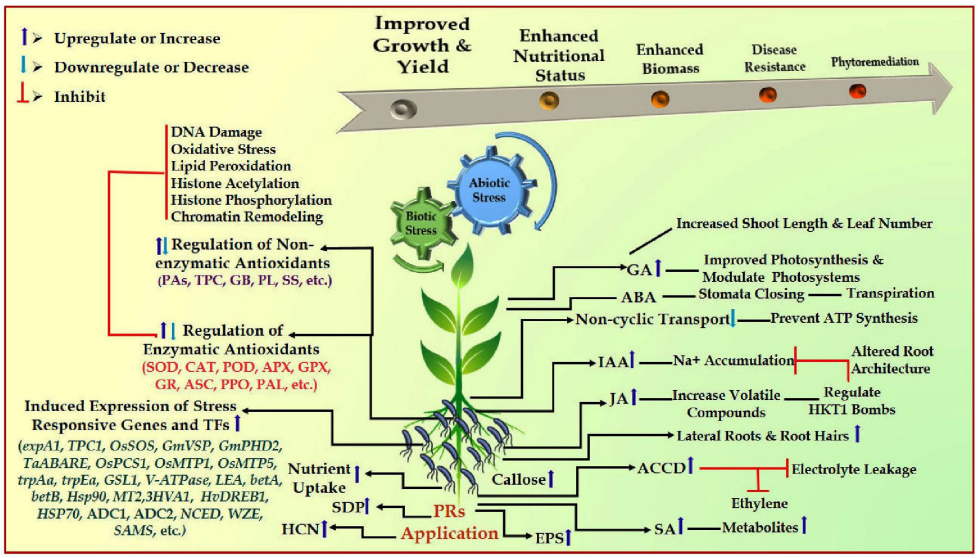

FIGURE 1
Schematic representation of PRs-mediated abiotic and biotic stress resistance mechanism in plants. ABA, abscisic acid; JA, jasmonic acid, GA, gibberellins, IAA, indole-3-acetic acid, SA, salicylic acid, EPS, exopolysaccharides, HCN, hydrogen cyanide; ACCD, 1-aminocyclopropane-1-carboxylate deaminase; SOD, superoxide dismutase; CAT, catalase; PAL, phenylalanine ammonia-lyase; APX, ascorbate peroxidase; POD, peroxidase; ASC, ascorbate; PPO, polyphenol oxidase; GPX, glutathione peroxidase; GR, glutathione reductase; Pas, polyamines; TPC, total phenolic content; PL, proline; SS, soluble sugar; HSPs, heat shock proteins; HKT—High-affinity K^+ transporters; *expA1,* expansin*; TPC1,* calcium transporter; ADC1 and ADC2, putrescine synthesis; *OsPCS1,* phytochelatin synthase; *OsMTP1,* gene related to metal transport; *OsMTP5*, gene related to expulsion of excess metal*; trpAa*, and *trpEa,* genes related to tryptophan biosynthesis; *betA* and *betB*, genes related to betaine biosynthesis; *GmVSP* and *GmPHD2,* stress responsive genes; *GSL1,* gene related to cell wall synthesis; V-ATPase, Vacuolar-H^+ -pyrophosphatase; LEA, late embryogenesis abundant; NCED, WZE and SAMS = transcription factors.

TABLE 1 PR-mediated abiotic stress reduction in crop plants and their tolerance mechanism.

Stress condition	Plant	PR strains	PRs-mediated possible tolerance mechanism	Source
Cold	Tomato	*Streptomyces* sp. TOR3209	Upregulation of genes related to biosynthesis of abscisic acid (ABA), stress-related metabolism and photosynthesis	Ma et al., 2023
Cold	Maize	*Lysinibacillus fusiformis* YJ4, *L. sphaericus* YJ5	Upregulation of genes related to osmolytes, phenolic content, superoxide dismutase (SOD), catalase (CAT), phenylalanine ammonia-lyase (PAL), indole-3-acetic acid (IAA), and gibberellic acid (GA_3)	Jha and Mohamed, 2023b
Cold	Wheat	*Bacillus* spp. CJCL2, *B. velezensis* FZB42	Downregulation of ABA and lipid peroxidation encoding genes *ABARE* and *4-HNE*, upregulation of gene related to Expansin *(expA1)*, Cytokinin *(CKX2)*, and Auxin *(ARF)*	Zubair et al., 2019
Drought	Barley	*Providencia rettgeri*	Increased production of IAA, siderophores (SDP), proline(PL), exopolysaccharides (EPS), and reduced level of malondialdehyde (MDA)	Ferioun et al., 2023
Drought	Chickpea	*Stenotrophomonas* sp. CV83	Upregulation of genes related to antioxidant enzymes SOD, POD, ascorbate peroxidise (APX), and lipoxygenase	Sharma et al., 2023
Drought	Maize	*Cronobacter* sp.Y501	Constrain ABA signaling, increase IAA biosynthesis, decrease MDA, SOD, CAT, peroxidase (POD) activity	Gao et al., 2023
Drought	Rice	*Pseudomonas putida* AKMP7	Polyamines (PAs)homeostasis through biosynthesis, back-conversion and catabolism of PAs	Nikhil et al., 2023
Drought	Soybean	*Bacillus pumilus* SH-9	Downregulation of ABA, upregulation of SOD, POD, APX, glutathione (GSH), EPS and SPD	Shaffique et al., 2023
Drought	Wheat	*Enterobacter bugandensis* WRS7	Overexpression of genes related to antioxidants (*CAT*, *APX*, *GPX*), osmolyte (*P5CS*, *P5CR*, *TPS1*), stress hormone (*NCED, WZE, SAMS, ACS1*) and *ACO* encoding proteins for ABA, ethylene, and calcium transporter (*TPC1*)	Arora and Jha, 2023
Heat	Tomato	*Bacillus safensis SCAL1*	Increased level of ACCD, EPS, IAA, gibberellic acid (GA_3), kinetin, SOD, CAT, POD	Mukhtar et al., 2023
Heat	Maize	*Bacillus* spp. AH-08, AH-67, SH16 and *Pseudomonas* spp. SH-29	Upregulation of heat shock proteins (HSP1, *HSP18, HSP70, HSP101*), CAT, POD, and carotenoids	Ahmad et al., 2023
Heat	Mustered	*Bacillus aryabhattai* NSRSSS-1, *B. licheniformis* SSA 61, *Bacillus* sp. MRD-17	Increased production of IAA, GA, CAT, SOD, APX, phenolic content and reduction in PL, and soluble sugar (SS)	Kiruthika et al., 2023
Heat	Wheat	*Bacillus safensis*	Elicited expression of ADC1 and ADC2 linked to putrescine synthesis, modulated expressions of HSPs, upregulate redox enzymes and antioxidants associated with ascorbate (ASC)-GSH cycle, enhanced GB, SS, and phenols	Sarkar et al., 2021
Heavy metal	Barley	*Rhodospirillum* sp. JY3	Enhanced production of POX, CAT, SOD, GSH, ASC, polyphenols, phytochelatins, glutaredoxin, thioredoxin, peroxiredoxin	Alsiary et al., 2023
Heavy metal	Barley	*B. glycinifermentans* IS-2	Modulation of endogenous phytohormones and uptake of essential elements (K, P)	Belhassan et al., 2024
Heavy metal	Maize	*Agrococcus terreus* (MW 979614)	Augmented levels of antioxidant enzymes (SOD, POD), and nutrient uptake	Shahzad et al., 2023
Heavy metal	Maize	*Serratia* CP-13	Upregulate IAA, osmolytes (SS, PL), antioxidants and downregulate MDA, ABA, and Cd uptake	Tanwir et al., 2023
Heavy metal	Rice	*Serratia marcescens* DB1	Decreased expression of genes related to phytochelatin synthase (*OsPCS1)*,metal transport *(OsMTP1)*, expulsion of excess metal (*OsMTP5)*	Bhatta et al., 2023
Heavy metal	Tomato	*Serratia* sp. D23, *Sphingomonas* sp.	Upregulation of defense genes (*Hsp90*, *MT2* and *Nramp 3*)	Wei et al., 2022
Salt	Barley	*Siccibacter* sp. C2	Overexpression of *HVA1*, *HvDREB1, HvWRKY38*, *HvP5CS* genes	Sayahi et al., 2022
Salt	Chickpea	*Bacillus* sp. BSE01	Maintained levels of ACC, ABA and K^+/Na^+ ratio, enhanced production of, antioxidant enzyme, PL and decreased activity of NADPH oxidase	Basu et al., 2023
Salt	Lettuce	*Bacillus velezensis* JB0319	Induce SOD, POD activity and decreased MDA	Bai et al., 2023

(Continued)

TABLE 1 Continued

Stress condition	Plant	PR strains	PRs-mediated possible tolerance mechanism	Source
Salt	Maize	*Pseudomonas* sp. MHR6	Induce production of EPS, reduce MDA and electrolyte leakage (EL)	Liu et al., 2022
Salt	Mustered	*Pseudomonas fluorescens*	Augmented production of glycine-betaine (GB), PL, SOD, CAT, APX and GR	Khan et al., 2023
Salt	Oat	*Bacillus* sp. LrM2	Induced production of ACCD, non–enzymatic antioxidants, ASC, GSH, dehydroascorbate	Zhang et al., 2023
Salt	Rice	*Pseudomonas promysalinigen* RL-WG26	Induce biosynthesis of tryptophan (*trpAa, trpB, trpC, trpD, trpEa*), IAA (*iaaM, iaaH*), betaine (*betA, betB, betT*) and inhibit ethylene biosynthesis (acdS) related transcripts	Ren et al., 2024
Salt	Rice	*Lysinibacillus fusiformis, L. sphaericus, Brevibacterium pityocampae*	Increased expression of JA,*OsNHX1*,*OsAPX1*, *OsPIN1, OsCATA* gene and reduced expression of ABA, salicylic acid (SA), and *OsSOS* gene	Asif et al., 2023
Salt	Soybean	*Streptomyces lasalocidi* JCM 3373	Induce expression of indole-3-carboxaldehyde (ICA1d), expression of stress-responsive genes (*GmVSP, GmPHD2, GmWRKY54)* and root growth related genes (*GmPIN1a, GmPIN2a, GmYUCCA5, GmYUCCA6)*	Lu et al., 2024
Salt	Tomato	*Bacillus halotolerans* Gb67, *B. subtilis* All3, *B. mojavensis* Gb7	Induced production of PAs, VCs, EPS and ACCD	Abdelkefi et al., 2024
Salt	Wheat	*Variovorax* sp. P1R9	Increased SOD, CAT activity and reduced thiobarbituric acid reactive substances ($TBAR_S$)	Acuna et al., 2024
Salt	Wheat	*Nocardioides* sp.	Induce expression of ACCD, *TaABARE*, TaHAk1, hkt1, *CAT*, *MnSOD*, *POD*, *APX*, *GPX*, and *GR* gene transcripts	Meena et al., 2023

et al., 2023; Verma et al., 2023). The formation of the enzyme ACC deaminase by rhizobacteria and reduction in ethylene level had been the main function for enhanced plant growth and resistance ability during different stresses (Bharti et al., 2014; Jalmi and Sinha, 2022).

Eco-physiological and omic responses of plant rhizobacteria required more attention and extensive field research demonstrations to increase stress resistance efficiency. Hence, the present article focused on the interactions between plants and rhizobacteria and their impact on tolerance to adverse agroclimatic variables for agricultural sustainability in an eco-friendly environment.

Impact of plant development, biomass, and productivity

Plant rhizobacteria (PRs) effectively improve plant morphological structures during adverse environmental conditions. Abiotic stresses, such as acidic and alkaline soil, insufficient water supply, low and high temperature, UV-radiation, soil flooding, and contaminated/toxic soil, affect agronomic, anatomical, cellular, and metabolic activities (Glick et al., 2007; Verma et al., 2020a, b). Higher levels of phytohormones, defense-related proteins and enzymes, antioxidants, and epoxypolysaccharides cause PGPR-induced resistance (Kaushal and Wani, 2016). It is accomplished by changing transcriptional and signaling processes, which lead to altered gene expression when PRs are present. Because PRs produce phytohormones that change root shape and improve root development, surface area, uptake, and accumulation of nutrients, plant productivity increases in the presence of PRs (Mellidou and Karamanoli, 2022). They can also increase total plant productivity by helping to induce ACC-deaminase activity in plants. The potential of PRs enhancing plant growth and development varies due to differences in their properties, such as ACC-deaminase activity, IAA generation, root colonization, P-solubilization, etc (Ghosh et al., 2018; Gupta and Pandey, 2019). The defense mechanisms of plants against unfavorable agroclimatic conditions depend on the variation in the development of roots (Khoshru et al., 2023). Different PGPR strains can enhance the overall root system by increasing the total number of root tips, surface area, and structure of the roots under stressful conditions (Brambilla et al., 2022). Lowering the ethylene content increases the plant's capacity to withstand stress by facilitating improved nutrition and water uptake capacity (Chieb and Gachomo, 2023) (Figure 1).

When under stress, PRs also improve the uptake of water and nutrients. The absorption of nutrients and antioxidant activities are associated with stress management. By diminishing the negative consequences of saline soil, inoculation with *Klebsiella oxytoca* (Rs-5) containing ACC-deaminase boosted plant establishment and increased the absorption of key mineral nutrients (Yue et al., 2007; Zahir et al., 2012). In a similar way, *Pseudomonas* spp. inoculation increased the antioxidative enzymatic activities and promoted the growth of plants during unfavorable climatic conditions (Fu et al., 2010; Jalmi and Sinha, 2022) (Table 1).

According to Zahir et al. (2009) and Orozco-Mosqueda et al. (2020), rhizobacterial strains have been explored to have a substantial influence on the improvement of a variety of plants,

TABLE 2 PR-mediated biotic stress reduction in crop plants and their tolerance mechanism.

Stress condition	Crop	PR strains	PRs-mediated possible tolerance mechanism	Source
Net blotch fungus (*Drechslera teres*)	Barley	*Paraburkholderia phytofirmans* B25	Upregulation of genes related to cell wall synthesis (*GSL1,GSL3*, and downregulation of genes related to defense (*CAT2, AOC, PRB*), phenylpropanoid pathway (*PAL2*, *F3'H*), isovitexin, and lipid compounds	Backes et al., 2021
Wilt disease (*Fusarium oxysporum)*	Faba bean	*Bacillus velezensis*, *B. paramycoides*, *paramycoides*	Induced production of hydrogen cyanide (HCN), siderophores (SPD), indole-3-acetic acid (IAA), abscisic acid (ABA), benzyl, kinten, ziaten, and gibberellic acid (GA_3)	El-Sersawy et al., 2021
Wilt disease (*Fusarium oxysporum*)	Maize	*Pseudomonas pseudoalcaligenes* (EU921258), *Bacillus pumilus* (EU921259)	Induce expression of β-1,3 glucanase genes, improved photosynthetic pigment, and cell membrane stability	Jha and Mohamed, 2023a
Wilt disease (*Fusarium oxysporum f.* sp. *pisi*	Pea	*Bacillus subtilis* (IS1), *B. amyloliquificiens* (IS6), *B. fortis* (IS7)	Upregulation of total phenolic compounds and enzymes of phenylpropanoid pathway	Raza et al., 2024
Sheath blight disease (*Rhizoctonia solani*)	Rice	*Bacillus velezensis*, *B. megaterium, B. toyonensis*	Increased activity of polyphenol oxidase (PPO), superoxide dismutase (SOD), catalase(CAT)	Patil et al., 2024
Leaf stripe disease (*Burkholderia*)	Sorghum	*A. chroococcum* Beijerinck 1901 (MCC 2351), *B. megaterium* (MCC 2336), *P. fluorescens* (NAIMCC B-00,340)	Decreased levels of malondialdehyde (MDA), proline, CAT, SOD	Rizvi et al., 2024
Speck disease (*Pseudomonas syringae* pv. tomato)	Tomato	*Pseudomonas koreensis* 5, *Bacillus mycoides* 68, *B. mojavensis* 36,*B. simplex* 47	High levels of proline, POD, CAT	Yildiz et al., 2023
Wilt disease (*Ralstonia solanacearum*)	Tomato	*Pseudomonas fluorescens* Pf3, *Trichoderma longibrachiatum* UNS11	Increased activity of peroxidase (POX), phenylalanine ammonia-lyase (PAL), and PPO enzymes	Konappa et al., 2020
Spot blotch disease (*Bipolaris sorokiniana*)	Wheat	*Bacillus subtilis* BS87	Increased levels of nutrient solubilization, SPD, IAA, HCN and decrease levels of SOD, POD, PPO, MDA, PAL, proline	Chandra et al., 2024
Fungal pathogens (*Alternaria alternata*, *Rhizoctonia solani*, *F. oxysporum*, *Ustilaginoidea virens*)	Wheat	*Beijerinckia fluminensis* BFC-33	Increased levels of carotenoid, PAL, PPO, β-1,3 glucanase and reduce proline, thiobarbituric acid reactive substances ($TBAR_S$) and electrolyte leakage	Al-Shwaiman et al., 2022

including cereals, legumes, and vegetables cultivated under challenging conditions. They also enhanced the production of exopolysaccharides and ACC-deaminase activity. PRs enhance plant growth in polluted soil by downregulating the level of ethylene (Dell'Amico et al., 2008). PRs with 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity may promote plant development during stress. Compared to uninoculated plants, the inoculated plants with PRs containing ACC-deaminase activity improved plant growth and yield considerably. *Pseudomonas* sp. and *Acinetobacter* sp. have increased IAA and ACC-deaminase production in saline soil and enhanced stress tolerance efficiency in barley and oats (Kang et al., 2019).

It can be indicated by the significantly increased levels of chlorophyll, total phenolics, flavonoids, soluble sugars, protein contents, and antioxidative enzymatic activities, as well as the higher expression of stress-related genes, that resulted from inoculating Cd-stressed with *Serratia marcescens* BM1 in *Glycine max* L. plants. *Phaseolus vulgaris* subjected to the rhizobacterial consortia experienced reduced stress caused by salinity and improved overall plant growth and photosynthetic pigments (Gupta and Pandey, 2019). In tomato plants, *Streptomyces* sp. has been shown to reduce stress and promote growth (Palaniyandi et al., 2014). It has been observed that *Burkholderia phytofirmans* helps plants under drought stress (Naveed et al., 2014). They generate exopolysaccharides (EPS) during water-deficit conditions, enhancing seed germination and growth. Of all the strains, *Pseudomonas fluorescens* has the highest capacity to produce EPS and ACC deaminase. The saline rice field was employed by Sultana et al. (2020) to isolate rhizobacterial strains, which they found to enhance stomatal conductance, transpiration, and photosynthetic CO_2 assimilation rate, all of which contributed to increased crop yield, fruit and grains quality. According to the latest research, *Azospirillum brasilense* Sp245 increased *Arabidopsis thaliana* growth, suggesting that MAMPs produced from plant-

rhizobacteria are essential for plant cultivation (Méndez-Gómez et al., 2021) (Tables 1, 2).

Photosynthetic leaf gas exchange and chlorophyll fluorescence efficiency

Plant-rhizobacteria enhance inoculated plants' photosynthetic response and leaf gas exchange capability during stress (Verma et al., 2020b; Jalmi and Sinha, 2022). By modifying the photosynthetic characteristics, osmolytes production, antioxidant machinery, and expression of stress-related genes, inoculating soybean plants with *Serratia marcescens* BM1 (PR) provides Cd tolerance to plants (El-Esawi et al., 2020). Under salt stress, *Bacillus amyloliquefaciens* SQR9 has demonstrated higher efficiency in photosynthesis and overexpression of the RBCS and RBCL genes in *Zea mays* plants (Chen et al., 2016). During bacterial strain inoculation, *Arabidopsis helleri* showed elevated photosynthesis and proteins associated with abiotic stress (Khan et al., 2021).

Enhanced photosynthetic pigments and the expression of important genes (*RBCS* and *RBCL*) regulating RUBISCO activities during stress condition (Sherin et al., 2022; Amaral et al., 2023). By modulating ion homeostasis, redox potential, photosynthetic CO_2 assimilation rate, and the expression of stress-related genes, maize plants inoculated with *Serratia liquefaciens* KM4 revealed enhanced growth and stress tolerance (El-Esawi et al., 2018). Reduced phenol, flavonoid, and leaf relative water content and photosynthetic responses in maize plants have resulted from salinity stress, which also decreased root damage and water uptake. However, inoculating maize under salt stress with *Serratia liquefaciens* KM4 enhanced LRWC, photosynthetic characteristics, and the biosynthesis pathways of phenols and flavonoids, enhancing plant stress tolerance efficiency. In comparison to uninoculated plants, rhizobacteria-inoculated maize and white clover have demonstrated enhanced photosynthesis, soluble proteins, sugars, and enzymatic activities following inoculation with HAS31 rhizobacteria (Han et al., 2014) (Figure 1; Table 1).

Uptake and accumulation of mineral nutrients and water balance

By altering the solubility and absorption of nutrients, PRs improve the bioavailability of nutrients in plants under abiotic factors. Through N_2-fixation, mobilization, and the promotion of N_2-fixers through their secretions, several rhizobacteria can reduce the volume of nitrogen (N_2) supplementation required for plant growth (Shah et al., 2022; Khoso et al., 2024). Additionally, they change the shape and surface area of the roots, improving nitrogen bioavailability (Olenska et al., 2020). Elevating ammonium transporters' expression improves nutritional absorption during stresses (Calvo et al., 2019). According to Gomez-Godínez et al. (2023), phosphorus (P) solubilizing PRs, such as *Azotobacter, Bacillus, Burkholderia, Erwinia, Pseudomonas, Serratia*, and *Rhizobium*, generate organic acids that chelate P-bound cations and make it available to plant roots. Furthermore, under Fe-deficient conditions, PRs assist in acquiring iron (Fe) by generating siderophores, which are low molecular weight organic molecules (Mohanty et al., 2021). Reducing metal ion availability and decreasing metal uptake, siderophores that generate PRs enhance plants' survival under heavy metal stress (Dimkpa et al., 2009; Kumar et al., 2021) (Tables 1, 2).

Ocimum basilicum L. has demonstrated the ability of PRs to enhance nutrient absorption and downregulate abiotic stresses (Rakshapal et al., 2013). Under salinity stress, PRs, such as *Pseudomonas* sp. and *Azospirillum* sp., increase nutrient availability, improving plant growth, biomass, and productivity (Noorieh et al., 2013). The application of rhizobial inoculants has been observed to trigger delayed senescence, as evidenced by higher potassium (K) ion levels and lower ethylene and cytokinin production. In plants with a higher K^+/Na^+ ratio, PRs boost the absorption of K^+ ions by synthesizing *AtHKT1*, a high-affinity ion channel that promotes stress tolerance (Mahmud et al., 2021) (Figure 1).

Biosynthesis of plant hormones and compatible solutes

Along with metabolites and signaling molecules, the majority of rhizobacteria produce phytohormones (Ahmad et al., 2022; Shah et al., 2022). Among these include gibberellic acid, cytokinins, indole acetic acid (IAA), and abscisic acid (ABA) (Tariq et al., 2023). IAA is produced by 80% of soil microorganisms, including *Pseudomonas* sp., *Bacillus* sp., *Burkholderia* sp., and *Rhizobium* sp (Khan et al., 2021). It has been shown that IAA-producing rhizobacteria stimulate crop production and plant growth when exposed to abiotic stress (Mellidou and Karamanoli, 2022). Numerous IAA-producing rhizobacteria increase root biomass, length, and surface area, which improves nutrient accumulation, uptake, and plant growth (Fasusi et al., 2023). Increased IAA levels also foster lateral roots' growth, minerals' absorption, and root exudates' formation. It is well known that some PRs, including *Arthrobacter, Azotobacter, Bacillus, Pseudomonas*, and *Pantoea*, synthesize cytokinins that enhance nutrient availability as well as plant tolerance responses (Shah et al., 2022) (Table 1).

According to dos Santos et al. (2020), gibberellin-releasing PRs such as *Azospirillum, Shingomonas, Bacillus amyloliquafaciens*, and *Bacillus pumilus* can also promote plant growth and yield. Regulation of abscisic acid also played a significant role in stress resistance capacity influenced by rhizobacteria (Herrera-Medina et al., 2007). When pepper (*Capsicum annum*) is inoculated with *Serratia nematodiphila* (that produces gibberellin), the plant expands more under low-temperature stress, releases more GA_4 and ABA, and lower salicylate and jasmonate activities (Kang et al., 2015).

The plant and bacterial species may impact the mechanism of ABA-mediated tolerance to stressful conditions. Under abiotic stress, specific PRs (strains of *Rhizobium* spp., *B. pumilus, B. lycheniformis, Achromobacter xylosoxidans*, and *Azospirillium*

brasiliense) serve as ABA-stimulators or ABA-producers (Salomon et al., 2014; Egamberdieva et al., 2017). It can assist plants minimize water loss by activating Ca^{+2} channels that cause stomatal closure (Goswami and Deka, 2020; Grover et al., 2021). Greater ABA biosynthesis has been observed in *Arabidopsis* plants inoculated with the spermidine-producing *B. megaterium* strain (Zhou et al., 2016). By upregulating the gene expression that regulates ABA production, the rhizobacteria inoculation of rice with *Pseudomonas fluorescens* enhanced the plant's resistance to stress. The upregulation of *TaWRKY* and *TaMYB* expression in ABA-signaling cascades has also been observed. It has also been suggested that specific rhizobacteria can use ABA as a carbon and energy source, limiting ABA uptake throughout the plant organs. These results indicated the changes in ABA-mediated signaling pathways as a means by which inoculated plants can survive abiotic challenges (Olenska et al., 2020; Mellidou and Karamanoli, 2022) (Figure 1).

It has also been demonstrated that using rhizobacteria minimizes the negative effects of ethylene generated under abiotic stress circumstances (Grichko and Glick, 2001; Nadeem et al., 2007; Zahir et al., 2008). Under abiotic stresses, rhizobacteria-inoculated plants have been demonstrated to modify ethylene biosynthesis-related gene expression (Lephatsi et al., 2021; Verma et al., 2021; Fadiji et al., 2022). Plants can be spared the toxicity of ethylene through the presence of rhizobacteria that contain ACC deaminase, which can hydrolyze ACC, the precursor of ET (Mellidou and Karamanoli, 2022).

The impact of *Paenibacillus lentimorbus* B-30488 inoculation on the reduction of abiotic stress in *Arabidopsis thaliana*, as well as by modifications in plant hormones and RSA-related gene expression. According to Khoshru et al. (2023), specific PRs also generate polyamines, which enhance root architecture and promote stomatal conductance and photosynthesis. The microbial community in the rhizosphere is mainly influenced by the exudates produced by plant roots, such as organic acids, mucilage, carbohydrates, sugars, and proteins, which also confer tolerance to inoculated rhizobacterial plants (Backer et al., 2018). Under abiotic stress, *Azospirillum* sp. has been demonstrated to accumulate appropriate solutes such as glutamate, proline, glycine, betaine, and trehalose (Saleena et al., 2002). *Phaenibacillus polymyxa* has been shown to possess the drought-responsive gene ERD15 (Timmusk and Wagner, 1999). Conjugated phytohormones and flavonoids in root tissue can be extracted or hydrolyzed by *Azospirillum*, releasing them in their active forms (Spaepen et al., 2007; Dardanelli et al., 2008; Saikia et al., 2010; Fahad et al., 2015).

The mechanisms of photosynthetic activity, hydraulic conductance, osmotic accumulation, and sequestering toxic ions are associated with rhizobacteria-stimulated resilience to stress (Figure 1). Groundnut inoculated with *Bradyrhizobium* under drought conditions demonstrated stress resistance due to amino acids produced from the nitrogenase to catalyzed the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3) ions (Delfini et al., 2010; Enebe and Babalola, 2018). Furthermore, nitrogenase assists the supply of nitrogen to inoculated legumes, and these plants have been shown to produce more leaves due to more root nodules (Ferreira et al., 2011). To avoid desiccation, lower toxicity, and promote root growth, PRs also generate polysaccharides (Arora et al., 2010). A vital aspect of stress mitigation under environmental stress at the plant rhizosphere consists of forming biofilm and exopolysaccharide. One fascinating strategy PRs employ to mitigate the impacts of heat stress in plants involves the induction of osmoprotectants and heat shock proteins (HSPs) (Enebe and Babalola, 2018). Under stressful conditions, pepper plants treated with gibberellin-producing rhizobacteria showed a reduction in the level of salicylate and jasmonate. When the bacteria *Burkholderia phytofirmans* occurs, tomato plants produce more phenolics, proline, and starch under stress (Issa et al., 2018).

In plants under abiotic stress, PRs also improve proline synthesis. *Arthrobacter, Bacillus*, and *Burkholderia* are the main rhizobacteria that synthesize proline. Better stress tolerance in rhizobacteria-inoculated plants is mostly due to increased dissolved sugar levels and solute storage. Other potential strategies to reduce oxidative stress include stabilizing membranes, protein–protein complexes, and osmolytes, such as proline, glycine betaine, amino acids, and total sugars (Chieb and Gachomo, 2023).

Influence of enzymatic, non-enzymatic, and lignin biosynthesis

The synthesis of the enzyme ACC deaminase is a well-known mechanism for rhizobacteria-led abiotic stress tolerance (Etesami et al., 2015; Gupta and Pandey, 2019). By lowering ABA levels, plants inoculated with ACC-producing PRs expand more rapidly; the growth hormones regulate the synthesis of secondary metabolites (Kang et al., 2019). By promoting the activity of antioxidant enzymes (SOD, APX, and CAT) and upregulating the genes involved in the ROS pathway, it enhanced stress tolerance (Habib et al., 2016). Because ethylene causes stress-induced H_2O_2 accumulation and apoptosis induction, ACC deaminase-producing PRs provide plants resistance against abiotic stress by lowering ethylene synthesis. It has been observed that inoculating different crops under stress with strains that include ACC-deaminase enhances plant development (Li et al., 2017; Singh and Jha, 2017; Namwongsa et al., 2019; Danish et al., 2020; Mellidou et al., 2021; Mellidou and Karamanoli, 2022).

Plant-to-microbe communication also occurs by an array of non-hormonal signaling molecules. Microbes produce volatile compounds (VOCs), signaling molecules that control plant growth and modify soil and plant health in response to stress (Ullah et al., 2021). Moreover, plants tolerate heavy metal stress due to rhizobacteria-releasing extracellular polymeric substances (EPS), which primarily help by lowering the metals' bioavailability in the soil (Mishra et al., 2017). Some species of *Bacillus, Azotobacter, Burkholderia, Enterobacter*, and *Pseudomonas* can reprogram plants' redox states, increasing their tolerance to environmental stresses. During stress, the overproduction of reactive oxygen species (ROS) changes redox states and causes DNA damage, proteins, and membrane fluidity, ultimately resulting in cell death. However, plants inoculated with PRs defended against abiotic stressors by activating their defense mechanisms.

Antioxidant enzyme activity enhanced in an array of growth-promoting rhizobacterial species to assist them in combatting oxidative stress (Mitra et al., 2021; Mellidou and Karamanoli, 2022) (Figure 1; Tables 1, 2).

Additionally, rhizobacteria are essential in reducing oxidative damage caused by various stressors, including heavy metals, water deficit, low and high temperatures, salt, and water scarcity. By lowering ROS levels in plant roots, rhizobacteria-induced antioxidant enzymes assist in reducing the stressors that plants experience in the environment. Additionally, they accelerate the growth rate in response to abiotic stressors by promoting the generation of antioxidant enzymes. Better stress tolerance in inoculated plants may be due to increased activities of antioxidant enzymes, such as catalase (CAT), ascorbate peroxidase (APX), or glutathione peroxidase (GPX) (Mellidou et al., 2021; Swain et al., 2021; Fadiji et al., 2022). Ascorbate peroxidase increased when tomato seedlings were inoculated with *Enterobacter* and subjected to abiotic stress. Gladiolus plants treated with rhizobacteria revealed increased levels of CAT and SOD activities as compared to their control group (Figure 1).

Tomato seedlings inoculated with *P. oryzihabitans* AXSa06 (having ACC deaminase) experienced mild oxidative stress and enhanced lipid peroxidation to trigger the antioxidant machinery (Mellidou et al., 2021). Under abiotic stress, tomato plants inoculated with a strain of *Sphingomonas* sp. revealed reduced lipid peroxidation, increased glutathione levels, and antioxidant enzyme activities (Halo et al., 2015; Mellidou and Karamanoli, 2022). In contrast, rhizobacteria inoculation has been demonstrated in additional studies to decrease the production of ROS-scavenging or stress-responsive enzymes that are important for plant protection in stressful environments (Gupta and Pandey, 2019; Goswami and Deka, 2020; Song et al., 2021; Verma et al., 2022a, b, c). The generation of defensive enzymes like chitinase and glucanase to the rhizobacteria stress-tolerance mechanism (García-Fraile et al., 2015). *Glycine max* plants inoculated with *Bacillus firmus* SW5 exhibit stress tolerance through alterations in root ultrastructure, antioxidant levels, and stress-related gene expression (El-Esawi et al., 2020). The production of oxalic acid, gluconic acid, and citric acid by stressed rhizobacteria plays a crucial role in the mobilization of heavy metals. Biofilm-forming rhizobacteria were inoculated into *Spartina densiflora* plants, resulting in increased levels of SOD, CAT, and APOX activities as well as a decrease in the induced oxidative stress index (OSI) (Perez et al., 2019; Khan et al., 2021; Bhat et al., 2022).

In *Cicer arietinum* plants, *Pseudomonas putida* MTCC5279 has been shown to reduce stress by enhanced ROS scavenging ability, modulation of membrane integrity, and accumulation of osmolyte (proline, glycine, betaine). These findings have also been validated by differential expression of genes involved in dehydration-responsive element binding, transcription factors expressed under abiotic stress, salicylic acid, jasmonate, transcription activation, SOD, CAT, APX, and GST (Tiwari et al., 2016; Chieb and Gachomo, 2023). In *Abelmoschus esculentus* plants, the presence of ACC-producing PRs was associated with increased activities of antioxidant enzymes (SOD, APX, and CAT) and up-regulated genes of the ROS pathways (CAT, APX, GR, and DHAR) (Habib et al., 2016). These pathways have also been linked to enhanced POD/CAT activity, decreased cell death, and increased glutathione levels for ROS scavenging. When *Dietzia natronolimnaea* was inoculated into wheat (*Triticum aestivum*), it was observed that the ABA-signaling cascade genes, ion transporters, salt overly sensitive (SOS) pathway, and antioxidant enzymes upregulated (Bharti et al., 2016) (Figure 1).

Conclusion and future prospects

Adverse environmental variables severely affect crop growth, development, and output and downregulate the overall socio-economic growth of sustainable agriculture. Different application strategies have been developed to challenge stress, its benefits, and its applications. Nowadays, the requirement for higher food grain productivity and safety, enhanced plant yield, fertility of soil properties, and agricultural sustainability are upregulating. The research demonstrations are shifting toward soil rhizospheric-bio-based engineering to facilitate a better pollution-free environment for combining plants and rhizobacteria. The application of PRs is more beneficial in overcoming stressed conditions besides providing other significant direct and indirect ways to upregulate overall plant responses. PRs are more convenient, economical, and eco-enviro-friendly and can be applied in small cultivating areas to large fields. Variations in the modifications of plant responses under stress have been observed in inoculated plants, and these variations are dependent on the PRs mode of action, which represents the multifactorial processes regulated in stressful environments. The positive symbiotic associanship that plants develop with microbial physiology is fundamental for the plant development, especially in terms of biotic and abiotic stresses. It is necessary to set up deeply extensive field research demonstrations to understand better the interaction between the PRs-mediated signal and the metabolic/molecular reprogramming that improves plant tolerance to unfavorable environmental variables. Multi-strain bacterial strains can be substantial if a single strain of bacteria is not more significant in reducing stress resistance efficiency. The application, duration, and applicability of inoculation are more crucial as unmanaged methods may lead to consistent and correct results. Its successful agro-commercialization will based on the involvement of plant physiologists, plant biologists, plant pathologists, biotechnologists, agro-industrialists, and farmers. A better and deep understanding of the action mechanisms and interactions of plants and associated plant rhizobacteria directly in the matrix of interest can be favored by the adoption of a holistic approach that uses "omic" applications.

Author contributions

KV: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Validation, Writing – original draft. AJ: Conceptualization, Data curation, Formal analysis, Software, Writing – original draft. X-PS: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Software, Supervision, Validation, Visualization, Writing – review &

editing. QL: Data curation, Formal analysis, Funding acquisition, Resources, Software, Writing – review & editing. LX: Data curation, Formal analysis, Resources, Software, Writing – review & editing. H-rH: Data curation, Formal analysis, Resources, Software, Writing – review & editing. K-CW: Data curation, Funding acquisition, Resources, Software, Supervision, Writing – review & editing. CS: Data curation, Formal analysis, Resources, Software, Writing – review & editing. JA: Data curation, Formal analysis, Resources, Software, Writing – review & editing. Y-RL: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was financially supported by the Guangxi Key R & D Program (Guike AB24010140), Guangxi Natural Science Foundation (2021GXNSFAA220022; 2023GXNSFAA026459; 2023GXNSFAA026460), Guangxi Innovation Teams of Modern Agriculture Technology (nycytxgxcxtd-2021–03), Guangxi Characteristic Crop Experimental Station (GTS2022022), National Key Research and Development Project (2022YFD2301102–07), The National Natural Science Foundation of China (31760415; 32060468), Fund of Guangxi Academy of Agricultural Sciences (2021YT011), Science and Technology Major Project of Guangxi (Guike AA22117002–1), Fundamental Research Fund of Guangxi Academy of Agriculture Sciences (2023YM55) and Key Research and Development Program of Nanning (20232060).

Acknowledgments

The authors would like to thank the Guangxi Academy of Agricultural Sciences, Nanning, Guangxi, China, for providing the necessary facilities for 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.

Publisher's note

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 13 June 2024
DOI 10.3389/fpls.2024.1419764

OPEN ACCESS

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RECEIVED 18 April 2024
ACCEPTED 28 May 2024
PUBLISHED 13 June 2024

CITATION
Siddika A, Rashid AA, Khan SN, Khatun A, Karim MM, Prasad PVV and Hasanuzzaman M (2024) Harnessing plant growth-promoting rhizobacteria, *Bacillus subtilis* and *B. aryabhattai* to combat salt stress in rice: a study on the regulation of antioxidant defense, ion homeostasis, and photosynthetic parameters.
Front. Plant Sci. 15:1419764.
doi: 10.3389/fpls.2024.1419764

Harnessing plant growth-promoting rhizobacteria, *Bacillus subtilis* and *B. aryabhattai* to combat salt stress in rice: a study on the regulation of antioxidant defense, ion homeostasis, and photosynthetic parameters

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Introduction: The ongoing global expansion of salt-affected land is a significant factor, limiting the growth and yield of crops, particularly rice (*Oryza sativa* L). This experiment explores the mitigation of salt-induced damage in rice (cv BRRI dhan100) following the application of plant growth-promoting rhizobacteria (PGPR).

Methods: Rice seedlings, at five- and six-weeks post-transplanting, were subjected to salt stress treatments using 50 and 100 mM NaCl at seven-day intervals. Bacterial cultures consisting of endophytic PGPR (*Bacillus subtilis* and *B. aryabhattai*) and an epiphytic PGPR (*B. aryabhattai*) were administered at three critical stages: transplantation of 42-day-old seedlings, vegetative stage at five weeks post-transplantation, and panicle initiation stage at seven weeks post-transplantation.

Results: Salt stress induced osmotic stress, ionic imbalances, and oxidative damage in rice plants, with consequent negative effects on growth, decrease in photosynthetic efficiency, and changes in hormonal regulation, along with increased methylglyoxal (MG) toxicity. PGPR treatment alleviated salinity effects by improving plant antioxidant defenses, restoring ionic equilibrium, enhancing water balance, increasing nutrient uptake, improving photosynthetic attributes, bolstering hormone synthesis, and enhancing MG detoxification.

Discussion: These findings highlight the potential of PGPR to bolster physiological and biochemical functionality in rice by serving as an effective buffer against salt stress–induced damage. *B. subtilis* showed the greatest benefits, while both the endophytic and epiphytic *B. aryabhattai* had commendable effects in mitigating salt stress–induced damage in rice plants.

KEYWORDS

abiotic stress, AsA-GSH pathway, auxin, *Bacillus*, ion homeostasis, osmotic stress, stress signaling

1 Introduction

The escalation of urbanization and industrialization across the globe has decreased the areas of available fertile agricultural land in conjunction with substantial increases in the global population (Sharma and Kumawat, 2022). This scenario has necessitated urgent improvements in agricultural productivity to meet current and future food demands. However, the intensifying environmental stress arising from global climate change is also adversely affecting crop yield by exacerbating stresses due to various abiotic factors, including salinity, drought, waterlogging, heat stress, cold injury, light stress, UV radiation, toxic metal/metalloid stress, ozone exposure, and even soil nutrient toxicity. Of these abiotic stresses, salinity affected area is showing expansion and is particularly concerning, as it is not only destructive to growing plants, but it also renders vast areas of agricultural lands unfit for crop cultivation (Khasanov et al., 2023).

Soil salinity is characterized by the excessive accumulation of salts, such as sodium (Na^+), chloride (Cl^-), potassium (K^+), and calcium (Ca^{2+}), in the soils, with Na^+ and Cl^- as the dominant ion species. Elevated salt ion concentrations in soil disrupt natural soil processes (e.g., soil nutrient imbalance, microbial activity inhibition, reduced water infiltration, soil structure degradation, etc.), ultimately impeding plant growth and productivity (Munns, 2011). Salinity influences every phase of a plant's life cycle, from germination to yield, by altering morphophysiological and biochemical processes (Roman et al., 2020). In particular, plants growing in saline environments produce high levels of reactive oxygen species (ROS). Plants have their innate ability to prevent the generation of ROS during normal photosynthetic and respiratory metabolism through antioxidant defense systems. However, overly-produced ROS under saline conditions overwhelms the inherent antioxidant defense systems, resulting in oxidative stress in plants (Basit et al., 2023). Salinity, therefore, creates challenges to sustainable agriculture and the production of sufficient food to meet global food requirements and ensure future food and nutritional security.

One strategy for overcoming the deleterious effects of saline soils is to use plant growth-promoting rhizobacteria (PGPR). These microbes have gained attention in recent years for their potential to enhance soil ecosystems and improve crop yields in stressful environments by colonizing the plant root system or rhizosphere and stimulating growth without incurring negative impacts on the surrounding environment. PGPR enhance plant growth either directly or indirectly by fixing atmospheric nitrogen, solubilizing essential nutrient elements (e.g., phosphorus [P], potassium [K], zinc [Zn]); producing phytohormones (e.g., indole-3-acetic acid [IAA]), exopolysaccharides (EPS), siderophores, 1-aminocyclopropane-1-carboxylate deaminase, and antioxidants; suppressing diseases through antibiotic production; bolstering plant resistance to biotic and tolerance to abiotic stresses; and promoting plant-microbe symbiosis (Chakraborty et al., 2021; Dame et al., 2021). The ability of PGPR to alleviate environmental stress effects in plants improves plant growth and stress tolerance; therefore, PGPR can serve as ecological engineers for climate-smart farming.

The PGPR bacterial genera include *Agrobacterium*, *Azospirillum*, *Arthrobacter*, *Azotobacter*, *Rhizobium*, *Bacillus*, *Erwinia*, *Bradyrhizobium*, *Burkholderia*, *Pseudomonas*, *Achromobacter*, *Enterobacter*, *Chromobacterium*, among others, but all induce plant tolerance to salinity and other abiotic stresses to promote overall plant growth under stressful conditions. For instance, *Bacillus* sp. is a notable PGPR that enhances the morphophysiological attributes of plants in ways that aid plant survival under stressful conditions. Applications of *Bacillus* sp. in the soil as well as in plants improve plant growth, enhance water retention, reduce ionic toxicity, suppress membrane damage, and maintain electrical conductivity to mitigate salt-induced damage (Ji et al., 2022; Hasanuzzaman et al., 2022b). Beneficial effects are recognized for both endophytic PGPR, such as *B. subtilis* (Woo et al., 2020; Hasanuzzaman et al., 2022b) and *B. aryabhattai*, as well as epiphytic PGPR, such as *B. aryabhattai* (Sultana et al., 2020, 2021), in promoting plant stress tolerance.

This study aimed to assess the effects of salt stress on rice physiology and growth, with a focus on evaluating the potential of *B. subtilis* and *B. aryabhattai* to mitigate oxidative damage under salt stress conditions. Rice is a staple food for over half of the world's population, making it crucial to ensure its resilience to environmental stressors like salinity. However, there is limited research on the specific roles of *Bacillus* species in alleviating oxidative stress in rice plants under salt stress conditions. Sea

levels rise as a consequence of climate change causing seawater flooding and making rice cultivation difficult in the coastal areas during dry seasons (January-May) (SRDI, 2010). Therefore, rice cultivation during this period provides additional production to meet the global demand for rice (Jahan et al., 2023). Hence, the aim of the present study was to assess salt stress effects on the physiology and growth of rice. The main goal was to explore the extent of damage inflicted on rice exposed to salinity stress and to determine whether the presence of the endophytic PGPR, *B. subtilis* and *B. aryabhattai*, and the epiphytic PGPR, *B. aryabhattai*, can mitigate oxidative damage in rice under salt stress conditions. The findings will contribute to the broader goal of understanding and enhancing PGPR-mediated salt stress tolerance in rice.

2 Materials and methods

2.1 Plant materials, growing conditions, experimental treatments, and design

Uniform and healthy seeds of a Zn-enriched rice variety (*Oryza sativa* cv. BRRI dhan100) containing a Zn content of 25.7 mg kg^{-1} were used in this experiment. Vigorously growing, uniform, and disease-free 42-day-old seedlings were then transplanted into Wagner pots (14 L) with soil containing BRRI (2020) recommended fertilizer doses (Urea: 138 kg ha^{-1}, TSP: 51 kg ha^{-1}, MoP: 63 kg ha^{-1}, Gypsum: 60 kg ha^{-1}, and $ZnSO_4$: 4 kg ha^{-1}). Five hills in each pot were maintained at a uniform distance until the reproductive stage and then thinned to two hills per pot (Figure 1). Three different PGPR suspensions were applied using seedling dipping and soil drenching methods: endophytic *Bacillus subtilis* (1×10^9 CFU mL^{-1}), endophytic *B. aryabhattai* (3×10^9 CFU mL^{-1}) and epiphytic *B. aryabhattai* (3×10^9 CFU mL^{-1}). The applications were made at three distinct growth stages: transplantation of 42-day-old seedlings, vegetative stage at five weeks post-transplantation, and panicle initiation stage at seven weeks post-transplantation. Five weeks after transplantation, the plants were irrigated twice with 50 mM and 100 mM NaCl solutions at seven-day intervals, whereas the control group was irrigated with only water. The experiment was conducted as a completely randomized design (CRD) with three replications.

2.2 Measurements of crop growth attributes

Crop growth attributes (plant height, leaf area, plant fresh, and dry weight) were measured at 62 days after transplanting. Plant height was calculated by measuring the length of five plants per pot from the base to the most extended leaf tip and then averaging the measurements. Leaf area was measured from five randomly selected leaves per pot using a length-width method (Francis et al., 1969). Fresh weight (FW) was determined by gently uprooting five hills per pot and weighing them. The uprooted plants were then oven-dried for 72 h at 80°C, and the dry weight (DW) of each plant was measured. The data were presented as the averages of the five measurements.

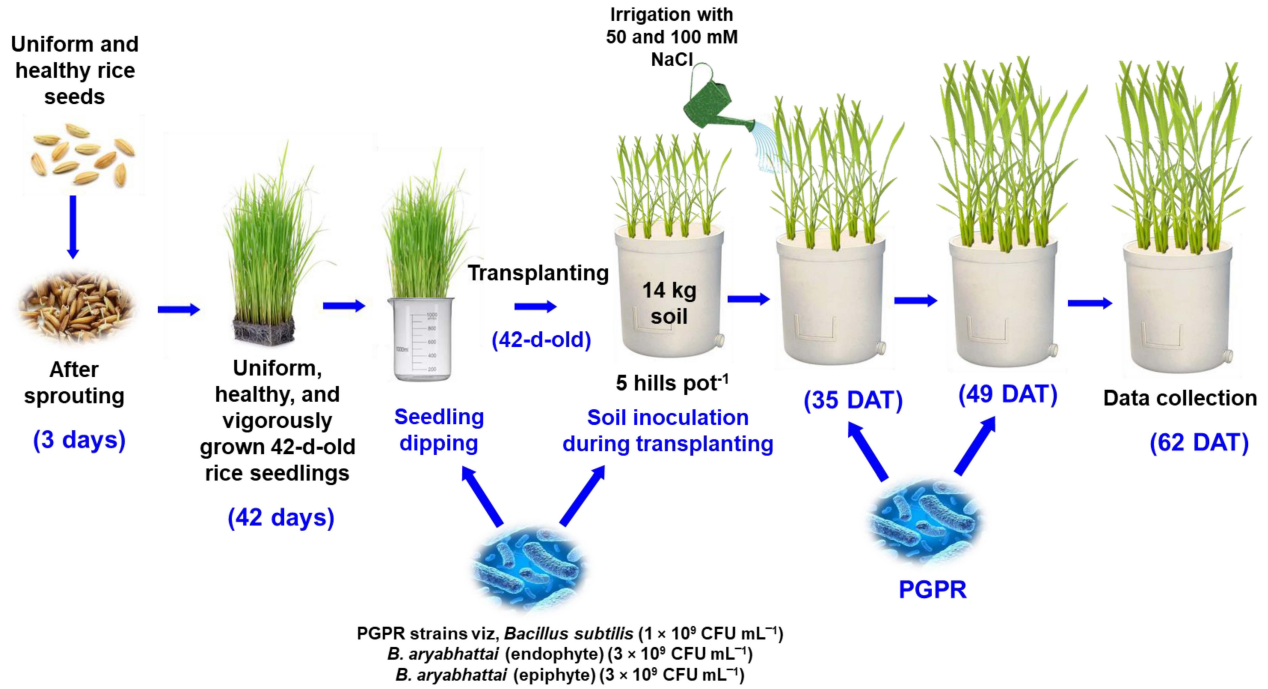

FIGURE 1
Schematic representation of rice plant growth conditions, salt stress impositions, and PGPR strain treatments.

2.3 Measurements of physiological and biochemical attributes

2.3.1 Relative water content and proline content

Leaf relative water content (RWC) was determined by measuring the FW of rice leaf blades. The leaves were then placed in water for 12 h for determination of the turgid weight (TW) and later oven-dried (48 h, 80°C) for measurement of leaf DW. The RWC was determined using the formula: RWC (%) = (FW–DW)/(TW–DW) × 100 (Barrs and Weatherley, 1962). The leaf proline (Pro) content was determined with a spectrophotometer (GENESYS 10S UV-Vis, Thermo Fisher Scientific Inc., Madison WI, USA) using 0.5 g of leaf tissue and the method described by Bates et al. (1973).

2.3.2 Ion content

Leaf Na^+ and K^+ contents were quantified using a portable ion meter (Horiba, Tokyo, Japan). Sap from fresh leaf samples was introduced into the calibrated sensor of the ion meter after rinsing the sensor with deionized water to eliminate residual dirt.

2.3.3 Chlorophyll content

For pigment extraction, 0.25 g of fresh leaf tissue from plants from each treatment was chopped and immersed in a water bath with 10 mL of 100% ethanol at 70°C until they turned white. The colored chlorophyll (Chl) chromophore was then measured spectrophotometrically at wavelengths of 663, 645, and 470 nm. The concentrations of Chl *a*, Chl *b*, and Chl (*a*+*b*) were determined using the method described by Arnon (1949).

2.3.4 Stomatal conductance

Stomatal conductance (g_s) was quantified from the surfaces of fully expanded leaves of individual plants from all experimental treatments using a leaf porometer (model SC-1, Decagon Devices, Inc., Pullman, WA, USA).

2.3.5 Chlorophyll fluorescence

A fluorimeter (Pocket PEA Chlorophyll Fluorimeter, Hansatech Instruments Ltd., Norfolk, UK) was employed to measure the Chl fluorescence of fully expanded leaf blades. The minimum fluorescence (F_o) was recorded in a simulated dark condition using clips. The maximum fluorescence (F_m) was obtained 15 min later by giving a light pulse of 3000 µmol m^{-2} s^{-1}. The photosystem II (PSII) activities were calculated using the following equation: $F_v/F_m = (F_m–F_o)/F_m$ where the variable fluroscence is denoted by F_v.

2.3.6 Indole-3-acetic acid concentration

The concentration of IAA was quantified using previously described methods (Gordon and Weber, 1951). Extracts were prepared from 0.5 g leaf material by grinding in an ice-cooled mortar and pestle in 2 mL 80% cold methanol, followed by centrifugation at 5,000×g for 5 min at 4°C. A 2 mL volume of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$) was then mixed with 1 mL of the supernatant and 2 drops of orthophosphoric acid. Two hours later, the optical density of the solution was measured spectrophotometrically at 530 nm. The IAA concentrations in the samples were determined using an IAA standard curve.

2.4 Estimation of oxidative stress indicators: malondialdehyde, hydrogen peroxide content, and electrolyte leakage (%)

The leaf malondialdehyde (MDA) content was quantified following the method of Heath and Packer (1968), with a slight modification (Hasanuzzaman et al., 2022a). A reaction mixture was prepared by mixing 4 mL of thiobarbituric acid (TBA) reagent (20% TCA + 0.5% TBA) reagent with 1 mL of supernatant. The supernatant was prepared by homogenizing leaf tissues (0.5 g) with 3 mL of 5% trichloroacetic acid (TCA) and centrifuging it at 11,500×g for 10 min at 4°C. Then spectrophotometric absorbance was recorded at 532 and 600 nm after incubating the mixture in a water bath at 95 °C for 30 min and cooling it quickly on ice. The final MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. The method of Yang et al. (2007) was used to determine H_2O_2 content. The reaction mixture was prepared by adding 3 mL of 5% TCA to 0.5 g leaf material and centrifuging, followed by adding 1 ml of 1 M potassium iodide and 3 mL of 50 mM potassium phosphate (K-P) buffer (pH 7.0). The H_2O_2 content was calculated after spectrophotometric readings at 390 nm and using an extinction coefficient of 0.28 μM^{-1} cm^{-1}. Electrolyte leakage (EL%) was measured following the method of Dionisio-Sese and Tobita (1998) and calculated using the following formula: EL = (EC_1/EC_2) × 100.

2.5 Quantification of ascorbate and glutathione content

Ascorbate (AsA) content was determined following the method of Nahar et al. (2016) by preparing leaf extracts in 1 mM ethylenediaminetetraacetic acid in 5% meta-phosphoric acid, centrifuging, mixing with 0.1 M dithiothreitol and distilled water, and neutralizing with 0.5 M K-P buffer (pH 7.0). The total and reduced AsA concentrations were measured spectrophotometrically at A_{265} and the dehydroascorbate (DHA) was calculated by subtracting the concentration of reduced AsA from the total AsA. The glutathione (GHS) content was determined by oxidizing the leaf extracts with 5,5-dithio-bis-2-nitrobenzoic acid and neutralizing with 0.5 M K-P buffer (pH 7.0) in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase (GR), followed by spectrophotometric measurement at A_{412}. The oxidized glutathione (GSSG) content was measured by neutralizing the extract with 2-vinylpyridine and K-P buffer. The final GHS content was estimated by comparison to standard curves for GSH and GSSG (Hasanuzzaman et al., 2022a).

2.6 Enzyme extraction and protein measurement

Enzymes were extracted using a previously described method (Hasanuzzaman et al., 2022a), which involved grinding of 0.5 g leaf tissue in a precooled mortar pestle with an extraction buffer containing 50 mM K-P buffer (pH 7.0) in 1 mM AsA, 5mM β-mercaptoethanol, 10% glycerol, and 100 mM KCl solution. The resultant leaf homogenate was centrifuged for 12 min at 11,500×g at 4°C. The clear supernatant was used to determine antioxidant enzyme activities and the free protein content was determined using the method of Bradford (1976).

2.7 Antioxidant enzyme activity determinations

Ascorbate peroxidase (APX; EC: 1.11.1.11) activity was determined using the method of Nakano and Asada (1981) and an extinction coefficient of 2.8 mM^{-1} cm^{-1}. Dehydroascorbate reductase (DHAR; EC: 1.8.5.1) activity was similarly assayed using an extinction coefficient of 14 mM^{-1} cm^{-1}. The method of Hossain et al. (1984) and an extinction coefficient of 6.2 mM^{-1} cm^{-1} were used to determine the monodehydroascorbate reductase (MDHAR; EC: 1.6.5.4) activity. The method of Hasanuzzaman et al. (2022a) and an extinction coefficient of 6.2 mM^{-1} cm^{-1} were used to measure glutathione reductase (GR; EC: 1.6.4.2) activity.

The activities of glutathione peroxidase (GPX; EC: 1.11.1.9), glutathione-*S*-transferase (GST; EC: 2.5.1.18), and catalase (CAT; EC: 1.11.1.6) were also measured as described previously mentioned method (Hasanuzzaman et al., 2022a), with a slight modification from Elia et al. (2003) for GPX determination. The extinction coefficients for GPX, GST, and CAT were 6.62 mM^{-1} cm^{-1}, 9.6 mM^{-1} cm^{-1}, and 39.4 mM^{-1} cm^{-1}, respectively. Lipoxygenase (LOX; EC: 1.13.11.12) activity was measured using the method by Doderer et al. (1992), with linolenic acid used as a substrate. The method of El-Shabrawi et al. (2010) was used to determine the superoxide dismutase (SOD; EC: 1.15.1.1) activity, using xanthine and xanthine oxidase as substrates. Peroxidase (POD; EC: 1.11.1.7) activity was determined following the method of Hemeda and Klein (1990).

2.8 Methylglyoxal content and glyoxalase enzyme activity determinations

The amount of methylglyoxal (MG) in leaf tissues was estimated using the method described by Wild et al. (2012). The leaf samples were homogenized with 5% perchloric acid, and the concentration of MG was determined by measuring the spectrophotometric absorbance at 288 mm and calculated using a standard curve. The activities of glyoxalase I (Gly I, EC: 4.4.1.5) and glyoxalase II (Gly II, EC: 3.1.2.6), were determined according to Hasanuzzaman et al. (2022a) and Principato et al. (1987) using extinction coefficients of 3.37 and 13.6 mM^{-1} cm^{-1}, respectively.

2.9 Statistical analyses

The data were presented as the mean ± standard deviation of three replications. Tukey's honestly significant difference (HSD) test at $p \leq 0.05$ was used to separate means in the statistical analysis by applying the one-way analysis of variance (ANOVA) technique using the CoStat v.6.400 (2008) computer software.

3 Results

3.1 Effects on the growth attributes

Plant height was reduced by 14 and 17% in response to 50 and 100 mM NaCl stress, respectively, when compared to the unstressed controls (no NaCl treatment). However, the application of *Bacillus subtilis* demonstrated superior performance than other strains by enhancing plant height significantly by 7 and 8% under 50 and 100 mM NaCl stress conditions, respectively, compared to the stressed alone plants. On the other hand, both the endophytic *B. aryabhattai* and epiphytic *B. aryabhattai* applications showed little to no change in plant height under similar stress conditions (Figure 2A).

In the presence of 50 and 100 mM NaCl stress, plant FW was decreased by 58 and 65%, respectively (Figure 2B), while the DW was declined by 39% and 47%, respectively, compared to the unstressed controls (Figure 2C). However, under 50 mM NaCl stress, treatment with *B. subtilis* (51%) and epiphytic *B. aryabhattai* (47%) led to a notable increase in FW compared to the non-inoculated plants, but this difference was not statistically significant under 100 mM NaCl stress (Figure 2B). Similarly, in terms of DW, both *B. subtilis* and epiphytic *B. aryabhattai* outperformed the endophytic *B. aryabhattai* in enhancing plant DW than the non-inoculated plants (Figure 2C).

Both salt stress levels significantly reduced the leaf area compared to unstressed controls (Figure 2D). Nonetheless, all PGPR strains were found to increase leaf area at both stress conditions but *B. subtilis* and epiphytic *B. aryabhattai* showed the greatest enhancements in leaf area by 22% and 19%, respectively, under only 50 mM salt stress (Figure 2D).

3.2 Effects on photosynthetic attributes

Chlorophyll *a* and Chl *b* contents in rice leaves were decreased significantly under both 50 and 100 mM NaCl stress conditions compared to the control (Table 1). This decline eventually led to the reduction of total Chl (*a*+*b*) content. However, salinity-stressed plants treated with PGPRs showed significantly increased amounts of photosynthetic pigment contents compared to non-treated plants under similar stress conditions. *B. subtilis* and epiphytic *B. aryabhattai* were most effective in restoring the Chl pigments in all cases specifically, under 100 mM NaCl stress. Moreover, among those PGPR strains, *B. subtilis* outperformed the latter by significantly enhancing Chl *a* (25%), Chl *b* (74%), and Chl (*a*+*b*) (43%) contents (Table 1). Though endophytic *B. aryabhattai*

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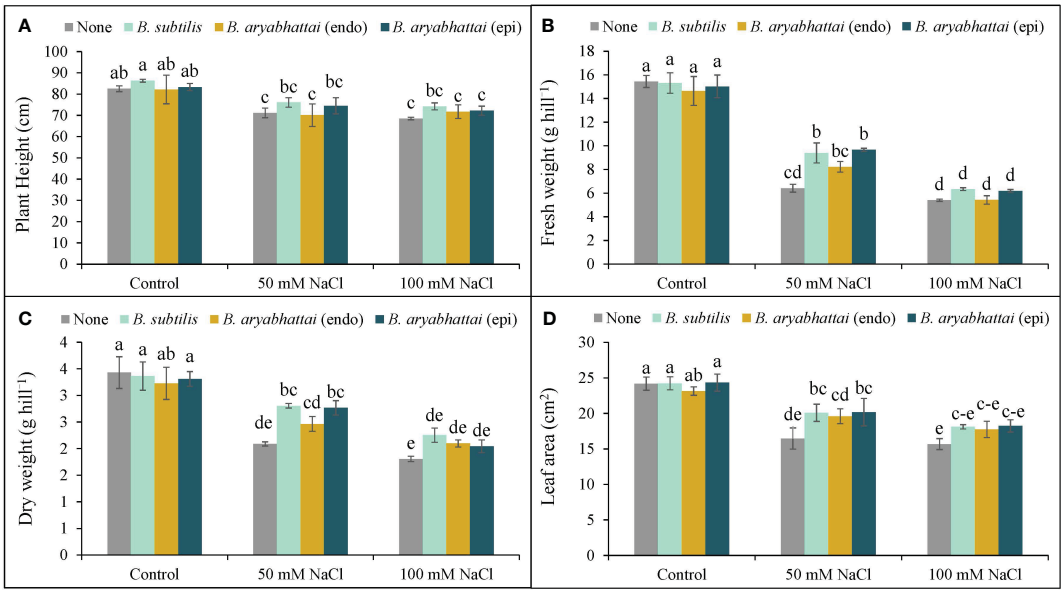

FIGURE 2
Variations in plant height **(A)**, fresh weight **(B)**, dry weight **(C)**, and leaf area **(D)** of rice plants under salt stress (50 or 100 mM NaCl) in the absence or presence of three PGPRs (*Bacillus subtilis*, epiphytic *B. aryabhattai*, and endophytic *B. aryabhattai*). Data are presented as mean ± standard deviation of three replications (n=3). Distinct letters on the bars show significant differences between treatments at $p \leq 0.05$ from Tukey's HSD test.

TABLE 1 Changes in photosynthetic attributes of rice plants under salt stress (S_1 = 50 mM NaCl; S_2 = 100 mM NaCl) in the absence or presence of three PGPRs (*Bacillus subtilis*, epiphytic *B. aryabhattai*, and endophytic *B. aryabhattai*).

Treatments	Chl *a* content (mg g^{-1} FW)	Chl *b* content (mg g^{-1} FW)	Chl (*a*+*b*) content (mg g^{-1} FW)	Stomatal conductance (mmol $m^{-2}s^{-1}$)	Chlorophyll fluorescence (F_v/F_m)
Control	1.34 ± 0.01 a	1.36 ± 0.10 a	2.70 ± 0.09 a	41.40 ± 1.50 a	0.77 ± 0.01 ab
B. subtilis	1.31 ± 0.01 a	1.49 ± 0.07 a	2.80 ± 0.09 a	40.90 ± 1.96 ab	0.76 ± 0.01 ab
B. aryabhattai (endo)	1.31 ± 0.00 a	1.35 ± 0.11 a	2.67 ± 0.11 ab	39.70 ± 0.58 abc	0.77 ± 0.01 ab
B. aryabhattai (epi)	1.33 ± 0.01 a	1.44 ± 0.07 a	2.78 ± 0.06 a	40.80 ± 1.50 ab	0.76 ± 0.01 ab
S_1	1.11 ± 0.07 bc	0.79 ± 0.05 d	1.89 ± 0.11 ef	35.90 ± 0.92 de	0.73 ± 0.01 cd
S_1+ *B. subtilis*	1.31 ± 0.02 a	1.11 ± 0.03 b	2.42 ± 0.05 bc	36.30 ± 0.23 cde	0.75 ± 0.02 bcd
S_1+ *B. aryabhattai* (endo)	1.19 ± 0.09 ab	1.04 ± 0.06 bc	2.23 ± 0.08 cd	35.50 ± 0.72 de	0.76 ± 0.01 ab
S_1+ *B. aryabhattai* (epi)	1.30 ± 0.03 a	1.07 ± 0.04 b	2.37 ± 0.07 c	35.85 ± 0.40 de	0.78 ± 0.00 ab
S_2	0.90 ± 0.07 d	0.51 ± 0.04 e	1.41 ± 0.05 g	34.70 ± 0.69 e	0.72 ± 0.01 d
S_2+ *B. subtilis*	1.13 ± 0.03 ab	0.88 ± 0.03 cd	2.01 ± 0.04 de	38.65 ± 0.40 a-d	0.76 ± 0.00 abc
S_2+ *B. aryabhattai* (endo)	1.03 ± 0.09 cd	0.76 ± 0.04 d	1.79 ± 0.06 f	37.80 ± 0.81 a-d	0.77 ± 0.01 ab
S_2+ *B. aryabhattai* (epi)	1.12 ± 0.02 bc	0.07 ± 0.05 d	1.94 ± 0.06 ef	36.90 ± 1.50 b-e	0.78 ± 0.01 a

Data are presented as mean ± standard deviation of three replications (n=3). Distinct letters on each column show significant differences between treatments at $p \leq 0.05$ from Tukey's HSD test.

escalated the photosynthetic pigment contents under both stress levels than the non-inoculated plants, the increments were not as significant as the other PGPR strains (Table 1).

Furthermore, g_s was decreased in a dose-dependent manner with increased salinity levels compared to the unstressed controls. The addition of all three PGPRs resulted in only a negligible increment in g_s under 50 mM NaCl stress compared to the salt-stressed plants (Table 1). Whereas, application of *B. subtilis* and epiphytic *B. aryabhattai*, increased the g_s significantly by 10% and 9%, respectively, under 100 mM salt stress (Table 1). A notable reduction (7%) in the F_v/F_m ratio was observed when plants were subjected to 100 mM NaCl stress relative to the control (Table 1). Though all the PGPR treatments restored the ratio in both doses of salt stress, the increment by epiphytic *B. aryabhtattai* was significant (8%) in 100 mM NaCl stress than the non-inoculated plants.

3.3 Effect on the physiological attributes

3.3.1 Osmotic adjustment and relative water content

The RWC was reduced under both 50 and 100 mM NaCl stress with a significant reduction (26%) under higher salinity dose compared to the unstressed controls (Figure 3A). However, PGPR treatments increased the RWC under both stress conditions, where, the improvement by *B. subtilis* was the highest (19%) under 100 mM NaCl stress compared to the salt-stressed controls (Figure 3A). Compared to the non-stressed controls, Pro content significantly increased in rice plants when exposed to increasing levels of salinity stress with the highest increment (327%) under 100 mM NaCl stress. The application of PGPR improved this condition by reducing the excessively generated Pro content in all treatments, where *B. subtilis* performed the best in reducing the Pro content (16%) compared to the salt-stressed controls under 100 mM salinity stress (Figure 3B).

3.3.2 Ion homeostasis

The application of 50 and 100 mM NaCl stress disrupted the ion homeostasis in rice plants, as evidenced by increased Na^+ accumulation as well as decreased K^+ accumulation, resulting in a 40 and 53-fold increase in the Na^+/K^+ ratio, respectively, compared to control plants (Figures 4A–C). Nevertheless, PGPR treatments reversed this imbalance by preserving ion homeostasis by significantly reducing Na^+ accumulation and enhancing K^+ uptake through rice plant roots. Among them, the greatest reduction (81%) in Na^+ was noted with *B. subtilis* inoculation under 100 mM NaCl stress, leading to a significant increase (67%) in K^+ accumulation (Figures 4A, B), which restored the Na^+/K^+ ratio by nearly 89% (Figure 4C) compared to the stressed plants.

3.3.3 Indole-3-acetic acid content

In comparison to the unstressed control, the concentration of IAA significantly decreased in rice plants exposed to increasing levels of salinity stress. Specifically, plants subjected to 100 mM NaCl stress demonstrated a significant IAA reduction (32%) compared to the controls (Supplementary Figure 1). However, the application of PGPRs ameliorated this condition by boosting the concentrations under both salinity conditions. Notably, among the three PGPRs, epiphytic *B. aryabhattai* was the most effective under both salinity levels, increasing IAA concentrations by approximately 49 and 92%, respectively, compared to stressed plants (Supplementary Figure 1).

3.4 Oxidative stress indicators

A significant rise in MDA content was observed with increasing salinity levels, where the highest (58%) lipid peroxidation was noted under 100 mM of NaCl stress compared to the controls (Figure 5A). Though PGPR treatment significantly reduced the MDA content in both stress conditions, *B. subtilis* outperformed other strains by reducing the MDA content by nearly 31 and 29% under 50 and 100 mM NaCl stress, respectively, compared to salt stress alone plants (Figure 5A).

Similarly, increasing levels of salinity doses corresponded with a rise in H_2O_2 levels, leading to membrane damage in rice plants. Under 100 mM NaCl stress, H_2O_2 levels rose substantially (69%) compared to the unstressed controls. However, PGPR treatment notably mitigated this effect with the greatest reduction (32%) in H_2O_2 level by *B. subtilis* under 100 mM NaCl stress, compared to the untreated plants (Figure 5B).

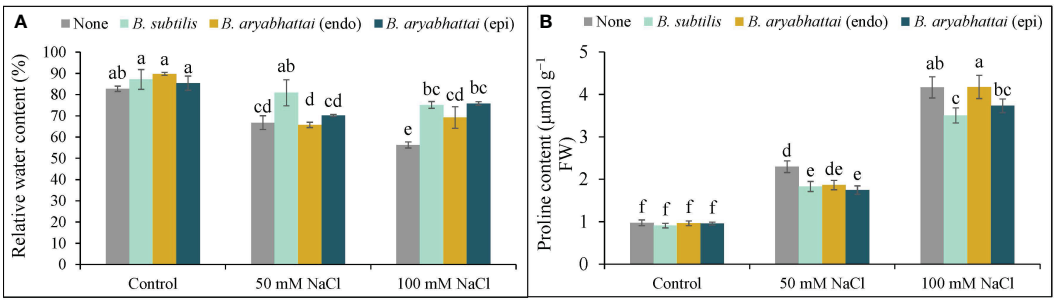

FIGURE 3
Changes in relative water content **(A)**, and proline content **(B)** of rice plants under salt stress (50 and 100 mM NaCl) in the absence or presence of three PGPRs (*Bacillus subtilis*, epiphytic *B. aryabhattai*, and endophytic *B. aryabhattai*). Data are presented as mean ± standard deviation of three replications (n=3). Distinct letters on the bars show significant differences between treatments at $p \leq 0.05$ from Tukey's HSD test.

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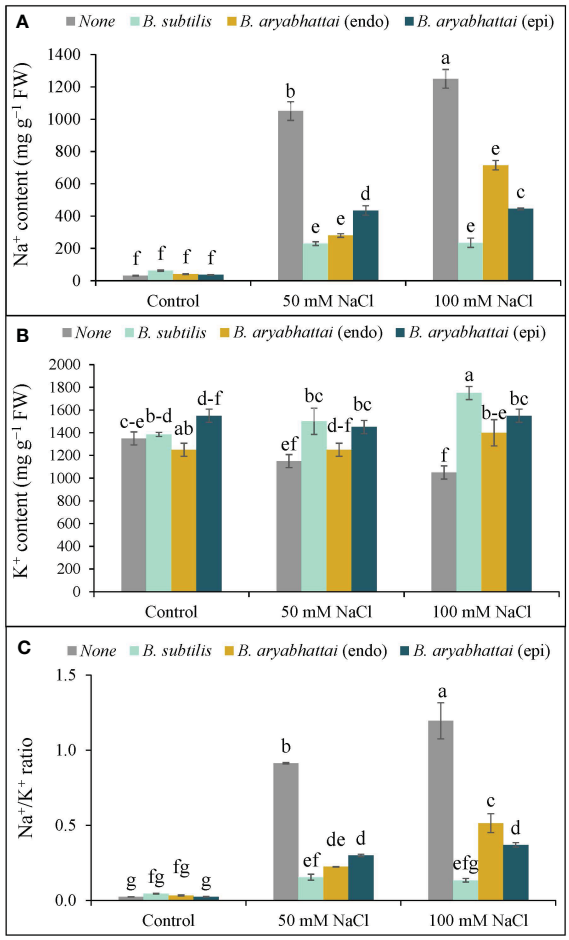

FIGURE 4
Variations in Na^+ content **(A)**, K^+ content **(B)**, and Na^+/K^+ ratio **(C)** of rice plants under salt stress (50 and 100 mM NaCl) in the absence or presence of three PGPRs (*Bacillus subtilis*, epiphytic *B. aryabhattai*, and endophytic *B. aryabhattai*). Data are presented as mean ± standard deviation of three replications (n=3). Distinct letters on the bars show significant differences between treatments at $p \leq 0.05$ from Tukey's HSD test.

Likewise, EL% was also increased under increasing salinity levels, mirroring the trend observed in MDA and H_2O_2 contents. The highest EL% (20%) was noticed under 100 mM NaCl stress, which was almost double the leakage occurring in plants exposed to 50 mM NaCl salt stress compared to the unstressed controls (Figure 5C). Application of PGPR decreased the leakage significantly under 50 and 100 mM NaCl stress compared to the salt-stressed controls, where the highest decrease (12%) was noted with *B. subtilis* treatment under 50 mM NaCl salt stress (Figure 5C).

3.5 Effects on antioxidant defense systems

3.5.1 AsA-GSH pools

Increasing salinity levels negatively affected AsA content with a significant reduction (53%) observed under 100 mM NaCl stress than the unstressed controls. However, the application of PGPRs mitigated this stress by increasing AsA content. *B. subtilis* was particularly effective than other PGPR strains, increasing AsA levels by 15 and 27% under 50 and 100 mM NaCl stress, respectively, than the non-inoculated controls (Figure 6A). The highest DHA content (89%) was observed under 100 mM NaCl stress and was approximately 1.5 times higher than that observed under 50 mM NaCl stress compared to the salt-stressed controls (Figure 6B). However, PGPRs ameliorated this effect, where *B. subtilis* notably reduced the DHA content (16%) at 100 mM NaCl stress than other strains compared to the salt-stressed controls (Figure 6B). Consequently, due to salt stress-induced reduction in AsA content and increase in DHA contents, the AsA/DHA ratio decreased than the non-stresses controls (Figure 6C). However, applying endophytic PGPRs restored the ratio under 50 and 100 mM NaCl stress, compared to plants only subjected to salt stress. Furthermore, among them, epiphytic *B. aryabhattai* increased the ratio (39%) under 50 mM NaCl stress, compared to the stressed controls. Except for *B. subtilis*, other PGPRs could not revert the increased AsA/DHA ratio under higher salinity levels (Figure 6C).

Compared to the control, GSH content increased by 33 and 94% under 50 and 100 mM NaCl stress, respectively (Figure 6D).

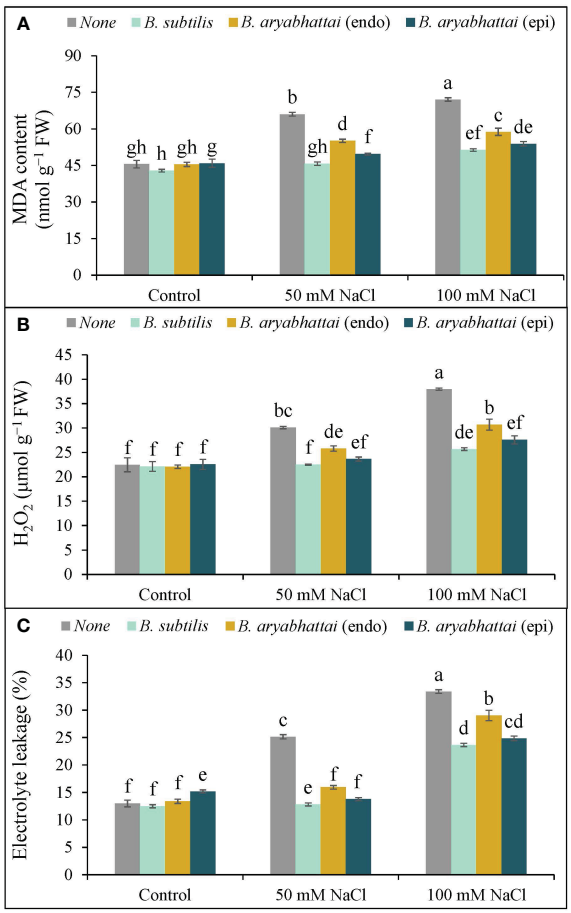

FIGURE 5
Variations in MDA content **(A)**, H_2O_2 content **(B)** and electrolyte leakage (%) **(C)** of rice plants under salt stress (50 and 100 mM NaCl) in the absence or presence of three PGPRs (*Bacillus subtilis*, epiphytic *B. aryabhattai*, and endophytic *B. aryabhattai*). Data are presented as mean ± standard deviation of three replications (n=3). Distinct letters on the bars show significant differences between treatments at $p \leq 0.05$ from Tukey's HSD test.

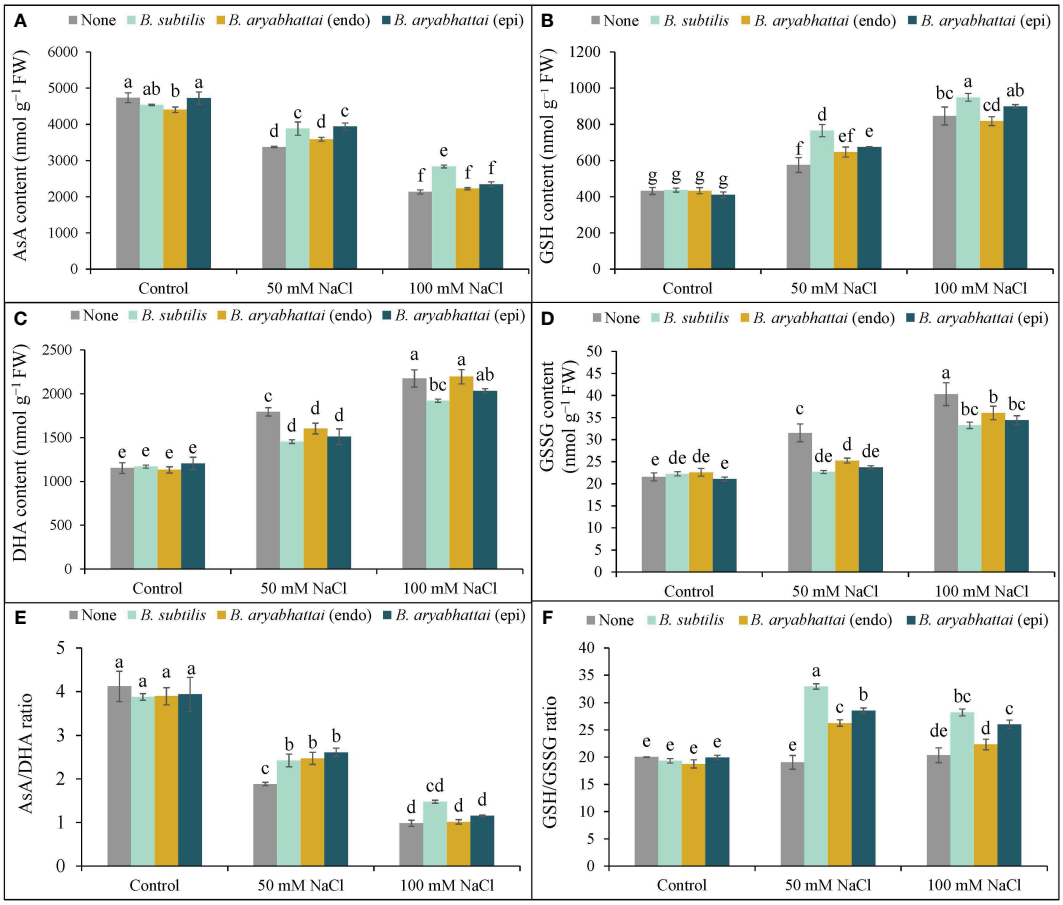

FIGURE 6
Variations in AsA content **(A)**, DHA content **(B)**, AsA/DHA ratio **(C)**, GSH content **(D)**, GSSG content **(E)** and GSH/GSSG ratio **(F)** of rice plants under salt stress (50 and 100 mM NaCl) in the absence or presence of three PGPRs (*Bacillus subtilis*, epiphytic *B. aryabhattai*, and endophytic *B. aryabhattai*). Data are presented as mean ± standard deviation of three replications (n=3). Distinct letters on the bars show significant differences between treatments at $p \leq 0.05$ from Tukey's HSD test.

The application of PGPRs further enhanced the GSH content under both salt stress conditions. The most substantial increase was found with *B. subtilis* application: a 25 and 12% increase at 50 and 100 mM saline conditions, compared to the salt-stressed plants only. The effect of other PGPRs was not significant at higher saline doses (Figure 6D). The level of GSSG content significantly increased by (87%) under 100 mM NaCl stress, compared to the controls (Figure 6E). However, PGPR treatments reduced the GSSG levels in salt-stressed plants. Application of *B. subtilis* gave the most significant reduction (28%) in 50 mM NaCl-stressed plants, compared to the salt-stressed controls (Figure 6F). However, the effect of PGPRs in reducing GSSG content was not significant at higher salt stress levels. The severity of the stress substantially decreased the GSH/GSSG ratio compared to the control. However, PGPR treatment recovered the GSH/GSSG ratio in salt-stressed rice plants with the most significant improvement (73%) in the ratio observed at 50 mM NaCl stress with the *B. subtilis* application (Figure 6F). The epiphytic PGPR, *B. aryabhattai*, performed better in increasing the GSH/GSSG ratio under both salt-stressed conditions, improving by nearly 50 and 28% under 50 and 100 mM NaCl stress, respectively. However, the endophytic PGPR, *B. aryabhattai*, was not as effective in reverting the GSH/GSSG ratio at both salinity stress levels, increasing the ratio by nearly 38% at 50 mM NaCl stress but showing a 3-fold lesser reduction under higher salinity stress. Therefore, among the three PGPRs, *B. subtilis* was most effective in restoring the AsA-GSH pool of salt-induced rice plants.

3.5.2 Antioxidant enzyme activities

A rise in APX activity was observed following the exposure to two different salinity levels with the most significant increase (250%) found under 100 mM NaCl stress, compared to controls, and was further increased by the application of PGPRs (Figure 7A). However, *B. subtilis* showed the best result among the other PGPRs, under higher salinity level by improving the APX activity by 26% than the stressed plants alone (Figure 7A). Similarly, MDHAR activity was also increased by 63 and 144% to the control under two different salinity doses, and further improvements were also noticed when rice plants were treated with three different PGPRs. However, among them, likewise APX activity, *B. subtilis* further improved the MDHAR activity (25%) than the salt-stressed alone plants (Figure 7B). A similar trend was also noticed in terms of the rise

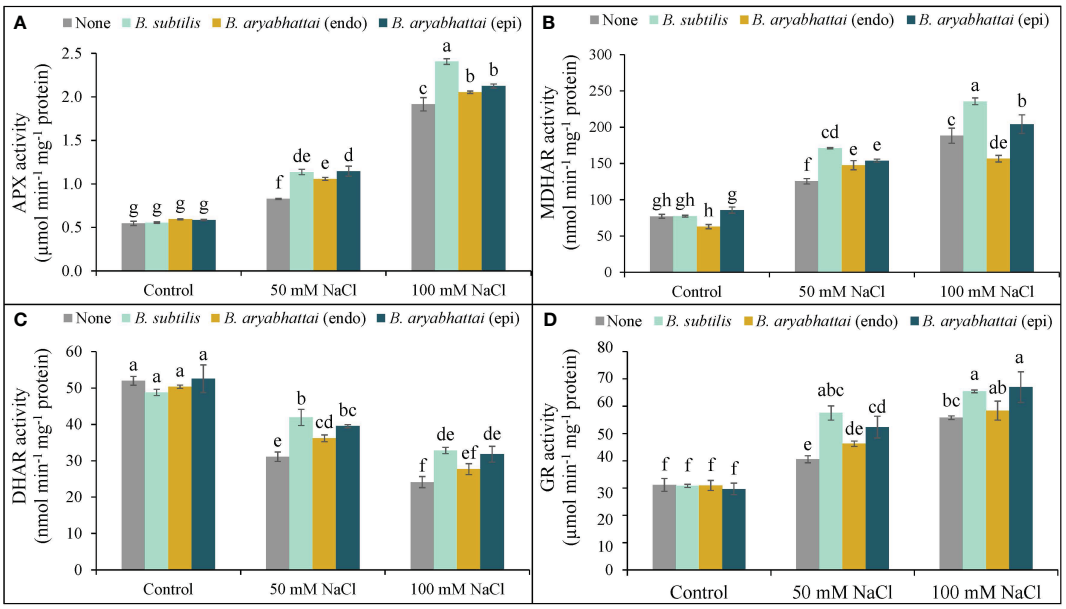

FIGURE 7
Changes in the activities of APX **(A)**, MDHAR **(B)**, DHAR **(C)**, and GR **(D)** of rice plants under salt stress (50 and 100 mM NaCl) in the absence or presence of three PGPRs (*Bacillus subtilis*, epiphytic *B. aryabhattai*, and endophytic *B. aryabhattai*). Data are presented as mean ± standard deviation of three replications (n=3). Distinct letters on the bars show significant differences between treatments at $p \leq 0.05$ from Tukey's HSD test.

in GR activity which was then further enhanced by the application of *B. subtilis*. However, here, both *B. subtilis* and epiphytic *B. aryabhattai* performed a significant role in increasing GR activity by 17 and 20%, respectively under 100 mM NaCl stress (Figure 7D). On the other hand, DHAR activity was noticeably reduced under 50 and 100 mM NaCl stress, compared to the unstressed controls (Figure 7C). But, in this case, both *B. subtilis* and epiphytic *B. aryabhattai* showed a significant acceleration in DHAR activity than the stressed plants with the highest increment (35%) by *B. subtilis* at 50 mM NaCl compared to the salt-stressed controls (Figure 7C).

Rice plants exposed to two different salinity levels showed a notable reduction in the activities of GPX and SOD relative to the controls (Figures 8A, D). The application of PGPRs reverted this situation by increasing both antioxidant enzyme activities but the performance was better under the lower salinity dose. However, as previously found, a similar trend of the better activity of *B. subtilis* was also noticed for GPX, where the improvement was 37% than the stressed alone plants under 50 mM NaCl (Figure 8A). In terms of SOD, all three PGPRs performances were significantly similar under both stress conditions (Figure 8D).

On the other hand, the application of PGPRs on salt-stressed rice plants had notable positive effects in terms of the other antioxidant enzymes, e.g., GST, LOX, CAT, and POD. The highest increment of GST, LOX, CAT, and POD activities was found by nearly 143, 160, 111, and 137%, respectively, under 100 mM NaCl stress in rice plants than the non-stressed controls (Figures 8B–F). Nevertheless, the PGPRs application further boosted their activities (GST, CAT, and POD), and here, both *B. subtilis* and epiphytic *B. aryabhattai* were found to have almost similar significant positive results. Additionally, both of these PGPR strains performed better in reducing the LOX activity by 43 and 38% under 50 mM NaCl stress, where the levels were prominently increased by 112 and 160% with increasing salinity doses (Figure 8C).

3.6 Glyoxalase system

Salt stress also affected the glyoxalase system of rice plants which was evident with the highest (59%) increase in MG content under 100 mM NaCl stress compared to the controls (Supplementary Figure 2A). However, *B. subtilis* along with the endophytic and epiphytic *B. aryabhattai* changed this situation by reducing the MG content, though, in this case, epiphytic *B. aryabhattai* performed better under 100 mM NaCl stress by reducing it by 39% than the stressed alone rice plants (Supplementary Figure 2A). On the other hand, the activities of Gly I and Gly II were sharply reduced under salt stress (Supplementary Figures 2B, C) in contrast with the control plants. However, both *B. subtilis* and epiphytic *B. aryabhattai* showed statistically similar results in boosting the Gly I and Gly II activities under two different salt stress levels (Supplementary Figures 2B, C).

4 Discussion

The initial response of plants to salinity stress involves osmotic shock and ionic imbalances, which disrupt water uptake, break down cell membranes, and inhibit stomatal opening; ultimately restrict cell division, cell enlargement, photosynthesis, plant growth, and development (Rajabi et al., 2024). In this experiment, salinity-induced decreases in photosynthetic attributes (Table 1) and increases in lipid peroxidation (Figure 5) resulted in a reduction

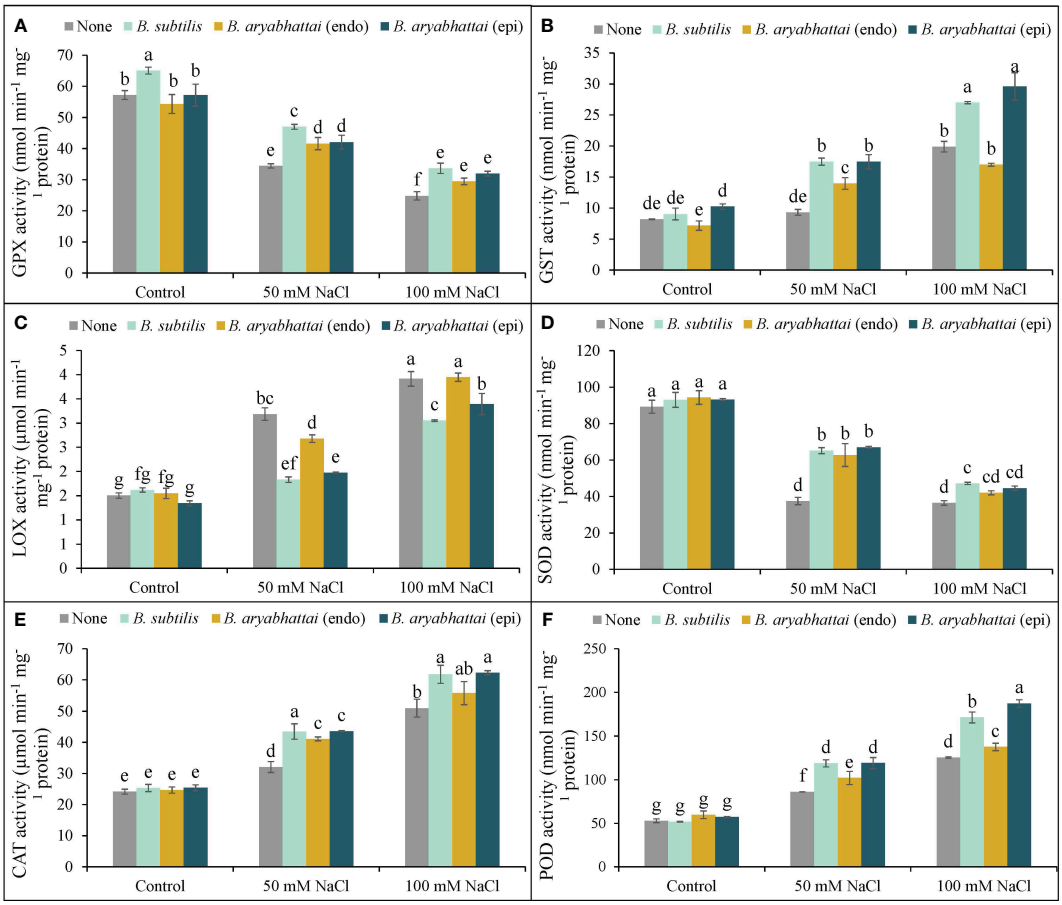

FIGURE 8
Variations in GPX **(A)**, GST **(B)**, LOX **(C)**, SOD **(D)**, CAT **(E)**, and POD **(F)** activities of rice plants under salt stress (50 and 100 mM NaCl) in the absence or presence of three PGPRs (*Bacillus subtilis*, epiphytic *B. aryabhattai*, and endophytic *B. aryabhattai*). Data are presented as mean ± standard deviation of three replications (n=3). Distinct letters on the bars show significant differences between treatments at $p \leq 0.05$ from Tukey's HSD test.

in plant growth parameters (Figure 2). However, application of PGPR strains alleviated salt stress and improved plant growth parameters by restoring photosynthetic efficiency and safeguarding the cell membranes. These improvements can be linked to PGPR-induced synthesis of IAA (Supplementary Figure 1). Auxin/IAA induces a variety of morphophysiological changes, such as increased root length, root surface area, nutrient uptake, and photosynthesis (Li et al., 2020; Iqbal et al., 2023). Moreover, microbial solubilization of iron (Rahimi et al., 2020) and magnesium (Ullah et al., 2022), combined with the stress-induced synthesis of siderophores by PGPRs may have led to the regeneration of the photosynthetic pigments, as well as restoration of F_v/F_m and g_s. Our findings concur with those of Wang et al. (2023), who reported that PGPR application improved the photosynthetic efficiency of rice under salt stress. Although siderophore synthesis by the PGPR strains was not investigated in this current experiment, previous studies (Sultana et al., 2021; Ghazy and El-Nahrawy, 2021) have provided evidence that these three *Bacillus* strains are capable of synthesizing siderophores. All these responses contribute to improving plant growth in stressful environments, in agreement with the findings of Shultana et al. (2020) and Shultana et al. (2021).

Salinity-induced osmotic and ionic stresses create imbalances in the ion homeostasis of plant cells (Hu et al., 2022; Zhao et al., 2023), as confirmed in the present study by the elevated Na^+/K^+ ratio. However, PGPR application restored the ionic and osmotic balance by decreasing Na^+ accumulation and increasing K^+ absorption by the roots (Figure 4), and by reducing Pro accumulation and enhancing RWC (Figure 3). One explanation could be that bacterial EPS obstructs Na^+ deposition on plant root surfaces (Shultana et al., 2020). These findings align with the study of Ji et al. (2022), which highlighted how wheat seedlings inoculated with PGPR under salt stress could stave off osmotic stress by regulating Pro and soluble sugar accumulation.

Excessively produced ROS induces oxidative stress in plants which is the secondary effect of salt stress. In this experiment, rice plants showed clear symptoms of salt stress-induced oxidative stress by increasing the stress indicators (Figure 5). However, to counteract the potential for ROS-induced damage, plants possess an intrinsic antioxidant defense mechanism containing enzymatic and non-enzymatic antioxidants, which is highly effective in preventing ROS production and regulating homeostasis, thereby safeguarding plant cells from oxidative damage (Rajabi et al., 2024; Yang et al., 2024). In this experiment, the balance between non-

enzymatic antioxidants (AsA/DHA and GSH/GSSG) ratios in the AsA-GSH pool was disrupted (Figure 6) due to salt-induced oxidative stress which matches the results of other studies (Soliman et al., 2020; Zhu et al., 2020). However, the application of PGPR restored the ratios, suggesting ROS detoxification under salt stress, in agreement with the results of Puthiyottil and Akkara (2021).

In addition to non-enzymatic antioxidants, plants also possess antioxidant enzymes, such as APX, DHAR, MDHAR, and GR, which catalyze crucial reactions to detoxify ROS and maintain the AsA-GSH pool under stress (Kanwal et al., 2024). In the present study, salt stress disrupted these enzyme activities, but PGPR application ameliorated the salt-induced oxidative stress by stimulating them (Figure 7). Similar results have also been reported by Ali et al. (2022) in rice under salt stress. Moreover, in the present study, increased CAT and POD activities and reduced SOD activities were noted under salt stress, possibly indicating preferential ROS scavenging and regulation of $-OH^{\bullet}$ radical formation, also reported previously (Hu, 2019; Mubeen et al., 2022). However, PGPR application enhanced SOD activity, as well as CAT and POD (Figure 8), which further supports the findings of Hu (2019) in wheat under salt stress. Glutathione peroxidase uses GSH and thioredoxins to detoxify H_2O_2 as part of the non-heme group of POD, indicating the benefits of upregulating GPX activities under stress (Hasanuzzaman et al., 2022b). The present experiment showed a clear increment in GPX activity after the application of PGPR strains in salt-treated rice plants, with *B. subtilis* showing the most significant effect, in agreement with the study by Hasanuzzaman et al. (2022b). On the contrary, epiphytic *B. aryabhattai* was the most effective PGPR at increasing GST activity, in agreement with findings by Shultana et al. (2021), indicating its potential as a modulator of antioxidant enzyme activities in rice under salt stress.

Plants produce a certain amount of MG under normal conditions as well, but the production increases under stress. Methylglyoxal detoxification by the glyoxalase systems occurs with the help of the GSH enzyme, which converts MG into S-D-lactoylglutathione (SLG) using Gly I, followed by the breakdown of SLG into D-lactic acid by Gly II (Hasanuzzaman et al., 2022b). The current study showed a trend toward elevated MG production under salt stress, coupled with reductions in Gly I and Gly II activities (Supplementary Figure 2), in agreement with the findings of a previous study (Alabdallah et al., 2024). However, application of PGPR strains increased the level of GSH, thereby detoxifying MG by enhancing Gly I and Gly II activities, as previously reported by Kapadia et al. (2022).

Taken together, the findings presented here for salt-stressed rice plants clearly indicate that PGPR strains have the potential to ameliorate salt stress in rice by enhancing antioxidant enzyme activities and regulating key cellular biochemical pathways. However, the efficacy of PGPR may depend on many other factors, such as the plant species or variety, stress types and intensity, and bacterial strain characteristics. The specific mechanism underlying the salt tolerance conferred by PGPR is also unclear and remains largely unanswered. The findings presented here for PGPR effects on salt tolerance in rice plants highlight the usefulness of PGPR in sustainable agriculture and the need for more research on the complex mechanisms underlying the capacity of PGPR to mitigate salinity.

5 Conclusions

Our study comprehensively evaluated the impact of salinity stress on various morphophysiological attributes of rice plants and highlighted significant reductions in growth, photosynthetic efficiency, and hormonal regulation, along with increased oxidative damage and ionic imbalance, as key features of salt stress in rice. The application of PGPR showed encouraging and promising potential for alleviating the detrimental effects of salt stress on rice. Specifically, PGPR treatment enhanced nutrient uptake, bolstered hormone synthesis, restored ionic equilibrium, and bolstered antioxidant defenses, culminating in notable improvements in plant growth. Notably, among the tested PGPR strains, *Bacillus subtilis* emerged as particularly effective in mitigating salinity-induced toxicity and boosting plant tolerance. *B. aryabhattai*, as both an endophyte and an epiphyte, demonstrated commendable effects in enhancing rice plant resilience to salt stress; however, *B. subtilis* set a benchmark for efficacy. These findings underscore the practical applicability of PGPR in sustainable agriculture and the need for further investigation into the intricate mechanisms underpinning their salinity-mitigating properties and their potential impacts on grain quality enhancement under saline conditions. Moving forward, field trials focusing on incorporating PGPR inoculation, particularly in conjunction with salt-tolerant rice varieties are needed, for elucidating their precise effects on yield-contributing parameters and economic benefits. Such studies will thereby, advance our understanding and application of these beneficial microbial agents in saline environments.

Data availability statement

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

Author contributions

AS: Data curation, Investigation, Writing – original draft. AR: Investigation, Methodology, Writing – review & editing. SN: Methodology, Resources, Writing – review & editing. AK: Writing – review & editing. MK: Methodology, Resources, Supervision, Writing – review & editing. PP: Conceptualization, Funding acquisition, Resources, Writing – review & editing. MH: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research is funded by the U.S. Agency for International

Development (USAID) under CFDA# 98.001 – through Feed the Future Sustainable Intensification Innovation Lab (Cooperative Agreement No. AID-OAA-L-14–00006) at Kansas State University.

Acknowledgments

The authors also would like to thank the Feed the Future Innovation Lab for Collaborative Research on Sustainable Intensification for supporting this research. The authors acknowledge the "Innovation Lab on Sustainable Intensification," project of Kansas State University for the assistance. The authors also acknowledge Farzana Nowroz, Md. Mahabub Alam, Md. Rakib Hossain Raihan, and A.H.M. Amir Faisal for their help during data collection. Contribution no. 24-248-J from the Kansas Agricultural Experiment Station is acknowledged.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1419764/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Original Research
PUBLISHED 01 July 2024
DOI 10.3389/fpls.2024.1408125

OPEN ACCESS

EDITED BY
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RECEIVED 27 March 2024
ACCEPTED 22 May 2024
PUBLISHED 01 July 2024

CITATION
del-Canto A, Sanz-Saez A, Heath KD, Grillo MA, Heras J and Lacuesta M (2024) Conventional management has a greater negative impact on *Phaseolus vulgaris* L. rhizobia diversity and abundance than water scarcity.
Front. Plant Sci. 15:1408125.
doi: 10.3389/fpls.2024.1408125

Conventional management has a greater negative impact on *Phaseolus vulgaris* L. rhizobia diversity and abundance than water scarcity

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Introduction: Drought is one of the biggest problems for crop production and also affects the survival and persistence of soil rhizobia, which limits the establishment of efficient symbiosis and endangers the productivity of legumes, the main source of plant protein worldwide.

Aim: Since the biodiversity can be altered by several factors including abiotic stresses or cultural practices, the objective of this research was to evaluate the effect of water availability, plant genotype and agricultural management on the presence, nodulation capacity and genotypic diversity of rhizobia.

Method: A field experiment was conducted with twelve common bean genotypes under irrigation and rain-fed conditions, both in conventional and organic management. Estimation of the number of viable rhizobia present in soils was performed before the crop establishment, whereas the crop yield, nodule number and the strain diversity of bacteria present in nodules were determined at postharvest.

Results: Rainfed conditions reduced the number of nodules and of isolated bacteria and their genetic diversity, although to a lesser extent than the agrochemical inputs related to conventional management. In addition, the effect of water scarcity on the conventional management soil was greater than observed under organic conditions.

Conclusions: The preservation of diversity will be a key factor to maintain crop production in the future, as problems caused by drought will be exacerbated by climate change and organic management can help to maintain the biodiversity of soil microbiota, a fundamental aspect for soil health and quality.

KEYWORDS

genomic fingerprinting, nodulation, organic management, common bean, strain diversity, yield

1 Introduction

Nitrogen-fixing bacteria are a widely distributed phylogenetic group of prokaryotic microorganisms that play a crucial role in the functioning of ecosystems since they are involved in the entry of nitrogen into the soils (Borges et al., 2016). These microorganisms take atmospheric nitrogen (N_2), the most abundant component of the atmosphere, and convert it to assimilable nitrogen for plants (NH_4^+), using the nitrogenase enzyme. Although most species can fix nitrogen in their free-living form, some of the microorganisms need to be associated with plants, thus symbiotic associations account for 50–70% of biological nitrogen fixation (BNF) in the world (Prasuna, 2014; Simon et al., 2014).

This symbiosis provides legumes a relevant ecological advantage as well as exceptional nutritional properties. Through BNF, legumes fulfil the N requirements needed for their growth (Masson-Boivin and Sachs, 2018; Song et al., 2024). This reduces the need of synthetic N fertilizers and improves the N content of the soils, increasing their fertility and, enabling crop development in N poor soils (Aserse et al., 2020; Lindström and Mousavi, 2020). Thereby, in farming systems, legumes are often used in crop rotation, as well as green fertilizers (Araújo et al., 2015; Aserse et al., 2020). Based on their ability to colonize low-N environments and represent an alternative for saving inputs and conserving resources, the Food and Agriculture Organization of the United Nations (FAO, 2013), considers legumes as one of the most promising components of the climate smart agriculture concept.

Drought is the most severe abiotic stress in agriculture, limiting crop growth and yields, and due to climate change, drought events are expected to increase in the early years, especially in southern Europe (IPCC, 2021). It is therefore essential to seek strategies to maintain food security in a sustainable way under water-limited conditions, and the selection of drought tolerant genotypes is one of the most important goals in breeding programs.

However, in the case of legumes, several authors have suggested that selecting drought tolerant rhizobia strains could be a more determining factor in drought tolerance than selecting a drought tolerant genotype (Mhadhbi et al., 2011; Sharaf et al., 2019). Thus, the establishment of symbiotic relationships with efficient rhizobia can alleviate the effects of stress in legumes (Igiehon et al., 2021; Omari et al., 2022; Oviya et al., 2023). This is the case of common bean, where it has been observed that symbiosis with drought-tolerant rhizobia improves plant tolerance to stress as well as legume yield and quality (Steiner et al., 2020; Rodiño et al., 2021; del-Canto et al., 2023), even under field conditions (Pastor-Bueis et al., 2019; Rodiño et al., 2021).

Rhizobia, are abundant in the soil of many ecosystems and show a great diversity at the species level, as well as great variability in their symbiotic efficiency (Borges et al., 2016; Lindström and Mousavi, 2020; Guanzon et al., 2023). Unfortunately, several abiotic factors such as drought can influence the survival, functioning and diversity of soil rhizobia, and thus, legume crop productivity (Benjelloun et al., 2019; Sharaf et al., 2019; Santillana Villanueva, 2021), because few strains of rhizobia show high tolerance to water stress (Sharaf et al., 2019; Sindhu et al., 2020). In addition, strain survival and competitiveness are not correlated with their N_2 fixation efficiency (De Souza et al., 2016; Kibido et al., 2019; da Silva et al., 2024), reducing the possibilities of establishing efficient symbiotic relationships.

Therefore, a higher diversity of legume nodulating bacteria in the soil will maximize the biological nitrogen fixation under stress conditions (Borges et al., 2016; Lindström and Mousavi, 2020; Martins et al., 2024). In this sense, greater soil microbial diversity will favour the adaptation of microbial populations to different environments, increasing the likelihood of survival of stress-tolerant microbial species and the establishment of effective symbiotic relationships (Wang and Young, 2019), contributing to greater crop resilience to stress. In addition, a greater biodiversity improves soil structure, nutrient cycling, and nutrient and water uptake, especially under drought conditions (Prudent et al., 2020).

Unfortunately, conventional agriculture related practices such as the use of herbicides and fungicides have a negative effect on the soil microbiota survival and diversity, reducing the efficiency of symbiotic relationships (Silva Neto et al., 2013; Da-Silveira-Cardillo et al., 2019; Rao et al., 2019). Additionally, the continued use of inorganic N-fertilizers causes the evolution of less-mutualistic rhizobia (Heath and Tiffin, 2007; Weese et al., 2015; Rao et al., 2019), and the decrease of nodules production (Heath et al., 2010; Regus et al., 2016). Thereby, with domestication and breeding in high-soil-N environments, the natural legume defences against less-effective rhizobia strains have been altered favouring less-cooperative rhizobia and reducing the agricultural benefits of the symbiosis (Weese et al., 2015).

Organic or sustainable farming, contrary to conventional production, promotes the biodiversity of agrosystems based on the concept that the greater biodiversity of the system, the greater health and resilience of ecosystems (Pimentel et al., 2005; Kremen and Miles, 2012; Wang and Young, 2019). The diversity of crops and their rotation system prevents the total depletion of nutrients from soils (Wolff and Killebrew, 2010; Hossain and Bakhsh, 2020) increasing soil fertility, and the diversity and activity of soil micro and macrobiotic communities (Herencia et al., 2020; Prudent et al., 2020). In this regard, studies about the abundance and diversity of rhizobia in soils, as well as the factors affecting both parameters, are of special importance to study the responsiveness of agrosystems to stresses (Omari et al., 2022).

According to different authors, the conservation of soil rhizobial diversity in agrosystems is a sustainable strategy of great interest to improve crop tolerance to stress by favouring the establishment of efficient symbiotic relationships even under water scarcity conditions (Wolff and Killebrew, 2010; Szparaga et al., 2019; Hossain and Bakhsh, 2020). In addition to this, there is interest in the search for indigenous inocula that are better adapted to local growing conditions and therefore more efficient in responding to stress conditions (del-Canto et al., 2023).

Abbreviations: AA, Arrocina de Álava; AK, Amarilla de Kuartango; BNF, biological nitrogen fixation; B, Borlotto de Vigevano; CL, Canela de León; CB, Cocco Blanco; DW, dry weight; FW, fresh weight; HF, humidity factor; L, Lingot; MPN, the most probable number; MU, Morada de Usánsolo; N, Negrita; NB, Negra de Basaburua; OM, organic matter content; PA, Pinta Alavesa; RL, Riñón de León; OV, Verde de Orbiso.

This is especially important in common bean under drought conditions as it is a very drought-sensitive crop (Embrapa, 2018; Nawaz et al., 2021) with a high frequency of inefficient symbiotic relationships (Michiels et al., 1998; Mwenda et al., 2023) that affect productivity. Considering the high nutritional value of common bean and that it is the grain legume for human consumption with the highest production worldwide (Beebe et al., 2013; FAO, 2021), the search for strategies to improve its productivity under conditions of low water availability is a challenge of great interest.

With this in mind, our hypothesis was that the type of management would have an effect on bean response to water stress, as greater microbial diversity could favour greater crop resilience. To test this, we analysed the effect of management (organic, conventional) on the production of several common bean genotype under water scarcity and how they affect the abundance, nodulation capacity and genotypic diversity of common bean rhizobia.

2 Materials and methods

2.1 Plant material

Twelve bushy genotypes of *Phaseolus vulgaris*, most of them of great economic interest in the North of Spain, were selected for the evaluation of the effect of water scarcity and management system in nodulation and rhizobia diversity. Of the twelve genotypes, four correspond to commercial genotypes and eight to locally adapted genotypes from different rainfall areas (Online resource Supplementary Table SI.1). The eight local genotypes, not studied to date, are traditionally grown on small family farms typically under rainfed conditions. Five of them are from the Basque Country (Northern Spain): Arrocina de Álava (AA), Amarilla de Kuartango (AK), Morada de Usánsolo (MU), Pinta Alavesa (PA) and Verde de Orbiso (VO). One is originally from Navarra (Northern Spain), Negra de Basaburua (NB), and two from Castilla y León (Central Spain), Canela de León (CL) and Riñón de León (RL). The other four are commercial genotypes commonly grown all around Spain: Cocco Blanco (CB), Lingot (L), Negrita (N), also marketed as "Frijol Negro", and Borlotto de Vigevano (B).

2.2 Location and soil characterization

The trials were performed in NEIKER experimental farm located in Arkaute, Álava (Spain), between May and August of 2015. Arkaute (WGS84: 42.850254, -2.621362) is located at 532 meters above sea level and has an oceanic climate, type Cfb (temperate oceanic climate or subtropical highland climate), according to the Köppen Geiger climate classification (1900), which is, a temperate and humid climate, in transition with the Mediterranean climate (information obtained from *Euskalmet, Euskal meteorologia agentzia*). The average temperature throughout the growing season was 17.8°C, with three days having a minimum temperature less than 5°C and 6 days having a maximum temperature that exceeded 35°C. The accumulated precipitation during the experiment was 116.5 mm.

The field experiment was conducted under conventional and organic management. Both types of soils were catalogued according to European standards and the current legislations of the Government of Spain (MAPA, 2023), which describe and classify the type of practices in each management system. The soil of the selected plots for the study presented close locations (one contiguous to the other), similar cropping histories (rotations between cereals, potatoes, vegetables and legumes), and generally were grown under irrigated conditions (Figure 1). The main differences in the management history of both soils were due to agrochemical supplies. In the conventional plots, different agrochemicals (herbicides, pesticides, and chemical fertilization) were frequently applied depending on the different types of crops and the requirements of each year. In the other hand, organic plots avoided the use of synthetic chemical products. The supply of nitrogen and nutrients was provided through organic amendments, according to the Council Regulation EC No 834/2007 of 28 June 2007. The conventionally managed soil has been worked under this type of management for more than ten years, while the organically managed soil has been worked in this way, without the use of synthetic chemicals, for five years. Although common beans were frequently grown in both soils, no plot had a history of commercial inoculation with rhizobia. Therefore, all the possible rhizobium inoculums were naturally occurring.For the soil characterization, eight soil samples 20 cm deep were randomly collected from each soil (conventional and organic) and analysed at the Fraisoro Agro-environmental Laboratory (Diputación Foral de Gipuzkoa) to study their physical-chemical characteristics, according to the official methods of analysis of the Ministry of Agriculture of Spain (MAPA, 1994). The Ph, electrical conductivity in calcium sulfate (EC, $\mu S \cdot cm^{-1}$) and effective cation exchange capacity (CEC, meq·100 ml^{-1}) were determined by ADAS method; the organic matter content (OM, %) was determined by the Walkley-Black method without heat input; the nitrogen (N) content (%), by Kjeldahl method; the phosphorous (P) content ($mg \cdot L^{-1}$), by the Olsen-Watanabe method performing an extraction in sodium bicarbonate at pH 8.5; the content of potassium (K, $mg \cdot L^{-1}$), calcium (Ca, $mg \cdot L^{-1}$) and magnesium (Mg, $mg \cdot L^{-1}$), by ADAS method with extraction in ammonium nitrate and subsequent reading in ICP-OES. The C/N balance was calculated based on the following formula:

$$C/N = (OM\ content/1.72)/N\ content$$

Where 1.72 is the factor of Van Bemmelen for the conversion of organic matter into Carbon. Finally, the granulometric characteristics of the soil (percentages of fine sand, coarse sand, silt, and clay) were determined according to the ISSS soil particle size fraction system.

2.3 Estimation of the number of viable rhizobia present in soils

The number of viable rhizobia present in the soils was estimated according to the methodology described by Hungría and Araujo (1994) with little modifications. The most probable number (MPN) of viable rhizobia is an indirect method for counting rhizobia

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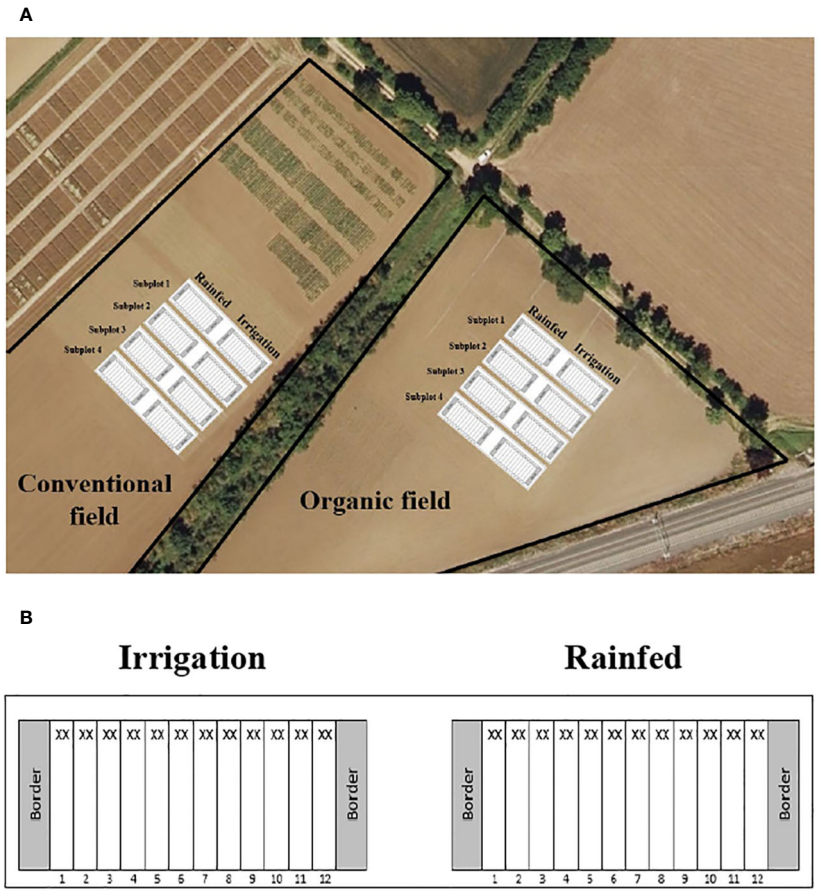

FIGURE 1
Location of the two crop fields of the study, including the experimental design scheme **(A)**, and detail the subplots **(B)**.

present in soils. This method assumes that an infectious or viable rhizobia is capable of developing a nodule. While a negative result, absence of nodules, indicates the absence of infectious rhizobia. For that, before the crop establishment, soil samples were taken from both conventional and organic management. The soil samples were passed through a 4 mm sieve. Then, 10 g of processed soil was diluted in 95 mL of diluent solution (phosphate buffer, pH 7.3) in a beaker with glass pearls on a horizontal shaker at slow speed at a temperature of 25°C for 30 minutes. From this soil solution, five serial dilutions were made (1 mL of previous solution and 9 mL of diluent solution), making four replications of each one. In addition, a small aliquot of the sieved soil sample was weighed (fresh weight, FW), and after drying in the oven at 80°C for two days, the sample was reweighed (dry weight, DW) in order to calculate the humidity factor (HF) and to compare the different soils regardless of its water content with the following formula:

$$HF = (FW - DW)/(DW - recipient\ \ tare)^{-1}$$

Previously, sterilized common bean seeds of Arrocina de Álava genotype (10 min in sodium hypochlorite 1%) were germinated in opaque cultivation jars containing 500 mL of nitrogen-free Fahraeus solution (Fahraeus, 1957). The seeds were held in a funnel, connected to the nutrient solution by a filter paper wick. Then, each of the seedlings were inoculated with one of the serial solutions of soil and grown in a growth chamber (Ibercex SA, Alcalá de Henares) under controlled conditions (12 h photoperiod, light intensity 500 µmoles of photons·m^{-2}·s^{-1}, 20/25°C temperature and 70/60% relative humidity, night/day respectively). After three weeks, the number of plants that developed nodules (positives) and those that did not (negatives) were counted. From these values, using a mathematical formula or a table of results (Hungría and Araujo, 1994), and considering the soil humidity factor (HF), the MPN of viable rhizobia was estimated, as well as the occurrence probabilities and lower and upper limits of the 95% confidence interval.

2.4 Experimental design and growth conditions

Irrigated and rain-fed treatments were performed in two subplots from each selected management plot. One subplot for conventional and the other for organic management. The 12 genotypes were sown by hand at a depth of 2 cm using a randomized block design with 4 biological replicates per water regime. A distance of 5 m separated each block, which consisted of two 10 m long rows with a 0.5 m between rows and 0.2 m separation between plants within the row, achieving a stand density of 100,000 plants ha^{-1}.

The water inputs were estimated controlling the irrigation time of the irrigation system (6 mm·h^{-1} flow), while the rainfall data were taken from the weather station Arkaute I (Euskalmet, 2023) located close to the experimental fields. Under rain-fed conditions, the seeds from common bean genotypes described above only received a minimum amount of water after sowing to assure the emergence and seedlings survival (12 mm). Afterwards the seedlings only received rainwater (116.5 mm). For the irrigated trials, three supplemental irrigations were supplied to plants during their growth (12 mm each). Therefore, the irrigated plots received a total contribution of 164.5 mm, while those under rainfed conditions received 128.5 mm, which is, a 22% reduction of water availability.

Under organic management, weeding control was mechanical and manual. In fields under conventional management, various herbicides were applied: preemergence herbicide Linuron 500 g·L^{-1} (Linurex ® 50 SC, Adama Chile SA), at a minimum recommended dose of 0.8 L·ha^{-1}; and post-emergence herbicide, pendimethalin 45.5% (Stomp® Aqua, BASF), at a minimum recommended dose of 2 L·ha^{-1}, at the V3 development stage, plants with the first trifoliate leaf (Fernández et al., 1986).

2.5 Nodule sampling

In the pre-flowering stage, R5 (Fernández et al., 1986), the nodules from three randomly selected plants from each experimental plots were collected, with four replicates of each, i.e. 12 nodules were harvested from each experimental condition (three plants of 12 genotypes, grown under two different water regimes in two different agricultural managements, with four replications) a total of 576 plants. Using a shovel, and measuring 20 cm from the center of the plat, the root system was excavated until a depth of 26 cm totalling a soil volume of 10.4 L per plant, similarly to Somasegaran and Hoben (1994), to ensure the harvest of practically the entire root system. Once in the laboratory, whole plants were carefully removed from the soil to obtain roots and nodules, and adhering soil was removed from roots by careful shaking. Then, the removed soil was carefully examined to recover any nodules left in the soil. Harvested nodules were washed in water and gently dried with paper. The nodules were counted and desiccated in bottles with silica gel at 4°C to preserve and use them in future research.

2.6 Yield quantification

At harvest, all plants from each block were counted, collected individually, dried, threshed, and cleaned separately to quantify the yield (Kg·ha^{-1}). Four biological replicates of each experimental conditions.

2.7 Endosymbiont isolation

The use of common bean "trap plants" grown in local field under rainfed conditions, could guarantee that isolated rhizobia have a certain competitiveness and possibly tolerance to water stress. Therefore, once the four most productive genotypes under rainfed conditions and a less productive one were selected, the bacteria present in their nodules were extracted from eight nodules randomly picked from each plot according to Sanz-Sáez et al. (2015). That is, 8 nodules per experimental condition, a total of 160 nodules (8 nodules of 5 genotypes grown under rainfed and irrigation conditions in two agricultural management systems). The nodules were first rehydrated by immersion in sterile distilled water for 2-4 h and surface-sterilized by immersing them in 70% ethanol (5 sec), and then in 50% sodium hypochlorite (5 min). Later, they were rinsed several times with sterile distilled water to remove the bleach residues. The nodules were crushed in a petri dish and 10-20 µL of autoclaved distilled water was added on the medulla of the nodule, aspirating, and expiring several times with the micro pipette to collect the bacteria (Somasegaran and Hoben, 1994; Howieson and Dilworth, 2016) and placing on a plate with solid TY medium (Beringer, 1974) to be grown at a constant temperature of 29°C.

Once the bacteria had grown, a single colony was chosen randomly and replated to a new solid TY plate for purification of rhizobia isolates. This operation was repeated for all observed colonies of different morphology and appearance from each plate. Using this method, 368 total isolates were obtained. These pure cultures were preserved at 4°C and were then transferred to 50% glycerol in TY, to preserve them at -80°C, in order to use them in future trials.

2.8 Isolated strain diversity by BOX+REP polymerase chain reaction genomic fingerprinting

Consensus sequences such as repetitive extragenetic palindromic sequences (REP), enterobacterial repetitive intergenic consensus (ERIC) and BOX elements, related to repetitive and conservative elements diffused in DNA, have been extensively used for rhizobial strain identification due to its ease, quickness, and high discriminatory power at infraspecific level (Kaschuk et al., 2006; Borges et al., 2016; Benjelloun et al., 2019). In our study, we used BOX and REP-PCR since the application of both PCR methods increases the accuracy when compared with only one PCR (Olive and Bean, 1999). The DNA extraction was performed according to Heath and Tiffin (2009) with modifications: 104 µL of liquid medium TY (Beringer, 1974) incubated with bacteria during 24-48 h at 28°C was centrifuged for 5 min at 16000 g and the pellet containing the cells was washed 3 times with 1 M NaCl and once with 100% ethanol and dried before extracting DNA. Then, the cells were re-suspended in 104 µL of sterile milli-Q water and homogenizing gently with the micropipette. The suspension was centrifuged 10 min at 16000 g and the aqueous phase was removed. For the DNA extraction, 30 µL of 10 mM tris-HCL (pH 8) and 1 µL of 20 mg·mL^{-1} protein kinase K enzyme (Invitrogen, Carisbad, CA) was added to the pellet and homogenized again with the micropipette and vortex. Then, 30 µL were transferred to a 0.2 mL PCR tube, and 55°C was applied for 4 h in order to let the protein kinase K to perform its function. After that it was

deactivated by heating the solution at 95°C for 10 min. DNA samples were quantified using a NanoDrop spectrophotometer (Thermo scientific, Wilmington, DE, USA) and diluted to 25 ng μL^{-1}. The DNA extraction was stored at 4°C.

BOX-PCR was performed according to the method described by Kaschuk et al., 2006 with modifications, using the BOX A1R primer (5′-CTACGG CAAGGCGACGCTGACG-3′; Versalovic et al., 1991). REP-PCR was performed according to the method described by Versalovic et al., 1991 with modifications, using the primers REP-1 (5′-IIIICGICGICATCIGGC-3′) and REP-2 (5′-ICGICTTATCIGGCCT AC-3′). Each PCR reaction was performed in a final volume of 10 µL containing: dNTPs 0.3 µL (10 mM); reaction buffer 1µL (10x BioLabs, New England); primer 0.5 µL (10 mM), for two primers in REP; Taq DNA polymerase 0.08 µL (5 $Um \cdot L^{-1}$); Betaine 0.5 µL (5 mM), DNA 1.5 µL; sterile milli-Q water to complete the volume. The amplification program was performed in a thermocycler S1000™ (Bio-Rad Laboratories, Inc.), applying an initial denaturing step (95°C, 7 min) with 30 cycles of denaturation (95°C, 1 min), annealing (53°C, 8 min for BOX-PCR and 40°C; 8 min for REP-PCR) and extension (65°C, 8 min); and a final extension cycle (65°C, 16 min). The PCR products were separated by horizontal electrophoresis on a 1.5% agarose gel (EEO-Mr<0.15) in TBE buffer (0.5x) at 90 V for 5 h, using a 1 Kb DNA marker (BioLabs, New England). The gels were stained with ethidium bromide, and visualized under UV light using a benchtop UV transilluminator (Bio-Doc-It™ UVP Imaging System).

2.9 Statistical analysis

The diversity of strains was analysed from the images of the gels (PCR fingerprints) using the free software GelJ v.2.0 (Heras et al., 2015) and transformed into a binary matrix. After analysing a large number of samples, the most consistent bands were selected, with 70% or more percentage of appearance. BOX and REP-PCR data were combined for each isolate.

From this BOX+REP binary matrix, Python software was used to build similarity matrices. Using the Jaccard coefficient and applying the UPGMA algorithm unweighted pair-group method with arithmetic mean (Sokal, 1973), the dendrogram and diversity indexes (Shannon and Weaver, 1949), richness (Margalef, 1958) and evenness (Pielou, 1977) were obtained. The graphic representations of the dendrograms were made with the free software iTOL (Interactive tree of Life, Leunic and Bork, 2019). Through these analyses, the different isolates were grouped according to the degree of similarity of their PCR fingerprints in different clusters. The number of clusters also indicates the strain diversity or diversity at infraspecific level. The number of clusters and their bootstrapping was calculated at 70% similarity. Due to the observed high variability, to obtain larger clusters, 35% similarity was also used (Grange and Hungria, 2004).

The nodule number and yield was analysed using the statistical package SPSS Statistics 24.0 (IBM Corporation, Armonk, NY, USA). The normality of the non-standardized residuals of the data was studied using the Shapiro-Wilk test and the homoscedasticity of the variance was studied with the Levenne test. As the water availability treatment was not randomized and organic and conventional management soils were separated, the behaviour of the genotypes was studied in the four experimental conditions: irrigated conventional management; rainfed conventional management; irrigated organic management; and rainfed organic management as it has been performed previously by Sanz-Sáez et al. (2019). For this, a one-way analysis of variance (ANOVA) was performed with genotypes as factor and replicates as random effect. When the genotype effect was significant, least square means *post-hoc* test was performed to compare means (Tukey or Kruskal Wallis). The management effect was also analysed separating the data according to the water regime through one-way analysis of variance (ANOVA), with agricultural management as factor and replicates as random effect. The graphic representations of the nodule number were made with SigmaPlot 15 (Systat Software, Inc.).

As one of the objectives of this research was to investigate the effect of water scarcity over the nodulation, the two management systems were treated as two different locations (environments) and the effect of water scarcity was analysed for each location separately, despite not being randomized. One-way ANOVA with water availability was treated as factor and replicates as random effect.

3 Results

3.1 Soil characterization

The properties and characteristics of soils cultivated under organic and conventional management were similar (Table 1). Both were clay-loam soils with a very light salinity, had a basic pH typical of limestone soils, contained an optimal carbon balance, and contained adequate nitrogen and phosphorus content with a medium magnesium content. However, both soils differed in their organic matter, magnesium and potassium content all being higher in the organic management soil, while higher calcium content occurred in the conventional management field.

3.2 Estimation of the number of viable rhizobia present in soils

The estimation of rhizobia cells existing in the sampled soils before sowing and the establishment of water treatment are shown in Table 2. The MPN shows the number of live rhizobia cells present per unit of volume in the matrix solution taking into consideration the soil humidity correction factor which allows for comparisons of the different soils regardless of water content. The MPN values were more than 20-fold higher in the organic (21.102) than in the conventional (0.609) management soil (Table 2).

3.3 Effect of water stress, agricultural management and legume genotypes on nodulation

The infection capacity of rhizobia, showed as nodule number per plant, was almost 58.25% lower, on average, in conventional

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TABLE 1 Physical-chemical characteristics of conventional and organic agricultural soils of the experimental fields.

	Conventional	Organic
pH	8.30	8.45
Electrical Conductivity ($\mu S \cdot cm^{-1}$)	1900	1700
C/N balance	3.44	7.62
Organic Matter (%)	1.12	1.43
Nitrogen (%)	0.19	0.20
Phosphorous ($mg \cdot L^{-1}$)	42.81	46.75
Potasium ($mg \cdot L^{-1}$)	164.0	340.00
Magnesium ($mg \cdot L^{-1}$)	85.20	127.20
Calcium ($mg \cdot L^{-1}$)	6850	6284
Sodium ($mg \cdot L^{-1}$)	34.50	29.90
Efective CEC (meq/100 mL)	20.76	16.81
Fine sand (0.2-0.02 mm, %)	34.34	48.50
Coarse sand (0.2-2 mm, %)	7.81	3.97
% Silt (0.02-0.002 mm, %)	24.53	29.40
% Clay (<0.002 mm, %)	33.32	18.10
Texture clasification	Clay-Loam	Clay-Loam

management than in organic management (Table 3). The number of nodules was also reduced by low water availability in both management systems although its effect was less evident than that of the management itself. Although there was not management by water availability interaction, rainfed conditions seemed to decrease the rhizobia infection capacity more in conventional (24.56%) than in organic management (20.30%) (Table 3).

The nodulation capacity also varied depending on the genotypes although all of them showed higher nodulation capacity in organic than in conventional management (Figure 2). The genotypes RL and MU showed a greater number of nodules under irrigation in both management systems, while CB, PA and CL showed the least in the conventional management, and B showed the least in the organic management field. Reduced water availability only decreased nodule number in RL and NB genotypes in the organic plots, whereas in conventional plots no significant effect of rainfed conditions was observed in any of the genotypes tested. RL was by far the genotype that developed a greater number of nodules under rainfed conditions in conventional management (Figure 2) but was more affected by water availability in organic management. Under organic management, the MU and L cultivars were those that showed greater nodulation under rainfed conditions, unlike CB, B and NB that were the less nodulated genotypes. The B and NB genotypes also showed less nodules in conventional management under rainfed conditions. Therefore, the genotypes that most times showed the highest nodulation capacity were the RL and MU, while the B and NB were the ones showing the lowest nodulation capacity.

TABLE 2 Most probable number of viable rhizobia in organic and conventional plots before sowing: HF, soil humidity factor; MPN, most probable number of viable rhizobia cells present in the soil matrix solution corrected with HF; P (%), probability of the combination occurrence if the experiment is repeated an infinite number of times with the same matrix solution; CImin and CImax, lower and upper limits of the 95% confidence interval.

	HF	MPN	P (%)	CI_{min}	CI_{max}
Conventional	1.0	**0.6**	28.2	0.2	2.3
Organic	1.2	**21.1**	0.2	9.3	52.8

3.4 Yield quantification

The yields obtained in conventional management were higher than those obtained with organic management. However, while rainfed conditions considerably reduced common bean production under conventional management, water scarcity did not significantly reduce organic bean production (Table 4).

On the other hand, it was observed that AA, N, NB and RL were the genotypes that ranked as most productive under different experimental conditions, also under rainfed conditions, while AK was one of the least productive (Table 4). Thus, those were the genotypes selected to study the diversity of the bacteria strains inside their nodules.

3.5 Isolated strain diversity by BOX+REP PCR genomic fingerprinting

From the 368 bacteria isolated from nodules of plants grown under different water availability in both organic and conventional management, BOX+REP PCR fingerprinting were obtained on 320 isolates, since some strains did not amplify with the primers used similarly to other literature (Judd et al., 1993; Mostasso et al., 2002; Kaschuk et al., 2006).

The genomic fingerprinting showed a high level of genetic diversity among the strains, considering a similarity of 70% in the clustering analysis, confirmed by the low final levels of similarity (Online Resource, Supplementary Table SI.2). With this level of similarity (70%) the vast majority of the generated clusters in the different experimental conditions were composed of a single strain and the values obtained from bootstrapping were generally greater than 0.60, thus, can be considered stable groups (Jain and Moreau, 1987; Kerr and Churchill, 2001). To obtain larger clusters, formed by a greater number of strains, the isolated strains were also grouped at a 35% similarity level (Figure 3, Tables 5, 6). Thus, larger clusters were obtained and formed by several strains, although bootstrap values remained below 0.60.

The number of isolated bacteria (Online resource, Supplementary Table SI.2, Supplementary Table SI.3), clusters, diversity (Shannon, H_0), richness (Margalef, R_1) and evenness (Pielou, E_1) of strains were greater in the nodules of the plants grown under organic management than under conventional management, regardless of the considered similarity level (Tables 5, 6, for 35% of similarity and Online Resource,

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TABLE 3 Mean values (± SE) and ANOVA results (p-value) of nodule number per plant of different common bean plants grown under different water availability (WA) conditions: irrigated and rainfed and in different management systems (M), conventional and organic.

Management	WA	Nodule number per plant
Conventional	Irrigation	6.80 ± 0.89 B
	Rainfed	5.13 ± 0.93 b
	Mean	**6.37 ± 0.75**
Organic	Irrigation	15.41 ± 0.95 A
	Rainfed	12.28 ± 1.11 a
	Mean	**15.26 ± 1.05**
ANOVA RESULTS		
Factors	P-value	
M	***	
WA	**	
M*WA	NS	

Capital letters were used to compare the water availability treatments in conventional management and lowercase letters in organic management (*p<0.05; **p<0.01 and ***p<0.001; NS, non-significant).

Supplementary Table SI.2, Supplementary SI.3, for 70% of similarity). Rainfed conditions also caused a drop, although not statistically significant, in the diversity indices (Shannon, Margalef and Pielou), number of isolates and obtained clusters, at both levels of similarity (Table 5 and Online resource, Supplementary Table SI.2). This can be observed when analysing both managements separately based on water availability (Figure 3). Nodules of plants grown in organic soils always showed higher strain genetic diversity (diversity indices and number of clusters), even under rainfed conditions, with respect to the most favourable conditions (irrigation) of conventional management (Figure 3, Table 5 and Online resource Supplementary Table SI.2).

In conventional management under irrigated conditions, 71 bacteria were isolated and grouped into 9 clusters, two of which represent 67.6% of the strains. Under rainfed conventional management, the isolated bacteria were reduced to 56 bacteria and 89.3% of them were grouped into one large cluster (Figure 3). Nevertheless, under organic management and irrigation conditions, 96 bacteria were isolated and grouped into 18 clusters, with the largest cluster representing 46.9% of the isolated bacteria. Under rainfed organic conditions, 97 strains were isolated and grouped into 15 clusters, three of which represented 71.1% of the isolated bacteria. Rainfed conditions reduced the number of isolated bacteria strains by 21.1% under conventional management but not in organic management soil. The genetic diversity of strains (number of clusters) was reduced by 33.3% in conventional management and by 16.7% in organic management due to low water availability (Figure 3).

Finally, when analysing the different genotypes (Table 6), AK showed the lowest number of isolated bacteria (54), the lowest indexes of genetic diversity and the lowest number of clusters. On the other hand, N and NB, showed the highest indexes of genetic diversity and a greater number of clusters (13 and 12 respectively). N was the genotype from which the greatest number of bacteria were isolated (71).

4 Discussion

The great diversity that rhizobia show at the species level is essential for *Phaseolus vulgaris* to stablish efficient symbiosis and maintain crop productivity in a sustainable way, especially under drought conditions (Lindström and Mousavi, 2020; Guanzon et al., 2023; da Silva et al., 2024). Rhizobia can play a more important role in the resistance to stress of the symbiosis relationship than the genotype of plant (Mhadhbi et al., 2011; Sharaf et al., 2019). Unfortunately, the symbiotic efficiency of rhizobia is highly variable and, on many occasions, establish inefficient symbiotic relationships with common bean having a negative impact on productivity (Michiels et al., 1998; Armenta-Borjóquez et al., 2016; Mwenda et al., 2023). Thus, greater microbial diversity in soils and the better the response of crops to stress will increase the probability of appearance of efficient stress-tolerant microbial species (Wang and Young, 2019) that can maximize biological nitrogen fixation under such conditions (Borges et al., 2016; Lindström and Mousavi, 2020; Liyanage et al., 2023).

The BOX and REP-PCR fingerprinting analysis showed a high level of genetic diversity among the bacteria isolated from nodules of all the sampled plants grown under the different agronomic conditions. These data confirm the great promiscuity of the common bean plants which are capable of nodulating with many different bacteria. Our results are comparable to those of other studies using similar techniques, in which most of the groups, clustered at 70% similarity, were formed just by one or two strains (Grange and Hungria, 2004; Chibeba et al., 2020; Odori et al., 2020).

In most clusters, the values obtained from bootstrapping were close to 0.60 at 70% similarity and even lower values were obtained at 35% of. These low bootstrap values are usually obtained in these type of dendrograms (Aulakh et al., 2020) due to both, the type of data (they are not related sequences), and to the large number of operational taxonomic units (OTUs, bacterial strains), which contribute to lower the value of the bootstraps (Efron and Tibshirani, 1993). However, despite the low bootstrap values, this technique is considered a powerful tool to detect strain diversity (Alberton et al., 2006; Kaschuk et al., 2006; Costa et al., 2018).

Water scarcity provokes reduction of nodule number per common bean plant (Berny-Mier et al., 2019; Aserse et al., 2020; Prudent et al., 2020). In this study, a reduction of only 22% in water availability affected the nodulation efficiency, reducing the number of nodules per plant and the number of isolated bacteria per nodule. However, it did not significantly affect the strain genetic diversity, although there was a tendency to decrease under water scarcity conditions.

Water scarcity is one of the factors that most affect the rhizobia survival in their free-life phase (Hungria et al., 2000; Sindhu et al., 2020; Santillana Villanueva, 2021) and the nodulation process, affecting chemotaxis, initiation, formation and development of nodules (Sindhu et al., 2020; Viti et al., 2021; Omari et al., 2022) thus reducing the number of nodules. In fact, under sufficiently

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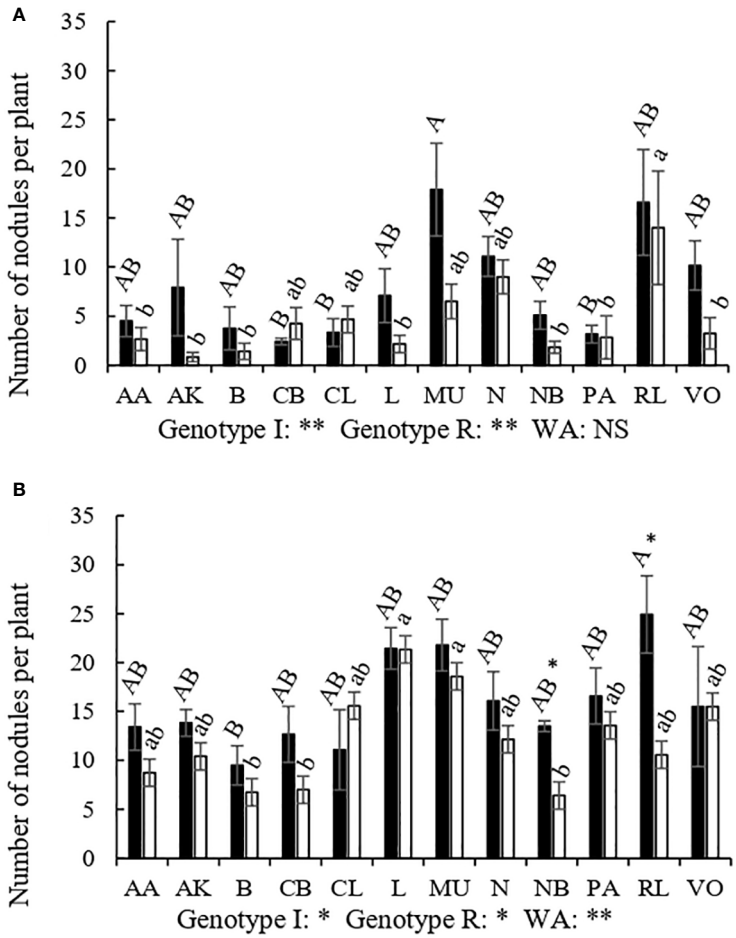

FIGURE 2
Number of nodules per plant of different genotypes of *Phaseolus vulgaris* (AA, Arrocina de Álava; AK, Amarilla de Kuartango; B, Borlotto; CB, Coco blanco; CL, Canela de León; L, Lingot; MU, Morada de Usansolo; N, Negrita; NB, Negra de Basaburua; PA, Pinta alavesa; RL, Riñón de León; y VO, Verde de Orbiso), under different water availability (WA) treatments in the field: irrigation (dark) and rainfed (light); both in conventional **(A)** and organic management **(B)**. Bars indicate the mean of each genotype for number of nodules. Different capital letters designate significantly different genotype means under irrigated conditions while lower case letters designate significant different genotype means for the rainfed treatment according to LSD *post-hoc* analysis. Asterisks withing the figures were used to show the effect of water in each genotype separately. Genotype I illustrate the p-value of the genotype effect under irrigated conditions; Genotype R illustrates the p-value of the genotype effect under rainfed conditions; WA, indicated the effect of water availability factor in each management (*p<0.05; **p<0.01 and ***p<0.001; NS, non-significant).

prolonged or severe drought conditions the nodule formation can be completely inhibited (Gerosa-Ramos et al., 2003). In drought-affected soils, the decrease of bacterial diversity and nodulation capacity also translates into a lower diversity of the nodule bacteriome, reducing the number of isolated bacteria and the genetic diversity of detected strains. Similar effects were described in soybean (Sharaf et al., 2019) and common bean (Cytryn et al., 2007).

The reduction of the bacterial diversity parameters due to lower water availability was observed in both managements, however, the effect was slightly higher under conventional management than that observed under organic conditions. In the latter, water scarcity had no effect on yield and higher nodulation, higher number of isolated strains and greater genetic diversity of strains were recorded even under rainfed conditions. These results would demonstrate that organic soil´s microbiota reflect wider environmental adaptation and superior competitive ability (Yan et al., 2017; Costa et al., 2018) and would confirm that these soils have greater resilience to adverse conditions such as water stress via greater microbial abundance and diversity (Borges et al., 2016; Lindström and Mousavi, 2020). As some authors suggest, the maintenance of the soil microbial genetic biodiversity is of great importance because it provides a major buffering capacity of the soil (Loreau et al., 2001) and it is related to soil health and quality as well as agricultural sustainability (Kaschuk et al., 2006).

Conventional plots showed lower abundance and diversity in the soil microbiome even before sowing and under irrigated conditions. This would be related to the practices and agrochemicals used in conventional agriculture (Rao et al., 2019), since the two sampled agricultural soils, conventional and organic, had similar edapho-climatic conditions and both presented similar cropping histories and soil characteristics.

The soil of conventional management plots was subjected to years of agrochemicals and N-fertilization inputs. Mineral fertilization reduces plant nodulation (Heath et al., 2010; Regus

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TABLE 4 Mean values (± SE) and ANOVA results (p-value) of the effect of water availability (WA) and genotype (G) on common bean yield (kg·ha^{-1}). both in conventional and organic management.

	Conventional yield (kg·ha-1)			Organic yield (kg·ha-1)	
	Irrigation	2746.2 ± 155 a		Irrigation	1649.5 ± 135.2 a
	Rainfed	2350.7 ± 111.2 b		Rainfed	1451 ± 87 a
	ANOVA RESULTS			ANOVA RESULTS	
	Factors	P-value		Factors	P-value
	WA	*		WA	NS
	Conventional yield (kg·ha^{-1})			Organic yield (kg·ha^{-1})	
Gen.	Irrigation	Rainfed	Gen.	Irrigation	Rainfed
AA	3807.8 ± 237.5	2679.5 ± 146.1 abc	AA	3107.3 ± 505.2 a	2395.7 ± 444.7 a
AK	2232.8 ± 450.8	1882.1 ± 381.3 bc	AK	1031.7 ± 158 b	1052.9 ± 153.9 b
B	2121.9 ± 379.7	1837.6 ± 246.3 c	B	1156.1 ± 163.5 b	951.4 ± 117.1 b
CB	2288.4 ± 291.1	2174.2 ± 334.2 bc	CB	867.2 ± 376.5 b	1139.7 ± 256.8 b
CL	2358.6 ± 377.5	1892.9 ± 296.9 bc	CL	1442.6 ± 196.3 ab	1189.7 ± 201.8 b
L	3462.8 ± 395.1	2092.7 ± 144.1 bc	L	1444.7 ± 208.2 ab	1750.8 ± 111.7 ab
N	2850.4 ± 686.5	2981.2 ± 398 ab	N	2179.6 ± 229.9 ab	1881.9 ± 344.4 ab
NB	3216.6 ± 416.7	2631.6 ± 251.8 abc	NB	1910.7 ± 381.7 ab	1562.7 ± 142.6 ab
PA	2890.8 ± 670.3	2424 ± 219.7 abc	PA	1384.7 ± 406.7 ab	1380 ± 242.5 ab
RL	3926.9 ± 343.2	3285.9 ± 509.7 a	RL	1884.5 ± 480.2 ab	1610.8 ± 42.9 ab
VO	2205.3 ± 254.9	2174.4 ± 215.4 bc	VO	1794.5 ± 658.1 ab	1160.2 ± 173.4 b
ANOVA RESULTS			ANOVA RESULTS		
Factors	P-value	P-value	Factors	P-value	P-value
G	NS	*	G	*	**

AA, Arrocina de Álava; AK, Amarilla de Kuartango; B, Borlotto de Vigevano; CB, Cocco Blanco; CL, Canela de León; L, Lingot; MU, Morada de Usansolo; N, Negrita; NB, Negra de Basaburua; PA, Pinta alavesa; RL, Riñón de León; and VO, Verde de Orbiso (*p<0.05; **p<0.01; NS, non-significant).

et al., 2016) and symbiotic efficiency (Heath and Tiffin, 2007; Weese et al., 2015; Gordon et al., 2016; Rao et al., 2019) because it is energetically cheaper for plants to reduce ammonium and nitrate than to fix N_2. This leads to a reduction on the presence and diversity of rhizobia over time (Moreira et al., 2006; Da-Silveira-Cardillo et al., 2019). In addition, during the experiment, two doses of herbicides, Pedimethalin and one of Linuron, were applied to conventional plots. There is quite a consensus on the negative effect of herbicides on bacterial diversity. Coinciding with our observations, García-Garijo et al (2014) detected that Imazamox drastically affected the nodulation and biological nitrogen fixation of common beans, and Darine et al. (2015) reported that Fusilade herbicide causes a decline in richness and structure of soil bacterial communities, mainly at the rhizosphere level. Furthermore, although the literature contains little information on the effects of pesticides on legume-rhizobia signal exchange, some *in vitro* work with 30 different pesticides showed that *Sinorhizobium meliloti* NodD was affected resulting in delayed nodulation and reduced biological nitrogen fixation in *Medicago sativa* (Fox et al., 2001).

Consequently, the use of agrochemicals leads to a loss of abundance and diversity in the soil microbiome (Rao et al., 2019), as well as of rhizobia (Silva Neto et al., 2013; Weese et al., 2015), reducing plant nodulation and genetic diversity of strains. On the contrary, organic amendments, such as manure, a common practice in organic management, usually increase the abundance and diversity of microorganisms and rhizobia (Rao et al., 2019; Li et al., 2022), promoting a greater plant nodulation (Herencia et al., 2020), as in our results.

This negative effect of agrochemicals on common bean symbiosis was even more drastic than that produced by a reduction of 22% in water availability. In fact, the use of agrochemicals reduced nodulation, the number of isolated bacteria and the genetic diversity of nodule bacteriome strains, while water availability only reduced nodulation, and no significant effect on strain diversity was observed.

Although in organic management no effect of water scarcity was observed in yield, confirming a greater resilience to water stress (Borges et al., 2016; Lindström and Mousavi, 2020), the yields obtained in this management were lower than in conventional management, probably due to the difficulty of weed control, since the physical and chemical conditions were not very different in both fields. Therefore, it is evident the need to study different strategies to

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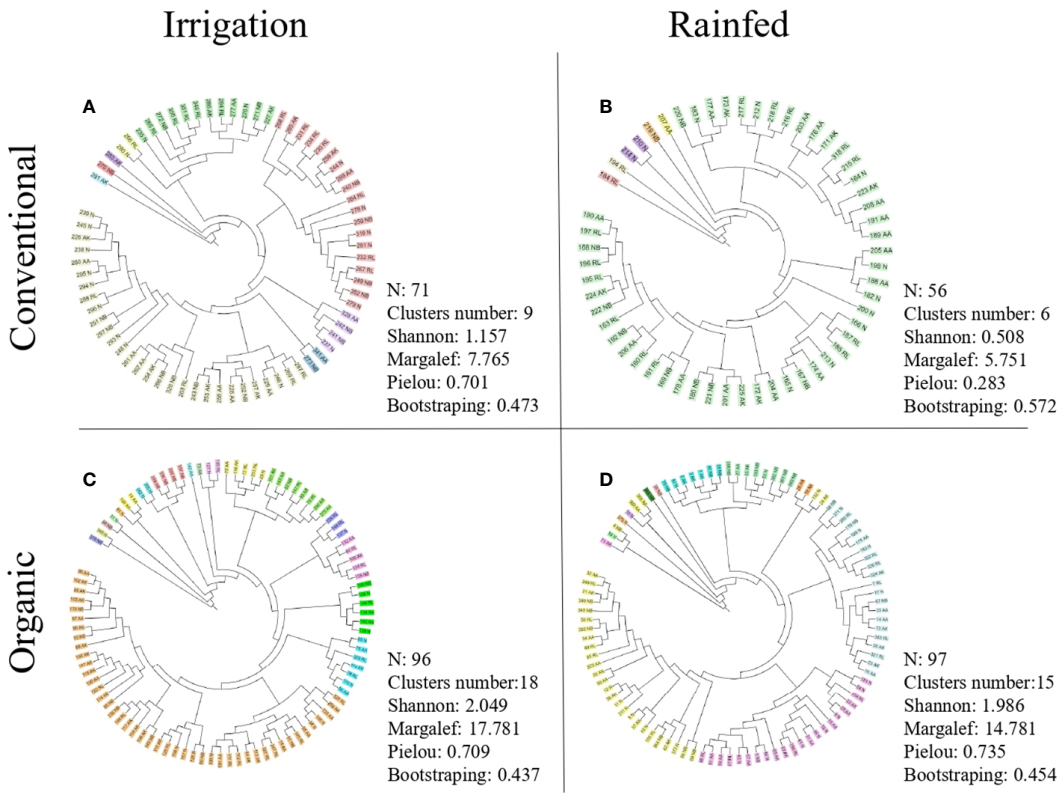

FIGURE 3
Dendrogram constructed from the BOX+REP PCR genomic fingerprinting. Number of isolated bacteria (N), number of clusters (represented graphically with different colours), diversity indices (Shannon, Margalef and Pielou) and cluster bootstraping obtained at a 35% similarity level in: conventional management under irrigation conditions **(A)**; conventional management under rainfed conditions **(B)**; organic management under irrigation conditions **(C)**; and organic management under rainfed conditions **(D)**.

TABLE 5 Number of isolated bacteria, average stability of clusters and mean values (± SE) and ANOVA results (p-value) of strain diversity indices (Shanon, Margalef and Pielou) and number of obtained clusters at a similarity level of 35% according to agricultural management (conventional and organic) and water availability (I, irrigated; and R, rainfed) (*p<0.05; **p<0.01 and ***p<0.001; NS, non-significant).

		Isolated bacteria	Cluster stability (bootstraping)	Diversity (Shannon´s index)	Richness (Margalef´s index)	Evenness (Pielou´s index)	Number of clusters
Conventional		127	0.51	1.27 ± 0.12 b	11.79 ± 0.32 b	0.51 ± 0.05 a	12 ± 0.30 b
Organic		193	0.46	2.17 ± 0.152 a	14.81 ± 0.70 a	0.69 ± 0.04 a	23 ± 0.69 a
Irrigation		167	0.44	2.13 ± 0,15 a	20.80 ± 0.77 a	0.70 ± 0.04 a	21 ± 0.77 a
Rainfed		153	0.44	1.79 ± 0.16 a	17.80 ± 0.56 a	0.62 ± 0.05 a	18 ± 0.54 a
Conventional	**I**	71	0.47	1.16 ± 0.115 a	7.76 ± 0.52 a	0.7 ± 0.06 a	8 ± 0.51 a
	R	56	0.57	0.51 ± 0.12 a	5.75 ± 0.40 a	0.28 ± 0.06 a	6 ± 0.37 a
Organic	**I**	96	0.44	2.05 ± 0.27 a	17.78 ± 1.24 a	0.71 ± 0.07 a	18 ± 1.24 a
	R	97	0.45	1.98 ± 0.17 a	14.78 ± 0.76 a	0.73 ± 0.05 a	15 ± 0.75 a
			ANOVA RESULTS				
			Factors	P-value			
			M	**	**	NS	**
			WA	NS	NS	NS	NS
			M*WA	NS	NS	NS	NS

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TABLE 6 Number of isolated bacteria, strain diversity indices (Shanon, Margalef and Pielou) and number, structure and average stability of clusters obtained at a similarity level of 35% according to agricultural management (C, conventional; O, organic), water availability (I, irrigated; R, rainfed), and genotype (AA, Arrocina de Álava; AK, Amarilla de Kuartango; N, Negrita; NB, Negrita de Basaburua; RL, Riñon de Leon).

			Isolated bacteria	Diversity (Shannon´s index)	Richness (Margalef´s index)	Evenness (Pielou´s index)	Number of clusters	Cluster stability (bootstraping)
AA	C	I	10	1.566	1.566	0.881	2	0.495
		R	15	0.720	3.631	0.519	4	0.409
	O	I	21	2.001	8.672	0.911	9	0.529
		R	19	1.979	4.660	0.931	5	0.473
		Total	**65**	**1.966**	**10.768**	**0.820**	**11**	**0.404**
AK	C	I	10	1.089	3.566	0.571	4	0.594
		R	6	0.451	1.442	0.650	2	0.562
	O	I	20	0.613	2.666	0.558	3	0.612
		R	18	1.132	3.654	0.817	4	0.549
		Total	**54**	**0.884**	**5.749**	**0.493**	**6**	**0.494**
N	C	I	18	0.937	2.654	0.853	3	0.482
		R	11	1.121	3.583	0.809	4	0.518
	O	I	18	2.000	8.654	0.910	9	0.562
		R	24	1.831	7.685	0.881	8	0.815
		Total	**71**	**1.735**	**12.765**	**0.676**	**13**	**0.488**
NB	C	I	16	1.180	3.639	0.851	4	0.516
		R	9	0.684	2.545	0.622	3	0.550
	O	I	16	1.581	5.639	0.882	6	0.543
		R	21	1.234	5.672	0.689	6	0.590
		Total	**62**	**1.527**	**11.758**	**0.615**	**12**	**0.472**
RL	C	I	17	1.401	4.647	0.871	5	0.552
		R	15	0.485	2.631	0.442	3	0.621
	O	I	21	0.996	3.672	0.719	4	0.461
		R	15	1.323	3.631	0.954	4	0.499
		Total	**68**	**1.602**	**9.763**	**0.696**	**10**	**0.441**

The row labeled **Total** shows the data obtained for each genotype without considering the different experimental conditions.

improve crop productivity in organic management to avoid yield losses, such us a better weed control, one of the prime causes of yield losses in organic management (Entz et al., 2001; Posner et al., 2008).

Finally, we also detected an effect of plant genotype on nodule number, as it has been observed in several publications (Berny-Mier et al., 2019; Goyal et al., 2021; Omari et al., 2022), although without interaction with the rest of the studied factors. In addition, we detected differences in the diversity of isolated strains across genotypes, as it was observed in soybeans (Sharaf et al., 2019) and in common bean (Shamseldin and Velázquez, 2020). Although some authors have not found effect of common bean genotype on rhizobial diversity (Grange and Hungria, 2004), other researchers have reported that the legume plants control the nodulation process (Ferguson et al., 2019), and the involvement of several *Phaseolus vulgaris* genes in strain-specific selection (Rípodas et al., 2019).

RL and MU were the genotypes that showed more nodules in both management practices, also under rainfed conditions. RL was also the second genotype with a higher number of strains, after the N genotype. On the contrary, NB, was the least nodulated genotype, but showed great strain diversity and was one of the most productive genotypes under limited water conditions. On the other hand, AK, a genotype selected as the least productive, showed few nodules and low number of isolated bacteria, with less strain diversity under rainfed conditions. These results agree with the findings of other authors (Aserse et al., 2020) that suggest that the number of nodules is not always related to biological nitrogen fixation efficiency (Zurdo-Piñeiro et al., 2009; Flores-Félix et al., 2019; Shamseldin and Velázquez, 2020). However, the number of nodules gives an idea of the abundance and/or the viability of the rhizobia present in soils, and it has also been stated

that more drought-tolerant strains show higher nodulation capacity under drought conditions (Aulakh et al., 2020; Omari et al., 2022; del-Canto et al., 2023).

The results show that the rhizobia isolated in this work, are efficient inocula able to nodulate under rainfed conditions as was demonstrated with some of them (del-Canto et al., 2023) and it would be interesting to test their efficiency under field conditions, following successful formulations such as those described by Pastor-Bueis et al. (2019).

5 Conclusion

Rainfed conditions reduced the number of nodules per plant and the number of isolated bacteria, however, the use of agrochemicals products related to conventional management had a greater negative effect than that observed by a reduction of 22% in water availability, and also affected the strain genetic diversity of the nodule bacteriome. In addition, while water limitation did not have an effect on the organic management yield, it was reduced in conventional management. Consequently, the effect of rainfed conditions on the conventional management soil was greater than observed under organic conditions.

These results would confirm that the use of agrochemicals leads to a loss of rhizobia abundance and diversity while organic management practices maintains higher values of rhizobia abundance, nodulation and diversity, even under rainfed conditions. This maintenance of diversity will be a key factor in the future, as problems caused by drought will be exacerbated by climate change. Maintaining microbial diversity implies broader environmental adaptation, superior competitive ability and greater resilience to adverse conditions. Therefore, it is necessary to develop sustainable and environmentally friendly agricultural systems, free of agrochemicals that allows for maintaining or even increasing the biodiversity of soil microbiota, a fundamental aspect for soil health and quality.

Data availability statement

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

Author contributions

AD: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. AS: Conceptualization, Data curation, Formal analysis, Supervision, Writing – review & editing. KH: Conceptualization, Resources, Supervision, Writing – review & editing. MG: Investigation, Methodology, Writing – review & editing. JH: Software, Visualization, Writing – review & editing. ML: Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was founded by research projects from the Basque Government: Grupo de Investigación del Sistema Universitario Vasco IT1022-16 and T1682-22; and projects from the Basque Country Government: Dpto. de Desarrollo Económico e Infraestructuras (32-2016-00043) and Dpto. de Desarrollo Económico, Sostenibilidad y Medioambiente (37-2017-00047, 00049-IDA2019-38 and 0039-IDA2021-45). A. Del-Canto was the recipient of a predoctoral fellowship granted by the Education Department of the Basque Country Government, Spain.

Acknowledgments

We thank Mr. Gary Wayne Morgan for his contribution reviewing the manuscript for English Grammatical correctness. We also thank Agustín Miranda del Ama for collaborating in the nodule sampling.

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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1408125/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 02 July 2024
DOI 10.3389/fpls.2024.1422504

OPEN ACCESS

EDITED BY
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RECEIVED 24 April 2024
ACCEPTED 17 June 2024
PUBLISHED 02 July 2024

CITATION
Mal S and Panchal S (2024) Drought and salt stress mitigation in crop plants using stress-tolerant auxin-producing endophytic bacteria: a futuristic approach towards sustainable agriculture.
Front. Plant Sci. 15:1422504.
doi: 10.3389/fpls.2024.1422504

Drought and salt stress mitigation in crop plants using stress-tolerant auxin-producing endophytic bacteria: a futuristic approach towards sustainable agriculture

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Abiotic stresses, especially drought stress and salt stress in crop plants are accelerating due to climate change. The combined impact of drought and salt is anticipated to lead to the loss of up to 50% of arable land globally, resulting in diminished growth and substantial yield losses threatening food security. Addressing the challenges, agriculture through sustainable practices emerges as a potential solution to achieve Zero Hunger, one of the sustainable development goals set by the IUCN. Plants deploy a myriad of mechanisms to effectively address drought and salt stress with phytohormones playing pivotal roles as crucial signaling molecules for stress tolerance. The phytohormone auxin, particularly indole acetic acid (IAA) emerges as a paramount regulator integral to numerous aspects of plant growth and development. During both drought and salt stress conditions, auxin plays crucial roles for tolerance, but stress-induced processes lead to decreased levels of endogenous free auxin in the plant, leading to an urgent need for auxin production. With an aim to augment this auxin deficiency, several researchers have extensively investigated auxin production, particularly IAA by plant-associated microorganisms, including endophytic bacteria. These endophytic bacteria have been introduced into various crop plants subjected to drought or salt stress and potential isolates promoting plant growth have been identified. However, post-identification, essential studies on translational research to advance these potential isolates from the laboratory to the field are lacking. This review aims to offer an overview of stress tolerant auxin-producing endophytic bacterial isolates while identifying research gaps that need to be fulfilled to utilize this knowledge for the formulation of crop-specific and stress-specific endophyte bioinoculants for the plant to cope with auxin imbalance occurring during these stress conditions.

KEYWORDS

climate change, zero hunger, plant microbiome, phytohormones, IAA, bioinoculants, abiotic stress

1 Introduction

In the last few decades, the evidence of climate change due to harsh human activities has threatened global biodiversity, especially of plants because of their sessile nature (Penuelas et al., 2002; Rosbakh et al., 2017; Anthelme et al., 2021; Vecerova et al., 2022). Plants depend only on internal mechanisms to withstand stress and modifications in their external surroundings (Esmon et al., 2005; Knudsen et al., 2018). Plants encounter two primary forms of stress: biotic, caused by various pathogenic bacteria, fungi, nematodes, oomycetes, and herbivores, and abiotic, arising from factors like salinity, drought, radiation, heavy metals, and extreme temperatures (Gull et al., 2019). Among these, drought, and salt stress have affected almost 2000 million hectares of land globally (Beltagy and Madkour, 2012). Drought alone has an impact on 45% of the global agricultural land, and 19.5% of irrigated agricultural areas are classified as saline (Abdelraheem et al., 2019). Consequently, crop production is hindered on a global scale, posing a threat to global food security (Fahad et al., 2017). According to the Food and Agriculture Organization (FAO), over 870 million people worldwide are affected by food insecurity, hindering progress towards achieving "Zero Hunger", one of the 17 Sustainable Development Goals outlined by the International Union for Conservation of Nature (IUCN) to be achieved by 2030 (FAO et al., 2022).

During drought and salt stress, plants experience water scarcity, ion toxicity, phytohormone imbalances, and increased production of reactive oxygen species (ROS), leading to considerable decreases in crop growth rate and the accumulation of biomass (Das and Roychoudhury, 2014). Plants deploy a myriad of mechanisms, encompassing osmotic adjustment, antioxidant defense, stomatal regulation, root system modification, transcriptional regulation, and phytohormone regulation, to effectively address stress. Phytohormones play pivotal roles serving as crucial signaling molecules for stress tolerance by activating multiple signaling pathways. Auxin, gibberellin (GA), cytokinin, ethylene, jasmonic acid (JA), and salicylic acid (SA) constitute the primary phytohormones crucial for regulating diverse biochemical and physiological processes governing plant growth and stress response (Abobatta, 2020; Sabagh et al., 2022). Auxin plays crucial roles during stress like improving root architecture by increasing lateral root number, expression of stress-related genes, metabolic homeostasis, and ROS detoxification (Shi et al., 2014). However, during both drought and salt stress, plants exhibit diminished auxin levels and reduced expression of auxin transporters which results in a disruption of auxin transport and distribution, leading to lowered stress tolerance (Park et al., 2007; Sun et al., 2008; Du et al., 2012; Liu et al., 2015). Crops can acquire supplementary auxin through various alternative methods. While the application of synthetic auxins is a prevalent practice, it comes with several drawbacks. These compounds exhibit high toxicity and are irritating to the eyes, skin, and respiratory system of farmers. Furthermore, their use can lead to unregulated or irregular plant growth tendencies, such as epinasty (Bhojwani, 2012; Keswani et al., 2020) Another alternative approach involves the contribution of plant-associated beneficial microorganisms, which have been reported to augment auxin levels in plants (Arshad and Frankenberger, 1991; Nassar et al., 2005; Tsavkelova et al., 2007; Shi et al., 2009; Keswani et al., 2020; Iqbal et al., 2023). Endophytic bacteria have been documented to promote plant growth in various crops including rice (Walitang et al., 2017), wheat (Yandigeri et al., 2012; Patel and Archana, 2017), maize (Riggs et al., 2001), potato (Nowak et al., 1995; Pavlo et al., 2011), cucumber (El-Tarabily et al., 2009; Shaalan et al., 2021), cotton (Bashan et al., 1989; Mohamad et al., 2022; Verma et al., 2022), tomato (Pillay and Nowak, 1997; Agarwal et al., 2020).

Endophytic bacterial diversity has been documented across numerous plant species with the Proteobacteria phylum being the most diverse and predominant (Santoyo et al., 2016; Afzal et al., 2019). The bacterial genera most frequently isolated include *Bacillus, Microbacterium, Pantoea, Burkholderia, Micrococcus and Stenotrophomonas*, with *Pseudomonas* and *Bacillus* being the prominent ones (Hallmann et al., 1997; Chaturvedi et al., 2016; Afzal et al., 2019).

Endophytes have been isolated from various tissues of the plant, with roots harboring the maximum number owing to their proximity to a microbe-rich soil environment (Figure 1A). Root rhizodermis cells produce a variety of metabolites, including sugars, purines, amino acids, inorganic ions, and vitamins while root cap cells produce polysaccharide mucilage, facilitating their selective entry into the plant interior (Quadt-Hallmann et al., 1997; Dakora and Phillips, 2002; Bulgarelli et al., 2013; Frank et al., 2017). Endophytes gain access to aerial tissues such as flowers, fruit, stems, and leaves through natural openings like stomata as well as via accidental wounds (Frank et al., 2017; Synek et al., 2021). Endophytes can be vertically transferred through seeds and pollen to the next generation and horizontally transferred by colonizing root and aerial tissues. Recent literature establishes the role of the plant microbiome, especially endophytic bacteria in boosting plant growth, and one of the mechanisms is by elevating auxin levels within plants in response to stress (Kushwaha et al., 2020; Siddique et al., 2022; Kaur and Karnwal, 2023).

This review aims to provide a comprehensive outlook on involvement of auxin in drought and salinity stress, focusing on the disruptions in auxin biosynthesis, transport, and signaling under these conditions. To address these imbalances, potential stress-tolerant endophytic bacteria capable of producing auxin are highlighted. However, translation of this knowledge is currently lacking due to certain limitations. Efforts to create crop-specific and stress-specific bioformulations are minimal. In this review, we try to outline a roadmap to drive these results into potentially useful products. We will discuss the efficient use of these bacterial isolates in the formulation of bioinoculants and how technological advancements in research can further enhance this approach towards sustainable agriculture.

2 Methodology

For this review, articles were sourced from the electronic databases Scopus, Web of Science, and Google Scholar. The search encompassed the entire span of these databases' archives

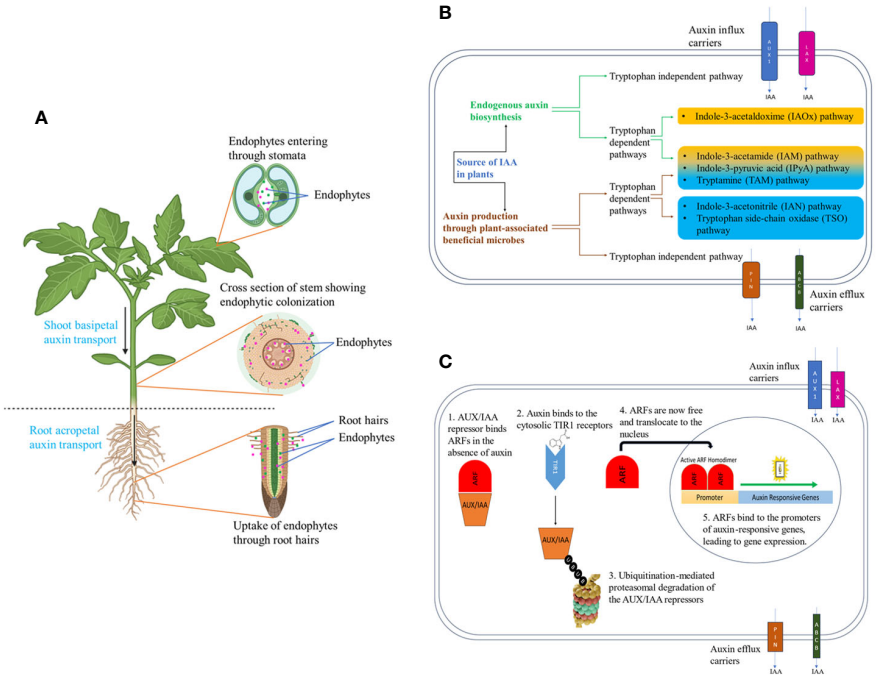

FIGURE 1
Endophytes of plants and auxin biosynthesis and signaling in plants. **(A)** The direction of polar auxin transport in plants and colonization of auxin-producing endophytes within the plant are depicted, **(B)** The biosynthesis pathways for both endogenous plant auxin and auxin produced by beneficial microbial associations, as well as the carriers responsible for auxin influx and efflux, are illustrated **(C)** Auxin signaling cascade in plants: 1. when auxin is absent, AUX/IAA repressors bind Auxin Response Factors (ARFs) in the cytosol, 2. When auxin is present, AUX/IAA repressors are degraded and ARFs move to the nucleus to activate auxin-responsive genes.

up to February 2024, followed by a comprehensive screening that involved manually reading the title and abstract of the retrieved literature.

The search for relevant articles was conducted using the following keywords: plant stress, auxin, drought stress, salt stress, endophytic bacteria, stress tolerance, plant growth promotion, bioinoculants, sustainable agriculture, nanotechnology, and nanoparticles. These terms were strategically combined using the Boolean operators "AND" and "OR" to refine the search scope to the topic of interest.

Only studies that evaluate auxin production and plant growth promotion capabilities of endophytic bacteria under conditions of drought and salt stress are included. Research focused on stress mitigation strategies of endophytic bacteria, rather than auxin production, and studies published in non-indexed journals are excluded.

3 Auxin in plants

Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA), and phenylacetic acid (PAA) are produced within plants, making them exclusive auxins categorized as "endogenous auxins" (Went and Thimann, 1937; Koepfli et al., 1938; Porter and Thimann, 1965). Furthermore, synthetic auxins remain pivotal as herbicides, with compounds such as 2,4- dichlorophenoxyacetic acid being widely utilized worldwide. The regulation of growth and development mediated by auxin involves multiple processes, including its biosynthesis, transport, perception, signaling, and conjugation, all working in concert to coordinate the plant's response. The process of auxin biosynthesis in plants is intricate and involves multiple pathways, as depicted in Figure 1B. According to their physiological status, different plants use different pathways but there are shared fundamental mechanisms across plant species due to the critical role of auxin in the plant life cycle (Mano and Nemoto, 2012). Auxin transportation across plant cells involves a combination of membrane diffusion and carrier-mediated transport mechanisms. Figure 1B highlights some of the influx and efflux carrier proteins involved in this process (Kramer and Bennett, 2006). Auxin can move both basipetally and acropetally from one part of the plant to another with the assistance of these carrier proteins (Figure 1B) (Blakeslee et al., 2005). Auxin is perceived by a cytosolic receptor known as TIR1, initiating a complex signaling cascade as depicted in Figure 1C leading to the regulation of auxin-responsive genes.

Among the natural auxins, IAA stands out as the primary auxin in plants, playing a critical role in regulating many facets of plant growth and development. IAA is involved in root development initiating lateral root and adventitious root formation (Yu et al., 2020), cell elongation (Cleland, 1987), gametophyte development (Zhang and O'Neill, 1993), development of fruit (Pattison et al., 2014), and tropisms (Muday, 2001). Endogenous auxin in plants exists in both active and inactive forms, with the active forms playing a crucial role in signaling and constituting the pool of

endogenous free auxin. For instance, only approximately 25% of the total quantity of IAA is present in its active form, while the majority exists as inactive forms like ester and amide conjugates, which do not actively participate in signaling (Ludwig-Müller, 2011). During abiotic stresses, the formation of these conjugates increases, leading to a decrease in the quantity of endogenous free auxin. To cope with this reduction, plant-associated endophytes supply free auxin during stress conditions, aiding plants in maintaining adequate auxin levels. Several auxin biosynthesis pathways have been identified in these plant growth-promoting endophytes (Figure 1B, Sukumar et al., 2013; Jahn et al., 2021).

3.1 Auxin and drought stress

When plants are subjected to drought stress, it typically leads to a notable decrease in the growth and yield of various crops. Auxin plays critical roles in mitigating drought stress through various mechanisms. In *Arabidopsis*, auxin upregulates antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and glutathione reductase (GR), and helps in decreasing the reactive oxygen species generated due to the stressful conditions. Auxin also upregulates different abiotic stress-related gene expressions like *RAB18, DREB2A, DREB2B, RD22, RD29A*, and *RD29B* and pointedly increases the formation of lateral root and shortens the length of the primary root during drought (Shi et al., 2014). A set of flavin monooxygenases known as YUCCAs has been discovered in various plants. These enzymes play a crucial role in tryptophan-dependent auxin biosynthesis by catalyzing the conversion of tryptamine to N-hydroxy tryptamine (Zhao et al., 2001). In Arabidopsis, *YUC7* can augment endogenous IAA levels and play several roles during drought stress. *yuc7-1D* overexpression studies had confirmed that upregulation of *YUC7* genes consequently upregulated drought resistance genes like *RD29A* and *COR15A* and increased auxin levels had modified the root system increasing lateral root numbers to tolerate the stress (Lee et al., 2012). In potato, *AtYUC6* overexpressed transgenic lines reduced ROS content significantly and improved phenotypic characters during drought conditions as compared to wild-type plants conferring the involvement of auxin in drought tolerance (Kim et al., 2013). In oilseed rape, *BnaYUC6a* overexpressing transgenic lines produced a high amount of auxin and consequently, drought-responsive genes including *ABA2*, *RD26*, and *RD29* expressed in high levels supporting auxin-mediated drought tolerance (Hao et al., 2022). In poplar and potato plants, the modulation of auxin levels has been achieved by regulating the expression of *YUCCA6* using both stress-inducible and constitutive promoters. This manipulation led to increased auxin levels and enhanced drought tolerance (Kim et al., 2013; Ke et al., 2015). During drought conditions in rice, there is an upregulation in the expression level of *OsPIN3t*, an auxin efflux carrier, indicating the role of polar auxin transport (PAT) in stress response. Consequently, it leads to the activation of drought-responsive genes, namely *OsAP37* and *OsDREB2A* (Zhang et al., 2012).

Multiple Gretchen Hagen 3 (*GH3*) family genes have been identified in different plants, including crops, where they significantly influence amide-linked IAA conjugate formation. GH3 enzymes add amino acid residues to free IAA molecules, forming conjugates that reduce the pool of active auxin available for signaling. Numerous studies have demonstrated that under drought stress conditions, the expression of these genes is upregulated (Yuan et al., 2013; Feng et al., 2015; Singh et al., 2015; Yao et al., 2023). For instance, in Arabidopsis, an activation-tagged wes1-D dwarf mutant exhibits a 44.6% reduction in free IAA levels, accompanied by a 621% increase in IAA-Asp conjugates under abiotic stress. The mutant exhibits dwarf phenotypic traits due to a markedly low level of free auxin. Additionally, its auxin-mediated lateral root development is notably impacted, resulting in a reduced number of lateral roots particularly under drought conditions. This observation underscores the significance of free auxin in the process of stress acclimatization. Further substantiating this, the application of a modest quantity of exogenous IAA has been demonstrated to augment the number of lateral roots (Park et al., 2007). One potential reason for the upregulation of *GH3* genes could be the elevated levels of abscisic acid (ABA) during drought conditions (Mittler and Blumwald, 2015) and exogenous ABA treatment also confirmed increased relative expression of GH3 genes (Park et al., 2007; Seo et al., 2009). The ABA signal transduction pathway interacts with auxin signaling, potentially suppressing auxin responses. Lowering auxin levels and hindering its signaling are anticipated to reduce growth rates in poplar plants (Popko et al., 2010). Under drought stress in rice, the expression of six *OsYUCCA* genes, and tryptophan biosynthesis anthranilate synthase genes were downregulated. Conversely, genes related to jasmonic acid (JA) biosynthesis were found to be upregulated in these conditions (Du et al., 2013). JA may act antagonistically to suppress the biosynthesis of IAA but this needs further investigation. Hence, when faced with drought stress, plants need an external source of auxin which can help the plant in tolerating this stress.

3.2 Auxin and salt stress

Increased soil salinity elevates the levels of Na^+ and Cl^- within plants, consequently raising the Na^+/K^+, which disrupts normal ionic functions within plants (Singh et al., 2014). Many plants have evolved various strategies to address these challenges including phytohormonal signaling.

The IAOx pathway of auxin biosynthesis (Figure 1B) involves *P450* genes such as *CYP79B2* and *CYP79B3*, which have been found to positively contribute to salt tolerance. Elevated expression of these specific genes promotes lateral root development in response to salt stress (Julkowska et al., 2017). Auxin influx plays a crucial role in proper plant development and is associated with responses to salt stress (Mellor et al., 2016). Key transmembrane transporter proteins facilitating auxin influx are AUX1 (Auxin Transporter Protein 1) and LAX (Like Auxin Resistant). These proteins participate in various processes, such as gravitropic responses and the emergence of lateral roots (Swarup et al., 2008). LAX3 proteins have been associated with the salt stress response, playing an active role in lateral root development (Mellor et al., 2016). Moreover, overexpression of

WRKY3 in *Solanum lycopersicum* results in elevated levels of *LAX3* transcripts. Remarkably, enhanced resistance to salt stress is exhibited by WRKY3 overexpression lines (Hichri et al., 2017). In response to salt stress, the expression of the *YUCCA* genes is intricately regulated (Korver et al., 2018). For example, when *Cucumis sativus* plants are exposed to salt stress, there is a regulatory interplay among *CsYUC10a*, *CsYUC10b*, and *CsYUC11* genes. Under 100 mM salt stress, *CsYUC10b* experiences an increase in expression, whereas *CsYUC10a* and *CsYUC11* exhibit notable downregulation. This opposing regulation is reinforced by a complementary expression observed in specific tissues. Together, these observations indicate that this opposing mechanism serves to establish a buffering system for endogenous auxin production in cucumber during the stress conditions. Furthermore, studies have confirmed that the overexpression of *CsYUC11* leads to higher concentrations of free IAA and enhances the salt tolerance mechanisms in transgenic Arabidopsis plants (Yan et al., 2016). The function of auxin receptors has been extensively investigated in salt stress-related conditions (Julkowska et al., 2017; Bouzroud et al., 2018). IAA regulates gene expression by directly interacting with TIR/AFB receptors, leading to the SCF E3-ubiquitin ligase-mediated proteasomal degradation of Aux/IAA transcriptional repressor proteins (Figure 1C, Gray et al., 2001). TIR/AFB receptors are actively involved in plant's response to salt stress. In Arabidopsis, a miR393-resistant variant of TIR1 (mTIR1) overexpression leads to enhanced salt tolerance. miR393, which targets TIR1 and AFB2 receptors for degradation is shown to increase in NaCl-induced salt stress. This degradation leads to the downregulation of auxin signaling and consequent repression of Auxin Response factor (ARF) genes (Figure 1C). However, the heightened expression of mTIR1 augments auxin signaling and bolsters plant resistance to salt stress by enhancing osmoregulation and augmenting Na^+ exclusion mechanisms (Chen et al., 2015). ARF transcription factors are key players in the auxin signaling pathway as these interact with the promoters of auxin-responsive genes (Lavy and Estelle, 2016). ARFs have been identified as crucial elements in several responses of the plants to abiotic stress (Wang et al., 2010; Hu et al., 2015). The role of ARF proteins has been investigated in rice and sweet potato under salt and drought stress. Overexpression of sweet potato *IbMP/ARF* in Arabidopsis enhances auxin signaling under both drought and salt stress (Kang et al., 2018). Genes like *OsARF11* and *OsARF15* in rice are upregulated by several folds under salt stress conditions implicating their role in this response (Jain and Khurana, 2009). Furthermore, the transportation of auxin across cells within a plant necessitates a well-coordinated auxin transport system. Among the key protein efflux carriers facilitating polar auxin transport (PAT), the PIN family proteins play a central role. However, the physiological and biochemical alterations induced by salt stress adversely impact PAT, posing a potential threat to the effective functioning of the auxin transport network. Salt stress triggers an increase in phospholipase D activity, leading to the localization of clathrin in the plasma membrane. This, in turn, initiates clathrin-mediated endocytosis of PIN2 proteins. Consequently, auxin redistribution occurs, causing the root tip to bend away from areas with higher salt levels, known as auxin-mediated halotropism (Galvan-Ampudia et al., 2013). The PIN protein family, especially the plasma membrane-located proteins like PIN1, PIN3, and PIN7, are essential for controlling auxin transport and adapting to salt stress. Notably, under salt stress conditions, there is a significant impairment in auxin transport, aligning with the detrimental effects of salt stress on root development. During salt stress, nitric oxide (NO) production is triggered which directs *PIN1*, *PIN3*, and *PIN7* downregulation in Arabidopsis. This decrease in expression results in reduced auxin transport and subsequently impacts auxin signaling (Liu et al., 2015). Free auxin levels are also affected during salt stress. Notably, *GH3* genes are activated during salt stress (Korver et al., 2018). Collectively, these alterations lead to a decrease in the endogenous free auxin levels, ultimately resulting in diminished plant growth.

A brief overview of the impact of drought and salt stress on plant growth through the involvement of auxin is depicted in Figure 2.

4 Auxin-producing endophytes and their potential use in drought and salt tolerance

Several researchers have extensively investigated auxin production, particularly IAA by plant-associated microorganisms, including endophytic bacteria (Kuklinsky-Sobral et al., 2004; Madhaiyan et al., 2004; Tsavkelova et al., 2007; Khan et al., 2014). However, both drought stress and salt stress are limiting factors in the growth of such organisms thus highlighting a need to find stress-tolerant endophytes. A logical step would be to look for plants that face such stresses regularly, namely xerophytic and halophytic plants, and study their microbiome (Bokhari et al., 2019; Rodríguez-Llorente et al., 2019; ALKahtani et al., 2020; Belaouni et al., 2022; Chebotar et al., 2022). Several researchers have isolated numerous auxin-producing stress-tolerant endophytic bacteria from such plants. These bacteria were further introduced into various crop plants subjecting them to diverse stress conditions. Promising bacterial isolates with the potential to promote plant-growth parameters like auxin quantity, seed germination, and root and shoot length have been identified (Govindasamy et al., 2022; Hwang et al., 2022).

4.1 Drought stress mitigation using auxin-producing endophytic bacteria

Drought stress causes a decrease in auxin concentration in plants, necessitating an increased supply to alleviate the stress and sustain growth. Several research groups have investigated auxin-producing drought-tolerant endophytic bacteria, and upcoming discussions will delve into recent research findings in detail (Table 1). *Opuntia ficus-indica*, a desert plant, has been identified as a valuable source of multiple drought-tolerant auxin-producing endophytic bacteria, having several plant growth-promoting characteristics. Among the several *Streptomyces* species isolated, *S. rameus* VL-70-PIII demonstrated the highest auxin production, reaching a peak of 200.82 µg/ml in a medium supplemented with 100 mg/ml L-tryptophan after a 5-day period. Upon inoculating

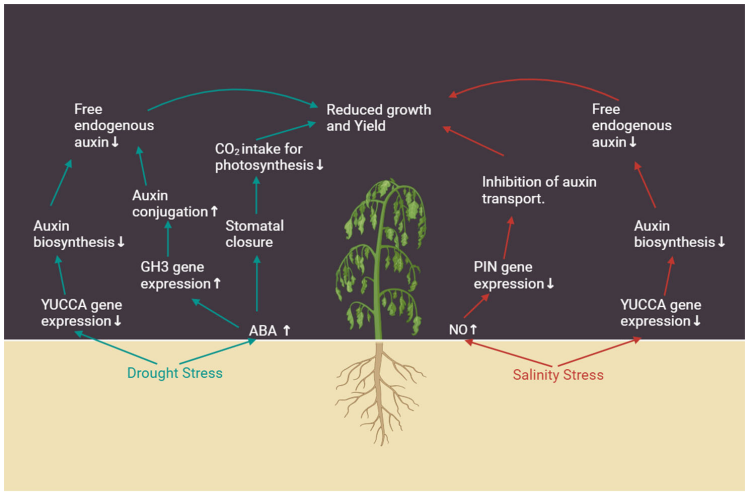

FIGURE 2
Impact of drought and salinity stress on various aspects of auxin signaling, and its consequences on gene expression and physiology. Arrows next to the text indicate increase (↑) or decrease (↓).

TABLE 1 Auxin-producing endophytic bacteria, along with their sources and applications in promoting plant growth and alleviating drought stress.

Sr. No.	Endophytic Bacteria	Source plant	Plant part used for isolation of endophytes	Inoculated plant	Plant response	Reference
1	*Streptomyces turius* VL-70-IX	*Opuntia ficus-indica*	Roots	*Triticum aestivum* (cultivar-nethravati)	Increase in rootlet numbers, root length, shoot length and total seedling length	(Govindasamy et al., 2022)
2	*S. levis VL-70-XII*					
3	*S. mutabilis* HV-18					
4	*S. mutabilis* HV-VIII					
5	*S. rameus* VL-70-PIII					
6	*Micrococcus luteus* strain 4.43	*Helianthus tuberosus*	Leaf and stem	*Helianthus tuberosus*	Improved plant height, total fresh weight and dry weight, root length and diameter, and harvest index	(Namwongsa et al., 2019)
7	*Bacillus aquimaris* 3.13					
8	*B.* sp. 5.2					
9	*B. methylotrophicus* 5.18					
10	*Staphylococcus* sp. Ceb1	*Curcuma longa*	Rhizome	*Vigna unguiculata*	Increased root length and number, and shoot length	(Jayakumar et al., 2020a)
11	*Shewanella putrefaciens* strain MCL-1	*Pennisetum glaucum, Brassica nigra, Cyamopsis tetragonoloba*		*Pennisetum glaucum* (variety Pusa Composite-443)	Improved seed germination percentage, plumule length, radicle length, and fresh weight. Upregulation of drought-responsive SbNAC1, PgAP2 and PgDREB2A genes	(Manjunatha et al., 2017, Manjunatha et al., 2022)
12	*Cronobacter dublinensis* strain MKS-1					
13	*Bacillus* sp. Acb9	*Ananas comosus*	Leaf	*Vigna radiata*	Increased shoot length, root length, and root numbers	(Jayakumar et al., 2020b)
14						

(Continued)

TABLE 1 Continued

Sr. No.	Endophytic Bacteria	Source plant	Plant part used for isolation of endophytes	Inoculated plant	Plant response	Reference
	Providencia sp. Acb11					
15	*Staphylococcus* sp. *Acb12*					
16	*Staphylococcus* sp. *Acb13*					
17	*Staphylococcus* sp. *Acb14*					
18	*Acinetobacter pittii*	*Sorghum bicolor*	Root	*Sorghum bicolor* (variety CO 30 and K 30)	Increase in seed germination percentage	(Umapathi et al., 2022)
19	*Pseudacidovorax intermedius*					
20	*Exiguobacterium* sp. Sch36	*Sporobolus speccatus*, *Cyperus laevigatus*	Root, Stem and Leaves	–		(Enquahone et al., 2022)
21	*Exiguobacterium* sp. Rch312					
22	*Alishewanella* sp. Rch14					
23	*Pantoea alhagi*	*Alhagi sparsifolia*	Whole plant	*Triticum aestivum* (cultivar Yumai 49-198)	Improvement in root and shoot length, plant fresh weight, and chlorophyll, MDA, and soluble sugar content in leaves	(Chen et al., 2017)
24	*Streptomyces coelicolor* DE07	*Aerva tomentosa*, *Acacia nilotica*, *Leptadenia pyrotechnica*, *Calligonum polygonides*, *Pennisetum glaucum*	Roots	*Triticum aestivum* (cultivar WR-544)	Increased root and shoot length, tiller numbers, fresh and dry weight of root and shoot, and yield	(Yandigeri et al., 2012)
25	*S. olivaceus DE10*					
26	*S. geysiriensis DE27*					

these strains to wheat seeds and keeping in drought conditions, *S. turius* VL-70-IX treatment led to a maximum increase in rootlet count. Furthermore, the co-inoculation of *S. levis* VL-70-XII and *S. turius* VL-70-IX resulted in the maximum increase in root length, while *S. mutabilis* HV-VIII showed the highest increase in shoot length (Govindasamy et al., 2022). In a separate study, an endophytic bacterium, *Pantoea alhagi*, isolated from Camelthorn plant *Alhagi sparsifolia*, exhibited drought-tolerant traits, thriving in media supplemented with 20% PEG and producing up to 17.73 µg/mL of IAA. When introduced to drought-stressed wheat seedlings, this strain effectively enhanced various plant growth parameters, including fresh weight, chlorophyll content, and soluble sugar content significantly (Chen et al., 2017). In a different study, three drought-tolerant actinobacteria from the roots of five distinct plant species (Table 1) significantly boosted growth and yield in another wheat cultivar, WR-544, by several folds. Instead of single isolate inoculation, co-inoculation with *Streptomyces olivaceus* and *S. geysiriensis* demonstrated maximum enhancement in growth and yield properties in drought-stressed wheat fields (Yandigeri et al., 2012).

The microbiome of medicinal plants has also been explored to identify drought-tolerant endophytic bacteria. For example, the rhizome of *Curcuma longa* harbored *Staphylococcus* sp. Ceb1, an endophyte capable of producing auxin along with other characteristics promoting plant growth. Surface-sterilized *Vigna unguiculata* seeds were germinated, treated with Ceb1, and then subjected to drought stress by withholding water for three weeks, after which water was resumed for one day before determining plant growth parameters. Compared to the control, there was an increase of 87.5% in root number, 208.4% in root length, and 55.54% in shoot length (Jayakumar et al., 2020a).

Researchers have explored the method of isolating stress-tolerant endophytes from crops and reintroducing them back into the same crops to enhance the uptake of these endophytes within the plant body, resulting in improved outcomes. These studies highlight the potential of native endophytes in stress tolerance. Manjunatha et al. (2017), isolated *Shewanella putrefaciens* MCL-1 and *Cronobacter dubliensis* MKS-1 from mustard, cluster bean, and pearl millet. Both MCL-1 and MKS-1 demonstrated the ability to promote growth. Following soaking in endophyte broth cultures for one hour, the sterilized seeds were placed on agar petri plates supplemented with 20% PEG for germination. Three days later, treatment with MCL-1 resulted in a 16.6% increase in plumule length, 9.02% increase in radicle length, and a 16.88% increase in fresh weight, while MKS-1 treatment led to an 18.8% increase in plumule length, 24% increase in radicle length, and a 21.63%

increase in fresh weight (Manjunatha et al., 2017). Further investigations conducted by the same research group affirmed that under severe drought stress conditions, endophyte-inoculated pearl millet plants treated with MCL-1 and MKS-1 demonstrated the ability to elevate auxin levels in pearl millet, resulting in a significant 68%–78% increase in IAA content, compared to uninoculated controls. Moreover, they noted a substantial upregulation, by several folds, of various stress-responsive genes such as *SbSNAC1*, *PgDREB2A*, and *PgAP2* under severe drought conditions in comparison to the endophyte-uninoculated control (Manjunatha et al., 2022). Eventually, the upregulation of these defense genes is the major target for auxin-mediated defense response against abiotic stresses as discussed in earlier sections. In another study, *Micrococcus luteus* 4.43, *Bacillus aquimaris* 3.13, *Bacillus* sp. 5.2, and *B. methylotrophicus* 5.18, isolated from *Helianthus tuberosus*, exhibited the ability to produce auxin while promoting the growth of *H. tuberosus* from planting to harvesting stages under water-stressed conditions. Among these strains, *M. luteus* produced the highest amount of IAA. Strains 4.43 and 3.13 notably enhanced fresh shoot weight and plant height, respectively, when plants received only 1/3 of their water requirement at 140 days. Strain 3.13 also increased shoot and root dry weight significantly under conditions of reduced water, both at 140 days (using only 2/3 of water) and at 60 days (using only 1/3 of water). Additionally, Strain 4.43 exhibited the greatest improvement in yield under conditions of limited water supply, specifically when only 1/3 of the water requirement was provided (Namwongsa et al., 2019). In a separate study, Umapathi et al. (2022), discovered that endophytic bacteria associated with sorghum roots have the ability to produce IAA and GA, while also enhancing various plant growth parameters under drought stress conditions. Particularly, *Pseudacidovorax intermedius* demonstrated the highest production of under -1 MPa PEG 6000 stress (Umapathi et al., 2022).

Economically significant plants like *Ananas comosus* have been utilized for isolating potent endophytic bacteria. Among the five isolated strains, *Providencia* sp. Acb11 exhibited the highest auxin production of 100 µg/ml under PEG (-1.5 MPa) conditions. Whereas, *Bacillus* sp. Acb9 produced 55 µg/mL IAA and *Staphylococcus* sp. Acb13 produced 10 µg/mL IAA under the same conditions. All strains contributed to the promotion of plant growth in *Vigna radiata* seedlings. *Bacillus* sp. Acb9 notably increased shoot length and root length by 34.8% and 153%, respectively. Additionally, *Staphylococcus* sp. Acb13 significantly enhanced the maximum root number by 160% compared to the control (Jayakumar et al., 2020b).

4.2 Salt stress mitigation using auxin-producing endophytic bacteria

Numerous studies suggest the involvement of endophytes that produce auxin in the tolerance to salt stress conditions (Table 2). Many such endophytes were isolated from halophytes. In one such study conducted by Hwang et al. (2022), *Priestia megaterium* Strain BP-R2 was isolated from the halophytic plant *Bolboschoenus planiculmis*. The bacterium was capable of producing approximately 25 µg/mL of IAA in NaCl concentrations ranging from 0.5 to 3.0% over a period of 48 hours. When inoculated in *Arabidopsis thaliana* (ecotype Columbia) plants under 250 mM NaCl conditions, it led to more than a 1.5-fold increase in leaf numbers, rosette diameter, fresh weight, and dry weight compared to control plants. Similarly, inoculation of the bacteria in *Brassica rapa* (pak choi) plants under 200 mM NaCl conditions resulted in a significant increase in plant height, width, leaf numbers, total leaf area, leaf length, width, and area per leaf, as well as root fresh weight, dry weight, and length as compared to control plants (Hwang et al., 2022). In another study, *Bacillus cereus* KP120, isolated from the halophytic plant *Kosteletzkya pentacarpos* produced significant amount of IAA after 15 minutes in LB medium supplemented with Tryptophan. When inoculated in Arabidopsis seedlings under 200 mM NaCl concentration, KP120 increased the IAA concentration by 35.83% in roots and 8.41% in leaves compared to control plants. Additionally, plant height, branch number, leaf number and root lengths increased by 182.24%, 53.84%, 14.28%, and 14.40% respectively as compared to the control group (Zhang et al., 2022). In another study, Khan et al. (2020), selected six bacterial endophytes from the root tissues of *Oenothera biennis* L., *Chenopodium ficifolium* Smith, *Artemisia princeps* Pamp, *Echinochloa crus-galli* (L.). Among these, *Enterobactor ludwigii* and *Curtobacterium luteum* produced 2.7 µg/mL IAA, whereas *Enterobacter tabaci, Bacillus cereus, Micrococcus yunnanensis*, and *Micrococcus curtobacterium oceanosedimentum* produced IAA in 1.1 to 1.6 µg/mL range. All the six strains of bacteria were tested for their effect on rice plants growing under 150 mM NaCl by inoculating the roots. *M. yunnanensis* increased shoot length by a maximum of 22.9%, *M. yunnanensis* and *C. luteum* increased root length by a maximum of 40%, *M. yunnanensis* increased fresh weight by a maximum of 25.7% and *C. oceanosedimentum* increased dry weight by a maximum of 29.1% and chlorophyll content by 52.1% in comparison to control (Khan et al., 2020). The shoot-associated endophyte, *Stenotrophomonas pavanii*, isolated from the halophyte *Seidlitzia rosmarinus* could produce a maximum of 20.5 µg/ml IAA when tryptophan was added to the media. Out of total 17 endophytes, 11 endophytes were capable of producing IAA and among them 10 were capable of promoting growth in cress-lettuce. *Pseudomonas fluorescens* showed the maximum increase in seed germination percentage, root growth, and shoot growth by 9%, 16.6%, and 11.7%, respectively under 100mM NaCl stress (Shurigin et al., 2020). In a separate study, *Oceanobacillus* sp.76, *Bacillus* sp. 7, and *Micrococcus luteus* 14 were isolated from *Cressa cretica, Salsola yazdiana* and *Salsola tomentosa*, respectively. These strains demonstrated the ability to germinate seeds of *Triticum aestivum* cv. Homa and *T. aestivum* cv. Mihan up to 91.66%, while control seeds failed to germinate under 300 mM NaCl stress. Furthermore, they significantly increased seedling, root, and shoot length in both wheat varieties under NaCl treatment up to 300 mM (Soltani et al., 2024). In their study, Zhao et al. (2016), investigated the effects of endophytes, associated with the halophytic plant *Salicornia europiea* in promoting the growth of *S. europiea* under salinity stress up to 500 mM. The auxin

TABLE 2 Auxin-producing endophytic bacteria, along with their sources and applications in promoting plant growth and alleviating salt stress.

Sr. No.	Endophytic Bacteria	Source plant	Plant part used for isolation of endophytes	Inoculated plant	Plant response	Reference
1	*Priestia megaterium*	*Bolboschoenus planiculmis*	Root	*Arabidopsis thaliana*, *Brassica rapa*	Increased fresh and dry weight, leaf numbers, total leaf area and average plant height	(Hwang et al., 2022)
2	*Bacillus cereus* KP120	*Kosteletzkya pentacarpos*		*Arabidopsis thaliana*	Upregulation of several SAUR family genes, YUCCA genes, ethylene synthesis, and signaling genes. Improvement in fresh and dry weight of shoot and root, plant height, root length, branch number and leaf number	(Zhang et al., 2022)
3	*Curtobacterium oceanosedimentum*	*Oenothera biennis L.* *Artemisia princeps* Pamp. *Chenopodium ficifolium* Smith. *Echinochloa crus-galli (L.) P.Beauv.*	Root	*Oryza sativa*	Increased shoot and root length, fresh and dry weight, and leaf chlorophyll content Upregulation of OsYUCCA1 gene, and OsPIN1 gene	(Khan et al., 2020)
4	*C. luteum*					
5	*Enterobactor ludwigii*					
6	*E. tabaci*					
7	*Bacillus cereus*					
8	*Micrococcus yunnanensis*					
9	*Kochuria palustris*	*Seidlitzia rosmarinus* Ehrenb. ex Boiss	Root, Shoot	*Lepidium sativum*	Improved root and shoot length, and seed germination percentage	(Shurigin et al., 2020)
10	*Staphylococcus succinus*					
11	*Staphylococcus epidermis*					
12	*Pseudomonas baetica*					
13	*Pseudomonas fluorescens*					
14	*Paenibacillus amylolyticus*					
15	*Stenotrophomonas pavanii*					
16	*Rothia terrae*					
17	*Planomicrobium koreense*					
181	*Planomicrobium soli*					
19	*Oceanobacillus* sp. *76*	*Cressa cretica*	Root	*Triticum aestivum*	Increased seed germination percentage, seedling length, and root and shoot length	(Soltani et al., 2024)
20	*Micrococcus luteus 14*	*Salsola tomentosa*	Shoot			
21	*Bacillus* sp. *7*	*Salsola yazdiana*	Root			
22	*Bacillus tequilensis*	*Salicornia europaea*	Stem	*Salicornia europaea*	Improved seed germination, shoot and root length, and fresh weight	(Zhao et al., 2016)
23	*Planococcus rifietoensis*		Stem			
24	*Variovorax paradoxus*		Root			

(Continued)

TABLE 2 Continued

Sr. No.	Endophytic Bacteria	Source plant	Plant part used for isolation of endophytes	Inoculated plant	Plant response	Reference
25	*Streptomyces heliomycini*	*Thymus roseus*		*Gossypium hirsutum* (variety Yumian-1)	Increased shoot and root length, and root and shoot fresh weight	(Mohamad et al., 2022)
26	*Nocardiopsis dassonvillei*					
27	*Alloactinosynnema album*					
28	*Bacillus subtilis*	*Cicer arietinum*	Root	*Pisum sativum*	Improved shoot and root length, fresh and dry weight of shoot and root, total pigment content, antioxidative activity, macronutrient concentration, and ethylene concentration	(Sofy et al., 2021
29	*Pseudomonas fluorescens*					
30	*Bacillus halotolerans*	*Lilium davidii* (variety *Unicolor*)	Root	*Lilium davidi* (variety Bright Diamond)	Improvement in plant height, leaf length, leaf width, root length and root dry weight	(Gao et al., 2022)
31	*Sphingomonas paucimobilis*	*Dendrobium officinale*	Root	–	–	(Li et al., 2023)
32	*Pseudomonas pseudoalcaligenes*	*Suaeda nigra*	Root, Aerial parts	–	–	(M. Sridevi et al., 2022)
33	*Bacillus licheniformis*	*Vigna radiata*	Root, Nodules	–	–	(Bhutani et al., 2022)
34	*Enterobacter cloacae S23*	*Arachis hypogaea* (variety VRI2)	Root nodules	–	–	(Ramakrishnan et al., 2023)
35	*Streptomyces pactum*	*Limonium sinense*	Root and Leaves	–		(Qin et al., 2014)
36	*Klebsiella pneumoniae* subsp. *rhinoscleromatis*	–	–	–		
37	*Serratia rubidea*	–	–	–		
38	*Pseudomonas brassicacearum* subsp. *brassicacearum*	–	–	–		
39	*Pantoea hericii*	*Limonium vulgare*	Root	*Vitis vinifera*	Increased in number of leaves, shoot length, dry weight of shoot and root	(Navarro-Torre et al., 2023)
40	*Pantoea anthophilla*	*Limonium daveaui*				
41	*Pantoea agglomerans*					
42	*Exiguobacterium* sp. Sch36	*Sporobolus speccatus*, *Cyperus laevigatus*	Root, Stem and Leaves	–	–	(Enquahone et al., 2022)
43	*Exiguobacterium* sp. Rch312					
44	*Alishewanella* sp. Rch14					

production capability of these endophytes was also assessed, and *Planococcus rifietoensis* exhibited the highest production, reaching a maximum of 1.2 µg/mL while tolerating up to 0.68 M NaCl concentration (Zhao et al., 2016).

Li et al. (2023), found that the root-associated endophyte *Sphingomonas paucimobilis*, isolated from medicinal herb, *Dendrobium officinale*, produces indole-3-acetic acid (IAA) through the indole acetamide (IAM), indole acetonitrile (IAN), and indole pyruvate (IPA) pathways. Additionally, this strain demonstrated tolerance to high levels of NaCl, up to 80 g/L (Li et al., 2023). In another study, conducted by Mohamad et al. (2022) three endophytic bacteria were screened from the medicinal plant, *Thymys roseus*, demonstrating the capability to produce auxin and tolerate up to 200 µM NaCl stress. Upon inoculation of these bacteria into cotton plants, *Streptomyces atrovirens* exhibited the greatest increase in root length and weight compared to the control, while *Alloactinosynne album* caused the maximum increase in shoot length and weight (Mohamad et al., 2022).

Numerous studies have shown the salt tolerance of various endophytic species from the *Pseudomonas* and *Bacillus* genera. In a study, *Pseudomonas fluorescens* and *Bacillus subtilis*, isolated from the roots of leguminous plant *Cicer arietinum*, could produce 5.98 and 8.11 µg/ml of IAA, respectively. *B. subtilis* exhibited greater potency, increasing shoot length, fresh weight of shoots, dry weight of shoots, fresh weight of roots, and dry weight of roots by 46.52%, 48.69%, 119.17%, 109.52%, and 141.27%, respectively, compared to the control in pea plants growing under 150 mM NaCl stress (Sofy et al., 2021). In a separate study, *Bacillus halotolerans*, an endophyte isolated from *Lilium davidii* var. unicolor, exhibited tolerance to up to 6% NaCl addition in LB media, along with confirmed auxin production ability. Upon inoculation in another *Lilium* variety, bright diamond, it led to an increase in plant height, leaf length and width, root length, and dry weight by 4%, 7.6%, 2.8%, 93.6%, and 138.7%, respectively (Gao et al., 2022). Multiple salt-tolerant and auxin-producing endophytes have been identified in *Limonium sinense*. *Streptomyces pactum*, isolated from *L. sinense* leaves, produced a maximum of 8.24 mg/L of IAA and demonstrated tolerance to 7% NaCl. It was also capable of increasing *L. sinense* seed germination by 12% under 500 mM NaCl conditions in comparison to the control (Qin et al., 2014). Utilizing microbial consortia has shown promising results for enhancing halotolerance. *Pantoea hericii*, *Pantoea anthophilla*, and *Pantoea agglomerans* were isolated from various halotolerant *Limonium* sp. and *P. anthophilla* produced a maximum of 11.78 mg/L IAA. These three isolates were inoculated as a consortium into grapevine plants under salt-stress conditions Consortium inoculated plants displayed significant increase in leaf numbers and shoot length and were able to withstand salt stress effectively. Additionally, after the stress was removed, the recovery rate of the inoculated plants was significantly higher compared to the control plants (Navarro-Torre et al., 2023).

Further, multiple studies have been carried out focusing solely on the isolation of auxin-producing salt-tolerant endophytic bacteria. A study by Sridevi et al. (2022), isolated and reported a novel endophyte, *Pseudomonas pseudoalcaligenes* from *Suaeda nigra*. This strain was capable of producing 43 µg/ml IAA after 48 hour incubation period and demonstrated tolerance to NaCl up to 8% (M. Sridevi et al., 2022). Another endophyte, *Bacillus licheniformis*, isolated from *Vigna radiata*, produced 27 µg/mL IAA after 30 minutes incubation with tryptophan and exhibited tolerance to a NaCl concentration of 15% (Bhutani et al., 2022). In another study, *Enterobacter cloacae*, isolated from the root nodules of groundnut was able to produce 0.37 µg/mL IAA under 7% NaCl stress (Ramakrishnan et al., 2023). Whether this exhibition of salt tolerance and IAA production observed in such studies proves to be useful for plants, needs further investigation.

5 Conclusion and future perspective

Despite extensive research focusing on the isolation and screening of potential endophytes through short-term experiments, there is a notable gap in studies that span throughout the entire cultivation cycle, from sowing to harvesting of the crops to observe the effects of the potential isolates on stress alleviation and crop yield improvement. In addition, subsequent steps post-identification using the endophytes such as bioinoculant development, patenting, and marketing are imperative to make these advancements available to farmers for application in crop fields. Furthermore, it is crucial to choose an appropriate carrier for endophyte protection and stabilization during transportation and storage. Therefore, comparative studies on formulations with various carriers should be conducted to maximize the product's effectiveness during use. To address these challenges, a suggested roadmap is delineated to guide translational research in ensuring global food security by developing bioinoculants for sustainable agricultural practices in the face of a rapidly changing climate (Figure 3).

A recent technological advancement in increasing agricultural productivity is the use of nanoparticles, including inorganic and organic nanomaterials. It has been reported that several endophytic bacteria produce nanomaterials, which have been demonstrated to help the plant endure abiotic stresses. Besides, using nanomaterial for bioinoculant development may enhance its effectiveness, bioavailability, and stability (Meena et al., 2021; Adeleke et al., 2022). However, the use of nanoparticles in auxin production by endophytes and auxin-mediated stress tolerance in crops needs exploration. The application of phytohormones directly using nanoparticles for plant growth promotion and defense induction has been recently explored. Recent studies have combined nanocarriers with hormones like SA, GA, JA, ABA, and IAA for the promotion of plant growth properties (Pereira et al., 2017; Clemente et al., 2018; Sun et al., 2018; Kumaraswamy et al., 2019; Korpayev et al., 2021; Gonzalez-Montfort et al., 2022; Wu et al., 2022). Future experiments that analyze the effect of nanoparticles on auxin production by endophytes and employ their use in the formulation of bioinoculants will be beneficial. This will promote studies to understand how these nanomaterials can modulate auxin biosynthesis, transport, and signaling in endophytes and plants under drought and salt stress conditions.

In conclusion, overall evidence suggests that the phytohormone auxin plays several roles in tolerating drought and salt stresses, and

FIGURE 3
Proposed roadmap for the development of stress-specific and crop-specific bioinoculants.

stress-tolerant auxin-producing endophytes can be a good source of supplemental auxin for stressed plants. This review extensively discusses and highlights potential isolates that can be used for bioinoculant development. Furthermore, the effectiveness of bioinoculants must be validated through extensive field trials in stress-affected fields before introducing the product to the market. Additionally, raising awareness among farmers to transition from conventional chemical products and using these bio-products is a crucial step towards sustainable agriculture.

Author contributions

SM: Conceptualization, Software, Writing – original draft, Writing – review & editing. SP: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors are grateful to the Vellore Institute of Technology, Vellore, Tamil Nadu, India for providing the facilities, support, and encouragement. SP acknowledges Vellore Institute Technology (VIT), Vellore, India, for the seed grant [sanction order no. SG20230036]. All the images are created in Biorender.com.

Conflict of interest

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

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frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 15 July 2024
DOI 10.3389/fpls.2024.1406913

OPEN ACCESS

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RECEIVED 25 March 2024
ACCEPTED 24 June 2024
PUBLISHED 15 July 2024

CITATION
Acharya BR, Gill SP, Kaundal A and Sandhu D (2024) Strategies for combating plant salinity stress: the potential of plant growth-promoting microorganisms.
Front. Plant Sci. 15:1406913.
doi: 10.3389/fpls.2024.1406913

Strategies for combating plant salinity stress: the potential of plant growth-promoting microorganisms

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Global climate change and the decreasing availability of high-quality water lead to an increase in the salinization of agricultural lands. This rising salinity represents a significant abiotic stressor that detrimentally influences plant physiology and gene expression. Consequently, critical processes such as seed germination, growth, development, and yield are adversely affected. Salinity severely impacts crop yields, given that many crop plants are sensitive to salt stress. Plant growth-promoting microorganisms (PGPMs) in the rhizosphere or the rhizoplane of plants are considered the "second genome" of plants as they contribute significantly to improving the plant growth and fitness of plants under normal conditions and when plants are under stress such as salinity. PGPMs are crucial in assisting plants to navigate the harsh conditions imposed by salt stress. By enhancing water and nutrient absorption, which is often hampered by high salinity, these microorganisms significantly improve plant resilience. They bolster the plant's defenses by increasing the production of osmoprotectants and antioxidants, mitigating salt-induced damage. Furthermore, PGPMs supply growth-promoting hormones like auxins and gibberellins and reduce levels of the stress hormone ethylene, fostering healthier plant growth. Importantly, they activate genes responsible for maintaining ion balance, a vital aspect of plant survival in saline environments. This review underscores the multifaceted roles of PGPMs in supporting plant life under salt stress, highlighting their value for agriculture in salt-affected areas and their potential impact on global food security.

KEYWORDS

climate change, glycophyte, ion toxicity, osmotic stress, PGPMs, salinity tolerance, salt stress

1 Introduction

Climate change poses a formidable challenge to global agricultural productivity, with agriculture being particularly vulnerable to shifts in weather patterns and climate conditions. A persistent increase in the average global temperatures has been recorded in recent years, posing significant challenges to agricultural productivity, food security, and environmental sustainability (Siegel, 2021). Climate change not only is limited to increasing average global temperature but also includes erratic rainfall patterns, heat waves, droughts, and flash floods, all of which adversely affect soil and water resources, agricultural workers, and rural communities (https://www.epa.gov/climateimpacts/climate-change-impacts-agriculture-and-food-supply). Regions that rely heavily on agriculture, such as South Asian countries, are particularly impacted by these climate-related challenges (Rhaman et al., 2022). The global population is projected to exceed 10 billion within the next 50 years (Glick, 2014), significantly increasing the demand for food production and placing additional strain on existing agricultural systems (Alexandratos, 2005; Cheeseman et al, 2016). This surge in population presents the dual challenges of boosting agricultural productivity amidst increasingly worsening environmental conditions. Among these challenges, drought and salt stress are the two major abiotic stressors that significantly reduce crop yields and threaten food security and livelihoods (Cheeseman et al, 2016).

Innovative strategies should be developed to address the food security crisis and meet the demand of the projected growing population in the climate change-induced environmental stresses (Wang et al., 2020). The approaches being used currently, including genetically modified organisms, have shown promise in mitigating the impact of drought and salinity stress (Askari and Pepoyan, 2012; Liang, 2016; Raza et al., 2023). However, regulatory constraints and environmental concerns are hurdles to widespread adoption and spread. Other candidate approaches include agronomic management practices (Majeed and Siyyar, 2020) and soil amendments (Bello et al., 2021). Organic amendments like biochar, bio-fertilizer, vermicompost, and vermiwash can improve the salinity tolerance of agricultural plants, leading to increased yields (Hoque et al., 2022). Additionally, seed priming and exogenous application of growth regulators can alleviate salt stress impacts in plants at various stages of development from germination to maturity (Tania et al., 2022). However, these approaches have limited effectiveness under harsh conditions, can be expensive, may vary in efficacy with different crop species, and may have environmental impacts.

Beneficial microorganisms colonize plants' rhizospheres or inside tissues, promoting growth, improving nutrient uptake, and conferring tolerance to various abiotic stresses (Ganesh et al., 2022; Vocciante et al., 2022). Unlike genetically modified organisms (GMOs), plant growth-promoting microorganism (PGPM)-based interventions offer a sustainable and environmentally friendly approach to improving crop resilience without genetic modifications or adverse environmental effects. This review highlights the significant contributions of microorganisms to sustainable crop production under challenging environmental conditions. By examining the mechanisms underlying PGPM-mediated salinity tolerance and their potential agricultural applications, we underscore the vital role of these microorganisms in addressing future agricultural challenges. We focus on PGPMs as a promising solution for overcoming the limitations of existing strategies in mitigating salinity stress. Harnessing the potential of PGPMs holds great promise for addressing the complex challenges posed by climate change and ensuring global food security amid increasing salinity stress. In this era of unprecedented threats to agriculture, it is imperative to develop innovative strategies to counteract these emerging challenges.

2 Salinity stress in plants and its impact on crop production and plant responses

Salinity, a major abiotic factor, severely affects the growth, development, and yield of various plants at different stages of their life (Khan et al., 2022). Soil salinization impacts agricultural productivity around the globe (Hu et al., 2022). Over 800 million hectares of irrigated land are impacted by soil salinity and are anticipated to be aggravated by both current irrigation practices and global climate change (Roy et al., 2014). The rising salinity in soils and water resources is contributed by natural incidents and/or human activities like irrigation water containing higher salts (Eswar et al., 2021). Saline soil with high Na^+ negatively impacts soil–water and soil–air relationships, directly influencing plant growth and productivity (Rengasamy and Olsson, 1991; Dexter, 2004). Increasing salinity stress modifies soil texture, causing decreased porosity, which causes reduced water uptake by plants (Lu and Fricke, 2023). Salinity not only disrupts the soil's physical structure but also significantly hampers the overall growth of plants, affecting shoots, roots, and reproductive organs. Salinity-induced modification of morphological, biochemical, and physiological processes in plants diminishes agricultural productivity. In addition, fluctuation in water dynamics, transpiration, nutritional equilibrium, stomatal conductance, and oxidative damage under salt stress collectively decrease crop yield. Moreover, salt stress hampers photosynthetic activity, impedes biomass accumulation, and disrupts source–sink dynamics, exerting a detrimental influence on yield-related variables and accelerating the

Abbreviations: ABA, Abscisic acid; ACC, 1-aminocyclopropane-1-carboxylate; APX, ascorbate peroxidase; ASC, ascorbate; BRs, brassinosteroids; CAT, catalase; CKs, cytokinins; DHAR, dehydroascorbate reductase; EC_e, electrical conductivity of soil saturation extract; EPS, extracellular polymeric substances; ET, ethylene; GAs, gibberellins; GIPC, glycosyl inositol phosphorylceramide; GMO, genetically modified organism; GR, glutathione reductase; GSH, glutathione; HKT1, high-affinity K^+ transporter 1; IAA, indole-3-acetic acid; JA, jasmonic acid; KSB, K-solubilizing bacteria; MDHAR, monodehydroascorbate dehydrogenase; MIP, major intrinsic protein; MOCA1, mono cation-induced $[Ca2^+]_i$ increase 1; NO, nitric oxide; PGPM, plant growth-promoting microorganisms; PIP, plasma membrane intrinsic protein; POX, peroxidase; Pro, proline; ROS, reactive oxygen species; SA, salicylic acid; SLs, strigolactones; SOS, salt overly sensitive; SOD, superoxide dismutase; TIP, tonoplast intrinsic protein.

senescence of essential organs (Khataar et al., 2018). Over time, the impact of salinity on plant productivity escalates, leading to economic losses and societal effects (Atta et al., 2023).

Plants can be categorized into two main groups based on their adaptive evolution: halophytes (salt-withstanding) and glycophytes (salt-sensitive) (Khan et al., 2022). The majority of crop plants belong to the glycophyte group and are adversely affected by elevated salt levels in the soil or irrigation water, impacting their growth, development, and yields (Shrivastava and Kumar, 2015). Salt-affected plants usually show dark green leaves, which are heavier and more succulent than typical plants of the same species (Amacher et al., 2000).

For instance, the impact of salinity on crop yields can be seen in specific examples. Beans experience no yield loss at an electrical conductivity of soil saturation extract (EC_e) of 1.0 dS m^{-1}, but show a 25% yield loss with EC_e = 2.3 dS m^{-1} and a 50% yield loss with EC_e = 3.6 dS m^{-1} (Amacher et al., 2000). Conversely, barley shows no yield loss with EC_e = 8 dS m^{-1}, a 25% yield loss with EC_e = 13 dS m^{-1}, and a 50% yield loss with EC_e = 17 dS m^{-1} (Amacher et al., 2000). It should also be noted that the salinity tolerance level of different cultivars of a specific species to salinity may show variation in responses and yields as observed in guar, alfalfa, and other crops (Sandhu et al., 2017; Kaundal et al., 2021; Sandhu et al., 2021, Sandhu et al, 2023).

Some stages of the plant growth are more susceptible to salinity stress than others (Sandhu and Kaundal, 2018). Several studies have illustrated that salinity stress leads to substantial yield losses in major crops during their reproductive stages. For example, salinity has been demonstrated to decrease plant height, the number of spikelets, spike length, grain weight, and overall yield (including both grain and straw) in wheat (Kalhoro et al., 2016). Additionally, the impact of salinity on grain yield depends on the stages of wheat development. For example, salinity diminishes grain yield by 39%, 24.3%, and 13.4% during anthesis, early booting, and mid-grain filling, respectively (Ashraf and Ashraf, 2016).

High soil salinity causes ionic toxicity and disrupts osmotic equilibrium in plants, causing plant nutrient imbalance and osmotic stress (Shrivastava and Kumar, 2015). Salt stress not only disrupts ionic homeostasis and enhances osmotic potential but also hinders several processes, including stomatal development, stomatal movement, and expansion of cells. In pea plants, it has been shown that the accumulation of ions in the apoplast contributes to cellular necrosis (Speer and Kaiser, 1991). Similarly, in rice, the salt accumulation in the apoplast disturbs cellular water relations, which leads to dehydration and subsequently causes wilting (Flowers et al., 1991). Yield losses in crops in response to salinity are primarily attributed to Na^+ and Cl^-. However, other ions also impact yield losses in crops. Toxicity impact varies among various ions and combinations of ions (Hawkins and Lewis, 1993; Sandhu et al., 2020). When the salinity level is low, it is easier for cellular machinery to transport salt ions into the vacuole to adjust to the flux of ions across the plasma membrane into the cell (Blumwald et al., 2000). In contrast, when the salinity level is high, the influx rates become elevated, disrupting the cellular ion homeostasis. It subsequently leads to the accumulation of cations like Na^+, sometimes Mg^{2+}, and Ca^{2+}, and anions like Cl^-, PO_4^{3-}, and SO_4^{2-} in the cytosol, stroma, and matrix rather than in the vacuole. Sodium ions impact plant development by not only delaying flowering but also hindering photosynthesis (Kim et al., 2007; Van Zelm et al., 2020). This impairment occurs through the inhibition of carbon-fixing enzymes and interference with proton-motive force. Furthermore, multiple studies have observed that the K^+/Na^+ ratio correlates with grain dry matter in wheat and other crops (Poustini and Siosemardeh, 2004; Kalhoro et al., 2016; El Sabagh et al., 2021).

Elevated levels of NaCl in the soil decrease water potential, consequently limiting the plant's access to water from the soil; this, in turn, triggers osmotic stress in plants (Acosta-Motos et al., 2017). Ions like Na^+ and Cl^- enter plants through the outer cells of the root (Van Zelm et al., 2020). Subsequently, these ions are transported from the xylem of roots to the shoots. The elevation of ions within plant cells initiates an ionic imbalance, leading to immediate osmotic stress, followed by ionic stress, subsequently ionic toxicity, and the generation of reactive oxygen species (ROS) (Munns and Tester, 2008). An increase in Na^+ due to salinity inhibits biosynthesis and activity of diverse metabolic enzymes, prompts stomatal closure, and diminishes photosynthesis. In response to salinity-induced osmotic stress, plants synthesize various compatible osmoprotectants and solutes, including mannitol, inositol, trehalose, polyamines, glycine, betaine, and proline to mitigate the severity of the salinity stress (Munns and Tester, 2008; Park et al., 2016; Van Zelm et al., 2020).

The intricate mechanisms through which plants perceive salts are not thoroughly comprehended. Salinity stress in plants triggers various signaling pathways, the combined effects of which confer salinity tolerance (Acharya et al., 2021). In response to salinity, MOCA1 (mono cation induced $[Ca2^+]_i$ increase 1), an extracellular salt sensor, detects Na^+ and a few other monovalent cations (Jiang et al., 2019). MOCA1 synthesizes glycosyl inositol phosphorylceramide (GIPC) sphingolipids in the plasma membrane. GIPCs, with the ability to bind to monovalent cations like Na^+, are implicated in the depolarization of cell-surface potential. It, in turn, triggers the opening of calcium-influx channels, leading to elevated intracellular Ca^{2+} levels. The activation of the salt overly sensitive (SOS) pathway follows the increase in intracellular Ca^{2+} (Zhu, 2002). Within this pathway, SOS3, upon binding with Ca^{2+}, interacts with SOS2 and stimulates its kinase domain (Kaundal et al., 2022). Subsequently, SOS1 is phosphorylated by activated SOS2, facilitating the transport of Na^+ from the interior to the exterior of the cell (Figure 1) (Quintero et al., 2011). The evidence described above indicates that both calcium and SOS signaling pathways are critical for plant's salinity tolerance. In addition to SOS pathway components (SOS1, SOS2, and SOS3), CIPK8, CBL 8, and CBL10 contribute to Na homeostasis under high salt stress (Figure 1) (Acharya et al., 2024).

ROS are important secondary messengers in response to diverse stress signaling pathways, including salt stress (Ma et al., 2012). The excessive generation of ROS in response to salinity leads to oxidative stress, which, in turn, causes damage to proteins, membrane lipids, and nucleic acids (Ma et al., 2012). To protect cellular components and macromolecules from the detrimental effects of oxidative stress-mediated damage, plants engage in the synthesis of both non-enzymatic and enzymatic antioxidants. Plants synthesize various non-enzymatic antioxidants, including ascorbic acid (vitamin C),

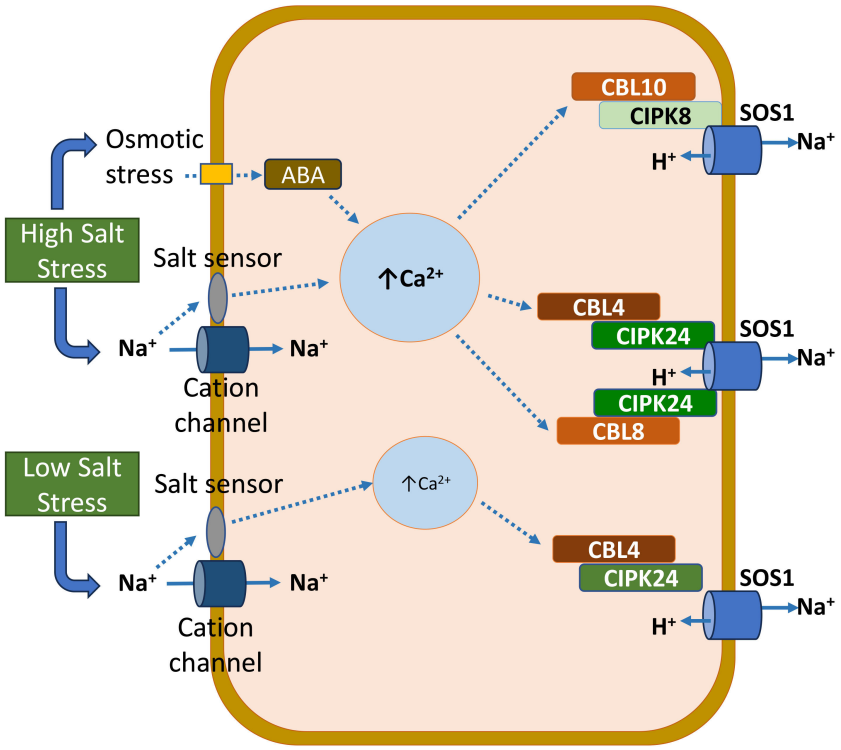

FIGURE 1
A model illustrating the role of the salt overly sensitive (SOS) pathway, including the SOS1, SOS2 (CIPK24), and SOS3 (CBL4) proteins, in maintaining Na^+ homeostasis under low- and high-salinity stress in plants. In addition to SOS1, SOS2, and SOS3, CIPK8, CBL 8, and CBL10 contribute to Na^+ homeostasis under high salt stress.

glutathione (GSH), and proline (Pro) (Gill and Tuteja, 2010). Enzymatic antioxidants are responsible for detoxifying ROS, encompassing superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and enzymes associated with the ascorbate (ASC)–glutathione cycle, such as monodehydroascorbate dehydrogenase (MDHAR), ASC peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Gill and Tuteja, 2010; Foyer and Noctor, 2011).

Plant hormones are alternatively known as phytohormones, which play essential roles in plant growth and development and play critical roles in response to biotic and abiotic stress. In general, phytohormones are classified into two groups: auxin, brassinosteroids (BRs), cytokinins (CKs), gibberellins (GAs), and strigolactones (SLs) are known as plant growth hormones, and abscisic acid (ABA), ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) are considered as plant stress hormones (Verma et al., 2016). The regulation of development, growth, and adaptation in plants under salinity stress is critically influenced by stress and growth hormones (Yu et al., 2020). A complex interplay occurs among plant stress hormones and plant growth hormones in response to salinity. These hormones play modulatory roles, engaging in complex crosstalk that significantly contributes to the plant growth adaptation during salinity stress (Yu et al., 2020). It should also be noted that the expression status of genes associated with phytohormone biosynthesis, transport, and signaling is an important determinant of salinity tolerance in plants (Acharya et al., 2022a, Acharya et al., 2022b).

3 Plant growth-promoting microbes to mitigate salinity stress

A diverse group of helpful microbes known as PGPMs inhabit the rhizoplane (root surface), rhizosphere (soil around roots), or endosphere (internal tissues). Generally, PGPMs include plant growth-promoting bacteria, rhizobia, and arbuscular mycorrhizal fungi. These microbes enhance plant growth in various ways, including producing indole-3-acetic acid (IAA), solubilizing phosphate for uptake, fixing nitrogen, producing beneficial enzymes like CAT (which helps to reduce oxidative stress), ACC deaminase (which reduces ET level that contributes to promote root growth), and producing siderophore (which chelates iron for plant use) (Mohanty et al., 2021). A detailed overview of the various ways PGPMs stimulate plant growth under non-stress conditions is thoroughly discussed in several recent reviews (Gahan and Schmalenberger, 2014; De Palma et al., 2022; Orozco-Mosqueda et al., 2023).

In this review, we focused on the role of PGPMs under salinity stress. Numerous research groups have discovered a wide array of PGPMs that alleviate salinity stress in plants. Various aspects of PGPM–plant interactions during salinity stress have been documented in previous research (Liu et al., 2022; Shrivastava and Kumar, 2015; Kaushal, 2020; Kumar et al., 2020; Mishra et al., 2021; Hoque et al., 2023; Mishra et al., 2023; Kumawat et al., 2024). In the following section, we categorize the various mechanisms through which PGPMs aid in mitigating salinity stress in plants.

3.1 Nutrient uptake and utilization

Essential nutrients are crucial for plant growth and yield, but their deficiency can negatively impact various aspects of plant development. During salinity conditions, elevated levels of sodium Na^+ and Cl^- limit the uptake of macronutrients, including nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), and magnesium (Mg) (Guo et al., 2020). It leads to a decreased availability of essential nutrients in plants, potentially triggering leaf senescence and inhibiting overall plant growth (Kumari et al., 2022).

Potassium-solubilizing bacteria (KSB) play a pivotal role in enhancing nutrient availability for plants, particularly in saline soils. Notable among these are bacterial species, such as *Pseudomonas* sp. and *Bacillus* sp., which can make K more accessible for plant uptake by solubilizing various silicate minerals (Supplementary Table 1) (Jaiswal et al., 2016; Vasanthi et al., 2018). Specifically, the PGPM strain *Burkholderia cepacia* SE4 has been shown to release K from soils, making it available to *Cucumis sativus* plants (Kang et al., 2014a). Moreover, the application of *Achromobacter piechaudii* to *Solanum lycopersicum* plants improved the uptake of K and P, while the application of salt-tolerant rhizobacteria, *Bacillus aquimaris*, in *Triticum aestivum* has been shown to enhance the uptake of K, P, and N in saline environments (Mayak et al., 2004; Upadhyay and Singh, 2015). Similarly, the application of *Azospirillum lipoferum* or *Azotobacter chroococcum* in *Zea mays* L under salinity enhanced K accumulation and provided salinity tolerance (Abdel Latef et al., 2020). In *Glycine max* seedlings subjected to salinity, inoculation with *B. firmus* (SW5) led to enhanced N and P accumulation and greater salinity tolerance, underscoring the significant role of *B. firmus* (SW5) in nutrient acquisition under stress conditions (El-Esawi et al., 2018). These findings suggest that salt-tolerant KSB can significantly enhance crop yields in saline soils.

Plant PGPMs also play a crucial role in increasing the accessibility of other essential minerals such as iron (Fe), zinc (Zn), and sulfur (S) to plants (Supplementary Table 1) (Gahan and Schmalenberger, 2014; Mishra et al., 2023). Iron is an essential micronutrient for plants as it is necessary for several metalloenzymes that are crucial in processes such as respiration and photosynthesis (Kobayashi et al., 2019). Salinity causes a deficiency of Fe that impacts plant growth, development, yield, and several other biological processes, including chlorophyll biosynthesis (Kobayashi et al., 2019). It has been documented that soil microbes play critical roles in accumulating Fe in roots and in transporting Fe in different plants (Masalha et al., 2000). Under conditions of low Fe availability, both microorganisms and plants produce siderophores—small organic molecules that selectively chelate ferric ions [Fe(III)], facilitating iron uptake (Ferreira et al., 2019; Timofeeva et al., 2022). The application of endophytic streptomyces has been demonstrated to significantly enhance the growth of mung bean and rice plants, leading to a notable increase in the biomass of both roots and shoots (Rungin et al., 2012). Furthermore, salt-tolerant siderophore-producing rhizobacteria (e.g., *Bacillus aryabhattai* MS3) have demonstrated the ability to promote plant growth in saline soils where Fe is limited (Sultana et al., 2020, Sultana et al., 2021). Therefore, siderophore-producing rhizobacteria are recognized as highly beneficial PGPMs, enabling plants to thrive in saline soils with limited iron availability (Ferreira et al., 2019).

Zinc (Zn) is a crucial plant micronutrient, essential for their development, growth, and yield (Saleem et al., 2022). Saline, sodic, and calcareous soil often cause Zn deficiency in plants (Tavallali et al., 2009; Daneshbakhsh et al., 2013). In the saline environment, the application of Zn is known to enhance sanity tolerance and stimulate proline metabolism (Mushtaq et al., 2023). Numerous studies have shown that PGPMs, including species such as *Trichoderma* sp., *Providencia* sp., *Anabaena* sp., and *Bacillus* sp., are capable of solubilizing Zn present in the soil (Upadhayay et al., 2022), which could be used for growth developments of plants including wheat (De Santiago et al., 2011).

Sulfur is a vital macronutrient crucial for the plant development and growth (Narayan et al., 2023). *P. putida* has been shown to play an important role in the S cycle in the conversion of organic S to an inorganic form that plants can uptake (Kertesz and Mirleau, 2004). Application of *P. putida*, *Pseudomonas fluorescens*, and *B. subtilis* provided salinity tolerance in soybean (Abulfaraj and Jalal, 2021). An *Enterobacter* sp., SA187, is known to promote alfalfa growth and yield in field conditions (De Zélicourt et al., 2018). Under salt stress, Arabidopsis plants showed symptoms resembling S starvation (De Zélicourt et al., 2018; Andrés-Barrao et al., 2021). However, when colonized with *Enterobacter* sp., SA187, these plants showed enhanced uptake of S and improved sulfur metabolism. This interaction also modulated the phytohormone signaling pathway and provided salinity tolerance (Supplementary Table 1) (De Zélicourt et al., 2018; Andrés-Barrao et al., 2021).

3.2 Synthesis of osmolytes and regulation

Salinity triggers osmotic stress in plants, leading them to produce various osmolytes that serve as osmoprotectants—like mannitol, inositol, trehalose, polyamines, glycine, proline, and betaine—to mitigate the severity of the salinity stress (Munns and Tester, 2008; Park et al., 2016; Van Zelm et al., 2020). In saline environments, PGPMs further support plants under osmotic stress by producing these osmoprotectants, thereby enhancing the plants' tolerance to salinity (Supplementary Table 1). For example, inoculation of PGPMs, *P. fluorescens*, and *B. subtilis* enhanced proline accumulation in cucumber plants under salinity stress compared to non-inoculated plants (Saberi-Riseh et al., 2020). Similarly, the application of salt-tolerant *Stenotrophomonas maltophilia* BJ01 enhanced proline accumulation in peanut plants, providing salinity tolerance (Alexander et al., 2020). Additionally, *B. amyloliquefaciens* NBRI-SN13 enhanced proline and total sugar accumulation in rice seedlings, in contrast to non-inoculated seedlings (Tiwari et al., 2017).

In capsicum, the application of a salt-tolerant rhizobacteria, *B. fortis*, improved proline accumulation and conferred salinity tolerance (Yasin et al., 2018). Chickpea plants inoculated with *Azosprillum lipoferum* FK1 accumulated osmolytes like betaine, glycine, proline, and soluble sugars in response to salinity (El-Esawi et al., 2019). Further studies indicated that while salt

treatment alone increased soluble sugars and proline content in maize plants, inoculation with *A. lipoferum* or *A. chroococcum* significantly boosted these levels, compared to plants treated with salt alone, enhancing maize growth under salinity (Abdel Latef et al., 2020). This suggests that the production of osmolytes and other components by these two PGPMs contributed to salinity tolerance and improved maize growth.

In soybeans, inoculation of *B. firmus* (SW5) not only increased the accumulation of osmoprotectants like glycine betaine and proline but also enhanced root architectural traits, including root length and volume, thereby improving salinity tolerance (El-Esawi et al., 2018). Moreover, an endophytic fungus, *Paecilomyces formosus*, known for producing Gas, provided salinity tolerance in cucumber by enhancing the accumulation of proline and other beneficial plant traits (Khan et al., 2012).

3.3 Enhancement of water transport

Aquaporins, integral membrane proteins from the major intrinsic protein (MIP) superfamily, form water-selective channels across membranes that play important roles in water transport and can also transport small neutral molecules (Kapilan et al., 2018). They play vital roles in cellular water transport as members of the plasma membrane intrinsic protein (PIP) and tonoplast intrinsic protein (TIP) families (Afzal et al., 2016). Aquaporins are key contributors to plant root hydraulic conductivity (Grondin et al., 2020). Expression of aquaporins is highly regulated by drought and salinity. An aquaporin gene *SpAQP1* of *Sesuvium portulacastrum*, a halophyte, was strongly induced in response to salt or drought treatment (Chang et al., 2016). Transgenic tobacco plants expressing *SpAQP1* demonstrated enhanced salt tolerance compared to wild-type and vector control plants, underscoring the role of aquaporin genes in salinity tolerance (Chang et al., 2016). In Arabidopsis, exposure to 100 mM NaCl leads to the downregulation of *PIP* and *TIP* aquaporin genes (Boursiac et al., 2005), a response also observed in other plants like cotton and tomato (Braz et al., 2019; Jia et al., 2020), significantly impacting root hydraulic conductivity (Siefritz et al., 2002). Conversely, the application of PGPMs has been shown to upregulate the expression of aquaporin genes, enhancing plant resilience to salinity. For instance, in maize, application of *Pantoea agglomerans* or *B. megaterium* upregulated aquaporin genes, improving root hydraulic conductivity and salinity tolerance (Marulanda et al., 2010; Gond et al., 2015). Similarly, barley seedlings treated with 200 mM NaCl exhibited reduced biomass and height alongside downregulated *HvPIP2;1* aquaporin gene expression (Zawoznik et al., 2011). However, inoculation with *Azospirillum brasilense* strain AZ39 induced *HvPIP2;1* expression, mitigating biomass and height reduction (Zawoznik et al., 2011). These observations suggest that *A. brasilense* strain AZ39 alleviates salinity stress possibly by upregulating the *HvPIP2;1* aquaporin gene, thereby enhancing root hair length, density, and improving water uptake. Furthermore, in response to salt stress, the mycorrhizae-mediated modulation of the expression of aquaporin genes has been reported in multiple plant species including *Phaseolus vulgaris*, *Lactuca sativa*, and *Robinia pseudoacacia* (Sharma et al., 2021). Mycorrhiza-mediated upregulation of aquaporin gene improved water status, K^+/Na^+ homeostasis, increased photosynthesis, enhanced expression of genes associated with ion homeostasis, including *SOS1* and *HKT1*, and alleviated salinity tolerance in black locust (*Robinia pseudoacacia*) (Supplementary Table 1) (Chen et al., 2017).

3.4 Regulation of ionic equilibrium

During salinity stress, toxic ions like Na^+ and Cl^- increase in the cytosol, and excessive accumulation of these ions causes toxicity. Na^+ not only imbalances the K^+/Na^+ ratio but also affects many physiological processes and functions of various proteins (Assaha et al., 2017). The SOS signaling pathway plays an important role to reduce Na^+ inside of the cell, and that, in turn, helps maintain Na^+ homeostasis (Park et al., 2016). Additionally, the high-affinity K^+ transporter 1 (HKT1) contributes to Na^+ homeostasis by removing Na^+ from the xylem and sending Na^+ back to the root (Kaundal et al., 2019). In rice, it has been shown that OsHKT1;4-mediated Na^+ transport in stems plays a role in excluding Na^+ from leaf blades during the reproductive growth stage in response to salt stress (Suzuki et al., 2016). Na^+/H^+ antiporters have been implicated in Na^+ and K^+ homeostasis and salt tolerance (Apse et al., 1999; Bassil et al., 2011). Moreover, the proton pump, AVP1, has been shown to play a role in salinity tolerance in different plants (Gaxiola et al., 2001; Lian et al., 2024).

Multiple PGPMs have been identified to induce expression of SOS pathway genes (Supplementary Table 1). Notably, volatile compounds produced by rhizobacteria *Alcaligenes faecalis* JBCS129 have been shown to upregulate expression of Arabidopsis *SOS1*, *HKT1*, *NHX1*, and *AVP1* under salt stress, assisting the plant in maintaining ion homeostasis during salinity stress (Bhattacharyya et al., 2015). The application of PGPM strain *Glutamicibacter* sp. YD01 in rice seedlings provided salinity tolerance by inducing expression of *OsHKT1* significantly and maintaining ion homeostasis (Ji et al., 2020). This strain also produces ACC (1-aminocyclopropane-1-carboxylate) deaminase and IAA, further supporting plant growth under stress.

In response to salinity, maize plants showed increased Na^+, decreased K^+, and reduced K^+/Na^+ ratio. However, salt-stressed maize plants inoculated with *A. lipoferum* or *A. chroococcum* showed reduced Na^+, enhanced K^+ accumulation, increased K^+/Na^+ ratio, and improved salinity tolerance, indicating that both *A. lipoferum* or *A. chroococcum* contribute to plant ion homeostasis in response to salinity (Abdel Latef et al., 2020). Similarly, inoculating white clover plant (*Trifolium repens*) with *A. brasilense* enhanced growth under salinity and reduced Na^+ content, enhanced K^+ content, and increased K^+/Na^+ ratio, suggesting that *A. brasilense* treatment contributes to ion homeostasis in plants under salinity (Khalid et al., 2017). Additionally, the application of *Variovorax paradoxus* 5C-2 to pea plants under salinity enhanced ion homeostasis by increasing K^+ uptake, decreasing Na^+ accumulation, and enhancing K^+/Na^+ ratio, leading to enhanced growth and salinity tolerance compared to uninoculated plants (Wang et al., 2016). Under salinity stress, rice inoculated with *C. albidum* strain SRV4 exhibited lower Na accumulation and higher K accumulation

compared to non-inoculated plants (Vimal et al., 2019). This improved tolerance to salinity indicates the positive role of *C. albidum* in maintaining ion homeostasis during salt stress.

3.5 Production of antioxidants

Various plant enzymes, including APX, CAT, GR, POX, and SOD, exhibit antioxidant activity (Upadhyay et al., 2012). Many PGPMs have been shown to boost the activity of these antioxidant enzymes. For instance, *Piriformospora indica*, a root-colonizing basidiomycete fungus, promotes growth and provides resistance against mild salinity stress in barley by activating the antioxidative capacity through the glutathione–ascorbate cycle (Waller et al., 2005). Similarly, applying *A. lipoferum* or *A. chroococcum* to maize plants enhanced the activity of CAT and POX POD, showcasing their positive regulatory roles in salinity tolerance (Abdel Latef et al., 2020). In soybean seedlings, *B. firmus* (SW5) inoculation elevates the activity of APX, SOD, CAT, and POD, alongside reducing H_2O_2 levels, indicating its contribution to enhanced antioxidative capacity (El-Esawi et al., 2018). Inoculating okra seeds with ACC-producing *Enterobacter* sp. UPMR18 improved seed germination and seedling growth under salinity conditions (Habib et al., 2016). This was accompanied by heightened ROS-scavenging activity of enzymes like APX, CAT, and SOD, indicating that these ROS-scavenging enzymes play a beneficial role in enhancing salinity tolerance through the action of PGPMs (Supplementary Table 1). The application of *P. putida* H-2–3 showed a higher activity of SOD and improved soybean plant growth under salinity and drought (Kang et al., 2014b). Additionally, a GA-producing endophytic fungus, *P. formosus*, aids in salinity tolerance in cucumber by accumulating antioxidants, among other beneficial traits (Khan et al., 2012). Application of *Curtobacterium albidum* strain SRV4 in rice under salinity showed the higher activity of antioxidant enzymes, including CAT and SOD, and provided tolerance to salinity compared to non-inoculated plants (Vimal et al., 2019).

3.6 Phytohormone synthesis and regulation

Phytohormones play vital roles in regulating plant growth, development, and various physiological processes (Acharya and Assmann, 2009; Acharya et al., 2013; Miransari and Smith, 2014; Acharya et al., 2017). In response to salinity stress, various phytohormones, including auxins, CK, ET, and GAs, are critical in helping plant to adapt (Kaundal et al., 2021; Acharya et al., 2022a, Acharya et al., 2022b). Several PGPMs have been identified that produce and excrete hormones that plants can absorb through their roots, enhancing plant growth or regulating hormone balance to improve salinity responses (Backer et al., 2018). For instance, application of a halotolerant PGPM strain, *Glutamicibacter* sp. YD01, equipped with ACC deaminase, has been shown to provide salinity tolerance by reducing ET in rice seedlings (Supplementary Table 1) (Ji et al., 2020). Furthermore, PGPMs are known to synthesize some phytohormones like auxin, CK, ET, GA, and SA, modulating physiological activity through molecular responses (Orozco-Mosqueda et al., 2023). Additionally, many organic compounds produced by PGPMs are known to influence plant physiological activities, underlining the significant role these microorganisms play in enhancing plant health and stress resilience. The next section explores how PGPMs influence the regulation of various phytohormones.

3.6.1 Auxin

Auxin, a crucial phytohormone, plays significant roles in plant growth and development, and particularly root development, including primary root elongation and lateral root initiation (Van Zelm et al., 2020). Additionally, auxin is vital in plant responses to salt stress; reduced auxin levels in roots under such conditions negatively impact root growth and architecture (Smolko et al., 2021). Specifically, salt stress, primarily through Na^+, inhibits auxin-mediated primary root elongation and impedes auxin-mediated lateral root initiation, emergence, and elongation (Van Zelm et al., 2020). In response to salinity, the ABA concentration increases, which further inhibits lateral root emergence and elongation (Van Zelm et al., 2020). An increase in Na^+ in roots reduces auxins and enhances ABA that, in turn, causes inhibition of lateral growth.

Many PGPMs are known to synthesize IAA, a physiologically active auxin, including species *Aeromonas veronii*, *A. brasilense*, *Enterobacter* sp., *Rhizobium leguminosarum, Actinobacteria*, *Frankia*, *Kitasatospora*, *Nocardia*, *Pseudomonas, Bacillus*, and *Streptomyces* (Supplementary Table 1) (Vessey, 2003; Kumar et al., 2020; Ganesh et al., 2022). The impact of the exogenous application of IAA is dependent on concentration; high concentrations accelerate the development of lateral roots and root hair formation while negatively affecting primary root growth (Vacheron et al., 2013). In contrast, a low dose of IAA may promote primary root growth (Vacheron et al., 2013). The application of PGPMs that produce auxin have been shown to induce plant growth by enhancing root growth and biomass (Backer et al., 2018). Additionally, many PGPMs indirectly influence auxin signaling pathways in plants. For example, some PGPMs with nitrite reductase activity, like *A. brasilense*, produce nitric oxide (NO), which is involved in lateral root development under stress conditions (Supplementary Table 1) (Wimalasekera and Scherer, 2022).

The uptake of IAA produced by PGPMs promotes primary and lateral root growth, as well as root hair proliferation, enabling plants to absorb more nutrients and minerals for improved growth and productivity. This indicates that the presence of PGPMs in soil, through the production of IAA, can significantly enhance plant growth compared to soils without PGPMs (Glick, 2014). Salt-tolerant rhizobacterial strains that produce auxin and proline mitigated salinity-induced growth inhibition of barley plants by regulating ion homeostasis and leaf water potential (Metoui Ben Mahmoud et al., 2020). A study showed that *Medicago truncatula* nodulated by an IAA-overproducing strain, *Sinorhizobium meliloti* RD64, showed improved tolerance to 300 mM NaCl (Supplementary Table 1) (Bianco and Defez, 2009). An IAA-

producing PGPM strain, *C. albidum* SRV4, provided tolerance in rice by improving growth, improving K uptake, and boosting antioxidative enzymatic activities (Vimal et al., 2019). IAA-producing PGPM *Pseudomonas* sp. provides salinity tolerance in cotton (Egamberdieva et al., 2015). Three IAA-producing halotolerant PGPMs isolated from halophytes, *Micrococcus yunnanensis*, *Planococcus rifietoensis*, and *V. paradoxus*, have been shown to provide salinity tolerance in sugar beet (Supplementary Table 1) (Zhou et al., 2017).

3.6.2 Gibberellins

GAs constitute a large group of phytohormones that positively regulate various aspects of plant growth, such as root and stem elongation, cell division, bolting, flowering, seed germination, and dormancy (Swain and Singh, 2005). DELLA (aspartic acid–glutamic acid–leucine–leucine–alanine) proteins, a sub-family of plant-specific GRAS (GIBBERILIC ACID INSENSITIVE, REPRESSOR OF *ga1–3*, and SCARECROW) transcriptional regulators, are critical components of the GA signaling pathway (Phokas and Coates, 2021). Abiotic stresses, such as salinity, are known to reduce GA levels, primarily by inhibiting the enzymes responsible for GA biosynthesis, highlighting the crucial role of GAs in plant stress resilience (Achard et al., 2006; Magome et al., 2008).

Many PGPMs are capable of producing GAs, which aid in plant growth enhancement (Backer et al., 2018). GA-producing bacteria, *Burkholdera cepacia* SE4, *Promicromonospora* sp. SE188, and *Acinetobacter calcoaceticus* SE370, provided salinity tolerance in cucumber plants (Kang et al., 2014a). Similarly, *Pseudomonas putida* H-2–3, another GA producer, improved soybean growth under salinity and drought (Supplementary Table 1) (Kang et al., 2014b). Further research has demonstrated that the application of the GA-producing endophytic fungus *Penicillium funiculosum* LHL06 can impart salt stress tolerance to soybean, by lowering plant levels of ABA and JA, and enhancing isoflavone biosynthesis (Khan et al., 2011). Additionally, the GA-producing endophytic fungus, *P. formosus*, provided salinity tolerance in cucumber by reducing stress hormone ABA and enhancing the accumulation of antioxidants and proline (Khan et al., 2012). A recent study shows that a GA-producing PGPM, *B. subtilis* ER-08 (isolated from a halotolerant plant), with multiple growth-promoting attributes enhanced the growth of fenugreek (*Trigonella foenum-graecum* L.) in response to salinity and drought stress (Supplementary Table 1) (Patel et al., 2023).

3.6.3 Cytokinins

CKs have been identified as both positive and negative regulators in the context of salinity stress tolerance (Liu et al., 2020). For instance, increased CK levels during salinity stress have been observed in plants like Arabidopsis, rice, tomato, and apple. Notably, the *OsCKX2* knockout rice mutant, which has a higher level of CK content, shows higher salinity tolerance compared to wild type (Joshi et al., 2018). Additionally, the application of INCYDE, a CK degradation inhibitor, has been shown to increase salinity tolerance in tomatoes, underscoring CK's beneficial role in salinity tolerance by suggesting that salt stress may reduce CK levels, thereby diminishing salinity tolerance (Aremu et al., 2014). Conversely, there are instances where increased CK levels have been associated with reduced salinity tolerance. Overproduction of CK in Arabidopsis showed reduced salinity tolerance (Wang et al., 2015). Furthermore, in Arabidopsis, CK negatively regulates the expression of *HKT1*, which is responsible for unloading Na^+ from the root xylem, which, in turn, causes an increase of Na^+ in the shoot (Mason et al., 2010). Additionally, reduced CK level due to increased degradation or reduced synthesis provided enhanced tolerance to salinity, including wheat and tomato (Avalbaev et al., 2016).

3.6.4 Ethylene

It is well known that ET is one of the important phytohormones that play key roles in several plant physiological processes, including salinity stress (Riyazuddin et al., 2020). Salinity and other abiotic stresses increase ET content, causing the stunted growth of plants (Chen et al., 2021). ACC deaminase, an enzyme that hydrolyzes ACC, the immediate precursor of ET, plays a vital role in reducing ET levels, thereby aiding plant growth under stress conditions (Shahid et al., 2023). PGPMs utilize ACC deaminase to reduce ET levels, which, in turn, helps to reduce the stress level induced by salinity or other stresses (Glick et al., 2007; Orozco-Mosqueda et al., 2020). For instance, *P. fluorescens* strain TDK1, which produces ACC deaminase, has been shown to confer salinity tolerance and increase yield in peanuts (Saravanakumar and Samiyappan, 2007). Similarly, inoculation with *V. paradoxus* 5C-2, an ACC deaminase-producing PGPM, has provided salinity tolerance in *Pisum sativum* L. cv. Alderman, leading to increased biomass and enhanced photosynthetic activity (Wang et al., 2016). In addition, various studies have documented that rhizobacteria that have functional ACC deaminase provide tolerance in various crops, including *P. fluorescens* LSMR-29 and *E. hirae* LSMRS-7 in *Vigna radiata* (Kumawat et al., 2024), *Arthrobacter protophoramiae* in *P. sativum* (Barnawal et al., 2014), *P. fluorescens* NBRC 14160 and *B. megaterium* NBRC 15308 in wheat (Fathalla and Abd El-Mageed, 2020), *Glutamicibacter* sp. YD01 in rice seedlings (Ji et al., 2020), *Aneurinibacillus aneurinilyticus* and *Paenibacillus* sp. in French bean (Gupta and Pandey, 2019), *Bacillus* sp. PM31 in maize (Ali et al., 2023), and *Hartmannibacter diazotrophicus* $E19^T$ in barley (Supplementary Table 1) (Suarez et al., 2015). Additionally, multiple species of ACC deaminase producing halotolerant PGPMs with additional growth-promoting properties isolated from halophytes, *P. rifietoensis*, *V. paradoxus*, and *M. yunnanensis*, have been shown to provide tolerance to salt stress in *Beta vulgaris* by reducing ET content (Zhou et al., 2017).

3.7 Biofilms

Biofilms are complex and structured communities of microorganisms, primarily bacteria that adhere to surfaces and are encased in a self-produced matrix of extracellular polymeric substances (EPS), comprising polysaccharides, proteins, nucleic acids, and other molecules (Di Martino, 2018). Biofilm-producing microorganisms gain a survival advantage in unfavorable conditions, including saline soils, where increased osmotic

pressure could otherwise lead to cell death through cytoplasmic lysis. The ability to form biofilms equips these microorganisms with a protective mechanism against saline environments and other abiotic stresses, effectively serving as barriers that enable them to withstand and thrive under harsh conditions (Yin et al., 2019).

Halotolerant PGPMs thrive in saline environments, establishing themselves around the root zone, and promoting plant growth and development (Ahemad and Kibret, 2014). They produce various beneficial chemicals and growth regulators in the rhizosphere. Among these, certain halotolerant PGPM strains have been discovered to enhance salinity stress tolerance in plants. For example, two halotolerant biofilm-forming PGPM strains, AP6 and PB5, affiliated with *B. licheniformis* and *P. plecoglossicida*, respectively, were found to produce IAA and ACC deaminase (Yasmeen et al., 2020). These strains contributed to salinity tolerance and led to better growth and yield of sunflower plants than the non-inoculated plants. It demonstrates the multifaceted benefits of biofilm formation, including the production of IAA and ACC deaminase, which contribute to improved plant growth, productivity, and salinity tolerance (Yasmeen et al., 2020). Furthermore, wheat seedlings inoculated with exopolysaccharide-producing bacteria have been shown to stimulate growth and provide salinity tolerance by restricting Na^+ influx (Ashraf et al., 2004). An exopolysaccharide-producing PGPM strain, *C. albidum* strain SRV4, provided tolerance to rice (Vimal et al., 2019).

4 Challenges in applying PGPMs in soil for improvement of crops

PGPMs have been extensively researched over the years, and many efforts have been made to leverage their potential for commercial use. Despite their significant promise for sustainable agriculture, their broad-scale implementation encounters various obstacles.

4.1 Inconsistent efficacy of PGPMs

PGPMs can be highly context-dependent, varying across different soil types and climates (Martínez-Viveros et al., 2010). One of the primary challenges in utilizing PGPMs is ensuring their survival and persistence in the soil environment. Soil conditions, such as temperature, pH, and the presence of competing microorganisms, can impact the viability of these microbes (Martínez-Viveros et al., 2010), making it difficult for farmers to predict and ensure positive outcomes from PGPM application, which, in turn, slows their widespread adoption in agriculture.

4.2 Specificity of action

While some PGPMs exhibit broad-spectrum benefits, many work optimally with specific plant species or crop cultivars (Dhawi and Hess, 2017; Pratush et al., 2018; Ma et al., 2020). Identifying the most effective strain for each crop–soil combination requires extensive testing, complicating large-scale implementation. Additionally, natural crop variability within a species further challenges finding a "one-size-fits-all" solution.

4.3 Issues in the development process

The development of PGPMs is based on screening assays in a laboratory setting, which measure specific PGPM activities such as IAA production, calcium phosphate solubilization, and siderophore production (Ganesh et al., 2022). However, the presence of these characteristics in microorganisms does not always guarantee effective PGPM function under field conditions. Conversely, microbes lacking these *in vitro* properties might possess alternative mechanisms for promoting plant growth, which are less well-understood. Because of this knowledge gap, such microbes risk being overlooked and discarded during the early stages of laboratory screening, potentially missing out on effective PGPM candidates (Cardinale et al., 2015).

4.4 Shelf life and viability

PGPMs are living entities with specific viability requirements, and owing to their structural and cellular composition, they have a relatively short shelf life (Arriel-Elias et al., 2018). Maintaining their effectiveness throughout production, storage, and application can be expensive, complex, and challenging, especially for small-scale farmers.

4.5 Regulatory hurdles and lack of standardization

Regulatory systems frequently lag scientific progress. Concerns about unintended environmental impacts and the complex nature of microbial communities can create regulatory hurdles, stalling commercialization efforts (Leggett et al., 2011). Additionally, the lack of consistent standardization in strain identification, characterization, and quality control for agricultural applications impedes broad adoption and undermines farmer confidence.

4.6 Economic benefit to farmers

A clear economic benefit demonstrated for farmers is crucial for widespread adoption. The microbes' application method must align with the farmer's equipment and agricultural practices. Factors like upfront costs, application complexity, and reliable performance data compared to conventional methods need careful consideration. Farmers commonly perceive PGPM formulations as costlier and less effective than chemical alternatives, which needs to be addressed.

4.7 Farmers' risk-taking ability:

Crop producers usually depend solely on farming for their livelihood and sustenance, with little to no extra financial runway between two crops. With such financial constraints, farmers are

unwilling to use nontraditional measures compared to tried-and-tested methods. This can pose a big hurdle in the widespread adoption of PGPMs.

More extensive work needs to be done by agricultural researchers and farmers to adopt PGPM formulations on a broader scale (Parnell et al., 2016). Educating farmers about the long-term benefits and building trust in PGPM technology is essential. Farmer education and awareness play a crucial role. Shifting from traditional practices to effectively utilizing PGPMs requires knowledge and training, which may be limited to certain regions. Addressing these challenges through continued research, improved formulations, streamlined regulations, and effective farmer education is crucial to unlocking the full potential of PGPMs and transforming agriculture toward a more sustainable future.

5 Conclusions

Salt stress disrupts various plant processes, including seed germination, seedling and root growth, development, early senescence, flowering, and yield, potentially leading to premature death. In saline environments, reduced water uptake causes osmotic stress due to changes in cell turgor. Plants synthesize osmoprotectants to cope, but these may be insufficient. Higher levels of Na^+ and Cl^- lead to ionic stress and imbalance, specifically affecting the K^+/Na^+ ratio. Gene expression changes may enable some tolerance, depending on salinity levels and plant genetics. Salinity negatively impacts the acquisition of essential nutrients like N, P, and K and induces oxidative stress by increasing ROS accumulation, which can be harmful. Although plants produce antioxidants, these may not fully counteract oxidative stress. Stress hormones like ABA and ET increase under salinity, inhibiting growth, while growth hormones like auxins and GAs are inhibited, further negatively impacting plant growth.

In a saline environment, halotolerant PGPMs can play critical roles in improving plant growth (Figure 2). Their natural availability or supplementation of PGPMs alleviates the impact of salinity stress on plant development, growth, and yield by influencing various aspects of plant life. They modulate nutritional, physiological, biochemical, and molecular aspects of plant life. PGPMs enhance water uptake during salinity by upregulating the expression of aquaporin genes and additional mechanisms. Many PGPMs also enhance the accumulation of osmoprotectants in plants, thereby enhancing tolerance to salinity. They contribute to reducing the Na^+ levels by upregulating genes that are involved in ion homeostasis, such as *SOS1* and *HKT1*, along with other genes playing roles in ion homeostasis. Halotolerant PGPMs also play critical roles in plant growth by enhancing the availability of essential nutrients, providing growth hormones like auxins and GAs, helping plants reduce stress hormones like ET through ACC deaminase, and enhancing the antioxidant capacity of plants. Employing PGPMs that produce ribosylated CK is beneficial, as this variant can move from the root to the shoot, promoting cell expansion and division without adversely impacting root growth, owing to its altered CK composition (Kudoyarova et al., 2019). Some PGPMs produce multiple hormones, enabling one to predict expected outcomes based on their respective functions. Specific combinations of PGPMs may be utilized according to the specific needs of a crop or plant to enhance salinity tolerance. Employing mathematical

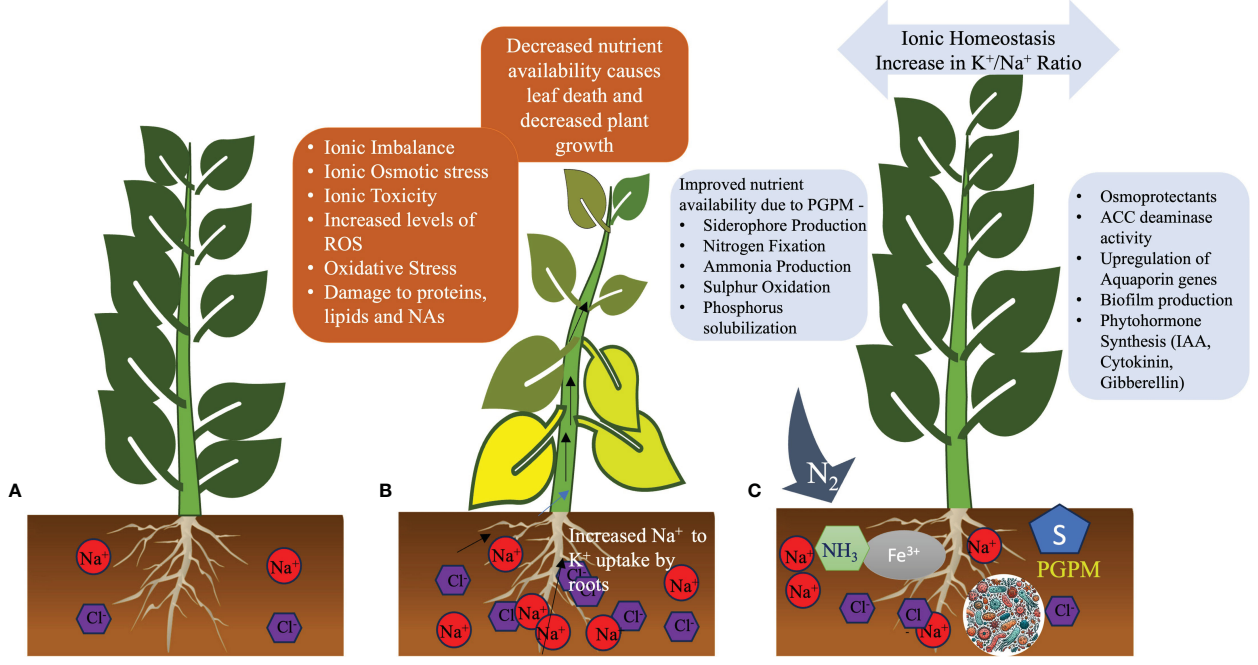

FIGURE 2
Roles of plant growth-promoting microorganisms (PGPMs) in enhancing salinity stress defense in plants. **(A)** A healthy plant in non-saline soil. **(B)** A plant facing saline conditions shows yellowing of leaves and stunted growth due to excessive ions in soil and tissues. **(C)** A plant in salt-affected soil treated with PGPMs regains health by mitigating the osmotic and ionic stresses induced by salinity.

modeling, one can predict which combinations of PGPMs would be most effective in imparting salinity tolerance to a specific crop with known traits, including its tolerance level of salinity, as well as its physiological, biochemical, and molecular tolerance traits. Additionally, specific traits of PGPMs could be improved using gene editing technology tailored to the specific needs of a user. PGPMs present promising opportunities for sustainable agriculture by enhancing yields and resilience and decreasing dependence on harsh chemicals. Nevertheless, further efforts are needed to translate the potential observed in laboratory studies into broad-scale field applications.

6 Perspectives

Despite recent progress on PGPM-mediated salinity tolerance in plants, many questions remain unanswered. For instance, do PGPMs contribute to enhancing Na^+ or salt-sensing mechanisms in plants? While literature suggests that many PGPMs provide general benefits like nutrient uptake, it is unclear if they are equally effective outside their natural range. Additionally, could some plant species be negatively impacted by specific strains of PGPMs?

Several reports indicate that PGPMs modulate gene expression in response to salinity. Do PGPMs induce epigenetic modifications in host plants under salt stress, affecting gene expression related to salinity tolerance? RNA-binding proteins (RBPs) are well-known regulators of gene expression at the post-transcriptional level. Given that PGPMs have been shown to provide salinity tolerance by regulating various genes, it would be highly interesting to investigate whether PGPMs specifically regulate gene expression through RBPs to promote salinity tolerance.

Author contributions

BA: Conceptualization, Formal Analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. SG: Formal Analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. AK: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. DS: Conceptualization, Funding acquisition, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing, Investigation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by the United States Department of Agriculture–National Institute of Food and Agriculture (grant number 2023–67014-40536), the United States Department of Agriculture–Agricultural Research Service, National Program 301: Plant Genetic Resources, Genomics, and Genetic Improvement (project number 2036–13210-013–000D), and SCBGP-Utah Department of Agriculture and Food grant (ID-205209–00001).

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/fpls.2024.1406913/full#supplementary-material

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frontiers | Frontiers in Plant Science

TYPE Review
PUBLISHED 19 July 2024
DOI 10.3389/fpls.2024.1420408

OPEN ACCESS

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RECEIVED 20 April 2024
ACCEPTED 28 June 2024
PUBLISHED 19 July 2024

CITATION
Tang H, Xiang G, Xiao W, Yang Z and Zhao B (2024) Microbial mediated remediation of heavy metals toxicity: mechanisms and future prospects.
Front. Plant Sci. 15:1420408.
doi: 10.3389/fpls.2024.1420408

Microbial mediated remediation of heavy metals toxicity: mechanisms and future prospects

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Heavy metal pollution has become a serious concern across the globe due to their persistent nature, higher toxicity, and recalcitrance. These toxic metals threaten the stability of the environment and the health of all living beings. Heavy metals also enter the human food chain by eating contaminated foods and cause toxic effects on human health. Thus, remediation of HMs polluted soils is mandatory and it needs to be addressed at higher priority. The use of microbes is considered as a promising approach to combat the adverse impacts of HMs. Microbes aided in the restoration of deteriorated environments to their natural condition, with long-term environmental effects. Microbial remediation prevents the leaching and mobilization of HMs and they also make the extraction of HMs simple. Therefore, in this context recent technological advancement allowed to use of bioremediation as an imperative approach to remediate polluted soils. Microbes use different mechanisms including bio-sorption, bioaccumulation, bioleaching, bio-transformation, bio-volatilization and bio-mineralization to mitigate toxic the effects of HMs. Thus, keeping in the view toxic HMs here in this review explores the role of bacteria, fungi and algae in bioremediation of polluted soils. This review also discusses the various approaches that can be used to improve the efficiency of microbes to remediate HMs polluted soils. It also highlights different research gaps that must be solved in future study programs to improve bioremediation efficency.

KEYWORDS

bio-sorption, genetic engineering, heavy metals, bioremediation, nano-particles

Introduction

The world's population is continuously growing up with a corresponding increase in food demands (Yaashikaa and Kumar, 2022). The recent increase in industrialization and anthropogenic activities are a serious threat to crop production owing to the fact they negatively soil fertility and productivity (Yaashikaa and Kumar, 2022). Various industries

excrete toxic heavy metals (HMs) that enter into the soil and negatively affect soil fertility, microbial activities, and crop productivity and these HMs also induce serious effects on humans (Table 1) by eating the contaminated foods (Asyakina et al., 2021; Debonne et al., 2021; Nizamutdinov et al., 2022). Global agricultural communities have serious concerns about contamination of agricultural soils with HMs. These HMs are very toxic and they can persist in the soils over a long time period. Different HMs including cadmium (Cd), lead (Pb), zinc (Zn) and copper (Cu) enter into agricultural soils with organic and inorganic fertilizers, while arsenic (As) and mercury (Hg) enter into agricultural soils through nearby located industrial enterprises (Uchimiya et al., 2020; Guan et al., 2022).

TABLE 1 Toxic effects of different heavy metals on human health.

Heavy metals	Toxic form	Health risks	References
Cadmium	Cd^{2+}	Cd toxicity reduce cell vitality, induce apoptosis, and damage the kidney, liver and bones.	Wang et al. (2021)
Cadmium	Cd^{2+}	High intake of Cd fractured the bones, kidney damage and liver infections along with reproductive dysfunctions.	Kim et al. (2019)
Arsenic	As	As toxicity developed dermal lesions (hyperkeatosis and pigment alterations) and lead to skin cancer,	Muzaffar et al. (2023)
Mercury	Hg	Hg toxicity enhanced heart rate, headache, hypertension, insomnia, alters nerve response, and impairs cognitive function and resulted in cardiac and renal dysfunctions	Eneh et al. (2023)
Lead	Pb	Pb toxicity is lethal to heart, kidney and nervous system. It also affect brain development and gastrointestinal tract of children.	Mishra et al. (2022)
Iron	Fe	Iron toxicity caused dehydrated condition that further develop abdominal pain, Vomiting, diarrhea and lethargy.	Singh et al. (2023)
Cooper	Cu	Cu toxicity caused gastrointestinal distress followed by abdominal pain, vomiting, and hypotension and it also affected the human brain, liver and kidney performances.	Leal et al. (2023)
Chromium	Cr^{3+}	Cr^{3+} toxicity reduced cell vigor and cause breast and liver cancer.	Chandra et al. (2020)
Aluminium	Al^{3+}	Al damaged central nervous system, kidney and liver dysfunction, and cause pulmonary fibrosis, osteomalacia and lung infections.	Obani et al. (2023)
Vanadium	V	Vanadium toxicity caused nausea and throat injury, rashes and blacken the teeth and tongue.	Briffa et al. (2020)
Mercury	Hg	Hg toxicity disturbed nervous, digestive, and immune systems and dysfunction the lungs, kidneys, skin, and eyes.	Demarco et al. (2023)
Lead	Pb	Pb toxicity damaged fetus brain and kidney along with circulatory and nervous system.	DeOliveira et al. (2023)

Heavy metals are known to accumulate in plants and they negatively affect the plant's physiological and biochemical processes and consequently cause serious yield losses (Yan et al., 2020). HMs reduce seed germination by negatively affecting the germination related processes which in turn reduce the overall stand establishment (Hassan et al., 2019). HMs also disturb the plant water status, membrane stability and increase the losses of important osmolytes through excessive production of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). Further, HMs also induce excessive reactive oxygen species (ROS) production which damages the proteins, lipids and DNA (Hassan et al., 2013).

Globally, different chemical, physical and biological methods are being used to remove the HMs from soils. Physical methods like thermal treatments, soil washing, vitri-fication, and chemical methods like the application of lime, organic amendments and phosphate compounds are being used to treat the HMs polluted soils (Gong et al., 2018). The physical and chemical methods are quick and efficient; however, they have major limitations. For instance, they are expensive and laborious and they can cause drastic changes in soil quality therefore, these methods offer no optimal solution to treat HMs polluted soils (Gong et al., 2018). Thus, in this context, biological methods offer an alternative solution owing to environmental friendly nature and they are less expensive. The biological methods involve the use of plants (phytoremediation) and microorganisms (bioremediation) to treat the HMs polluted soils (Khalid et al., 2017). The biological methods are economical and environment friendly, and they have appreciable applicability and efficiency as compared to physical and chemical methods (Yan et al., 2020). However, these methods also have some limitations like lengthy periods, environmental sensitivity, and contaminant toxicity (Liu et al., 2023). The use of microbes (bioremediation) got a great scientific attraction across the globe in recent times. The microbes remove the HMs from soil through different mechanisms including bio-sorption, bio-accumulation, bio-volatilization, bio-mineralization, oxidation and reduction, bio-leaching and production of bio-surfactants (Rahman and Singh, 2020). Micro-organism can protect from the negative effects of HMs; however, many HMs destroy membranes of microbial cells. Thus, the ability of microbes to survive under the effect of HMs is an area of decisive importance (Ayangbenro and Babalola, 2017). It has been reported that HMs toxicity and mobility is depend on the degree of oxidation of HMs (Haque et al., 2022). Microbe use HMs pollutants as a food source and change their redox potential (Faskhutdinova et al., 2021). Under HMs stress, some microbes also secrete different substances including polysaccharides, proteins, and lipids that can bind HMs ions and therefore reduce their availability (Martis et al., 2021).

Microbes also reduce the concentration of HMs in soil; for instance, *Aspergillus niger* showed an appreciable ability to for bioaccumulation of Cd and Cr (Khan et al., 2019), similarly,

Stenotrophomonas rhizophila also significantly removed Pb and Cu by 76.9% and 83.4% (Sun et al., 2021). Due to small size microbes also provide a large surface area to adsorb the HMs which reduces the overall availability of HMs (Rajput et al., 2022). Further, microbes also accelerate the bio-adsorption of toxic HMs which makes them an excellent amendment to remediate HMs contaminated soils (Srivastav et al., 2018). Microbes can also multiply quickly; thus, the use of microbes could be an important amendment to treat the HMs polluted soils (Singh et al., 2020). The recent advancement in microbial bioremediation techniques has shown promising results to remediate polluted soils. For instance, different bioinformatics are being used to develop more effective remediation technologies. These tools are using different databases to explore the underlying mechanisms of degradation (Zheng et al., 2018). Recently, bio-remediation also used genomics, transcriptomics, metabolomics, and proteomics which is added to the evaluation processes of *in-situ* bio-remediation (Villegas-Plazas et al., 2019). Moreover, genomic studies have also allowed us to analyze the genetic information of microbes within the cell which ensures to development of better microbes for remediation (Hakeem et al., 2020). Additionally, recent advancements in synthetic biology also showed promising results and genetically modified organisms (GMOs) have shown appreciable results in removing pesticides, and xenobiotics from the environment (Bala et al., 2022). There are many reviews available regarding the role of microbes in remediating metals polluted soils. Nonetheless, there is no comprehensive review available describing the role of microbes in remediating the antimony, arsenic, cadmium, chromium, mercury, lead, and nickel-contaminated soils. The aforementioned metals/metalloids are highly toxic and their concentration is rapidly increasing in the environment. Recently, bio-remediation got appreciable attention across the globe, therefore, we have discussed the role and mechanisms of microbes to remediate soils polluted by these toxic metals. The current review also discusses the different research gaps that must be filled besides the appreciable progress in the field of bio-remediation. This review provides insights to boost microbial functioning for the remediation of polluted soils.

Sources of heavy metals entry into soils

Recent industrialization is meeting population food demands but also posing a severe hazard to the environment by excreting poisonous compounds such as HMs (Aluko et al., 2021). These toxic HMs enter into the human food chain by eating the contaminated foods (Sayyed et al., 2019). Among HMs, As, Cr, Cd, Pb and Hg got a serious attention across the globe because their concentrations in many terrestrial, marine, and aerial systems exceed the safety threshold (WHO 1990; Rahman and Singh, 2019). HMs have both natural and anthropogenic origins and they can be found in the atmosphere, water, soil and biological organisms (Yin et al., 2021). HMs generation from human sources is permanent and constant while the generation of HMs from natural sources is also affected by natural sources (Armah et al., 2014). The major human sources of HMs are agriculture, industries and urbanization (Li et al., 2021). Textiles, tanneries, fertilizers, galvanizing factories, metallurgic factories, varnishes, pharmaceuticals and pesticide companies are major sources of HMs pollution (Verasoundarapandian et al., 2022).

In the mining process, a significant amount of waste rocks is produced which contains a low quantity of HMs. These HMs are carried into ground and water areas by biological and chemical leaching and are then enters into the human food chain (Li and Yu, 2015). Agriculture activities also add a significant amount of HMs into soil owing to the continuous use of inorganic chemicals. Natural phosphate contains impurities in the form of HMs, and different HMs such as As, Cd, Ni, Cr, and Zn have been identified in higher concentrations in over 200 phosphate fertilizers used worldwide (Nziguheba and Smolders, 2008). Likewise, pesticides also contained impurities in the form of HMs and it has been found that different pesticides contained Hg, As, Cu and Pb as an active elements. Different pesticides containing Hg (II) and Pb(II) has been banned owing to their higher toxicity (Kothe et al., 2010). The application of industrial and municipal wastewater is also common practice and the constant application of these waste waters also leads to the accumulation of HMs in soil (Ren et al., 2015; Li et al., 2017b). Electronic waste also has a significant contribution in HMs pollution. For instance, in China in electronic waste recycling site has a significant amount of Cd and Cu greater than the threshold levels (Wu et al., 2015). Heavy metals from natural sources include mineral deposition, eruption of volcanic pathogenic processes, and oceanic evaporation (Zhang et al., 2012). Mining is an important source of HMs release (Acosta et al., 2011), and in China mining is produces around 12 lakh ha of wasteland per year with an annual increase of approximately 47,000 ha (Zhuang et al., 2009).

Effects of HMs on agro-ecosystem

Soil biology is crucial for maintaining soil quality, which is critical for agricultural sustainability. Human activities are a major source of HMs and they disturb soil microbes, soil fertility, and productivity (Sharma et al., 2017). The survival of microbes is negatively corrected with prolonged exposure of HMs like Pb (Yuan et al., 2015). Similarly, coal mining activities also cause a decrease in microbial abundance, biomass, and variability (Nayak et al., 2015). Heavy metals also slow down the breakdown of litter resulting in uneven deposition of litter on the soil (Marschner and Kalbitz, 2003). Furthermore, HMs have a deleterious impact on the breakdown of stream litter (Hogsden and Harding, 2012; Ferreira et al., 2016). Moreover, HMs also induce a negative effect on soil microbes and a negative correlation has been reported between the concentration of HMs and microbial respiration (Nwuche and Ugoji, 2008). Depending on the soil parameters, substrate concentration, and HM exposure, heavy metals can either accelerate or inhibit N mineralization. The toxicity of HMs also disrupts the N transformation pathways which consequently affect the mineralization of HMs (Hamsa et al., 2017). Further, HM

pollution also induces a negative effect on N mineralization and nitrification and both these processes decrease with increasing the amount of HMs pollutants. Further, nitrification is considered to be more susceptible to HMs as compared to mineralization (Bewley and Stotzky, 1983). Moreover, HMs also affect the soil enzymatic activities and microbial abundance (Xian et al., 2015) and it has been found that HMs reduce the soil enzymatic and microbial activities and soil microbial abundance (Pan and Yu, 2011; Xian et al., 2015).

For instance, Li et al. (2020) documented that HMs reduced bioactivity, richness, and microbial diversity. They found that heavy metals (Cu, Cr, Ni, Pb, Zn, and Mn) showed total variations of 87.7%, 56.6%, 83.0%, and 55.1% α-diversity, and community composition, predicted by PICRUSt. In another study, it was documented that Pb stress altered the bacterial community structure. These authors found that Pb 2.5% and 5% increased *Actinobacteria* abundance by 118.56 and 147.25% while 5% Pb stress Bacteroidota and Myxococcota increased abundance by 280.76 and 138.54%, respectively (Meng et al., 2023). In another study, a significant change in microbial abundance and diversity was observed in Cd-polluted soil. Cadmium toxicity (50 mg kg-1) increased *Bacteroidota* and *Proteobacteria* by 2 and 0.3 folds while Cd toxicity decreased the abundance of *Acidobacteriota*, *Firmicutes*, *Chloroflexi*, *Myxococcota*, and *Gemmatimonadota* by 0.3, 0.5, 1.7, 2.2 and 2.4 folds (Bandara et al., 2022). The studies have documented that long-term exposure to heavy metals negatively affects soil health. For instance, Cheng et al. (2022) long-term Cd toxicity decreased the soil organic matter, nitrogen, phosphorus, and potassium availability. The other group of authors found that long-term As toxicity showed a negative showed a negative impact on soil enzymatic activities and soil properties. They found that As toxicity reduced the urease and dehydrogenase activities and soil nitrogen, SOM and clay were the main factors affecting the soil enzyme activity (Nurzhan et al., 2022). Some studies also reported that microbial species show resilience in response to HMs. For instance, Philippot et al. (2008) found higher resilience of nitrate reduction rates to Hg stress (100 mg kg^{-1}). Brandt et al. (2010) noted that soil bacterial communities showed structural and functional resilience to Cd exposure (0, 40, 150, and 500 mg·kg−1). They found that the observed increase in Cu tolerance against higher concentrations of Cu was involved in the phenotypic adaption and selection at the micro-diversity level. HMs-mediated disruption in soil microbial activities also negatively soil properties and microbial activities. For instance, soils contaminated with HMs are associated with insufficient nutrients, organic matter, and water retention capacity (Singh and Kalamdhad, 2011). The increase in toxicity of heavy decreases the microbial abundance and diversity and indirectly affects soil enzyme activities by changing microbial community synthesizing enzymes (Singh and Kalamdhad, 2016). Moreover, heavy metals also inhibit soil enzymatic activities and reduce the mineralization of SOM and nutrient nutrient cycle (Bakshi et al., 2018). Globally, different including physical, synthetic, and natural remediation techniques (*in situ* and *ex-situ*) are used to remediate polluted soils. The use of genetically modified microbes has received appreciable attention to cleanup metal-contaminated soils and improve stress tolerance (Narayanan and Ma, 2023).

Plant responses to heavy metals

Heavy metals seriously affect plants and the effects of HMs on plants can be seen from germination to senescence (Table 2). Seed germination is one of the most critical stages of plant life and a mediated decrease in seed germination declines seedling growth and subsequent stand establishment (Adrees et al., 2015). For instance, in a study, it was found that combined Cu and Cd stress reduce seed germination, growth of seedlings, and lateral growth rate (Neelima and Reddy, 2003). The exact mechanism through which HMs change seed physiology is not well understood, and different authors reported that HMs inhibit the activities of various enzymes that cause a reduction in seed germination (Figure 1). For instance, Hg induced a decrease in seed germination owing to the direct interaction of Hg with HS group proteins that leads to the formation of an S-Hg-S bridge thus causing a loss in enzymatic activities (Cui et al., 2014).

Apart from seed germinations, HMs also change the root architecture and this effect has been reported in plants. In particular, HMs decreased the root elongation (3-4 folds) and enhanced the formation of lateral roots (2-3 folds) in the presence of different HMs like Cu, Pb, Cr, Zn, and Cd (Sofo et al., 2017). The formation of lateral roots is the initial symptom of HMs toxicity which consequently impairs the uptake of nutrients and water thereby reducing subsequent plant growth (Rucińska-Sobkowiak, 2016). Along with root inhibition, HMS also causes a reduction in plant growth. HMs transport from roots to aerial parts and accumulates in plant cells which interfere with cellular metabolism and thus cause a reduction in plant growth (Shanker et al., 2005; Wang et al., 2020). As a result of their interactions with the central atom (Mg) of the porphyrin ring, heavy metals also break down the chlorophyll molecules, severely reducing photosynthesis and ultimately impairing plant growth (Yadav et al., 2014). Moreover, HMs like Cu also cause lignification of both roots and shoots which reduces biomass production owing to impaired cell development (Martins et al., 2020). Additionally, HMs hurt the water relationships which in turn affects a variety of physiological activities like photosynthesis and transpiration (Alsokari and Aldesuquy, 2011). A recent study showed that Cd stress (100 uM) decreased the plant height by 69% and 73% in sorghum cultivars JS-2002 and Chakwal sorghum (Hassan et al., 2019). Further, Cd toxicity also increased MDA concentration by 39% and 43% respectively in both cultivars (Hassan et al., 2019). In another study, it was witnessed that Pb stress decreased the photosynthetic rate, carbon dioxide concentration, transpiration rate, and WUE by 50.5, 73.2, 48.6, and 148.8% respectively (Qin et al., 2023). Heavy metal toxicity also negatively effect nutrient uptake by plants. For instance, *Fava bean* plants' Cd toxicity (150 mg/L) decreased the Ca and Mg concentration by 1.82 and 1.27 times while Cd toxicity (300 mg/L) decreased the Cd and Mg concentration by 2.278 and 2.25 folds (Piršelová and Ondrušková, 2021).In plants like *Helianthus annuus* and *Vigna radiata*, HMs (As) increased the number of stomata followed by the development of abnormal, arrested, and fused stomata (Gomes et al., 2011; Gupta and Bhatnagar, 2015). Heavy metals also affect the xylem vessels' parenchymatous and mesophyll cells and resultantly change the

TABLE 2 Toxic effects of different heavy metals on plants and soil health.

Heavy metals	Concentration of heavy metals	Growth media	Plant species	Effect of plants and soil	References
Chromium	120 µM	Soil	Grapevine	Cr toxicity reduced the root and shoot growth, tissue nutrient concentration, chlorophyll contents, leaf water status, quantum yield of photosystem II and soil microbial activity.	Nikolaou et al. (2022)
Lead	200 mg Kg^{-1}	Soil	Sunflower	Pb intensity reduced the soil fertility and water uptake along with a significant decrease in stem and root length, dry biomass and crop yield.	Alaboudi et al. (2018).
Cadmium	2 mg kg^{-1}	Soil	Rice	Cd stress increased ROS that destroyed the chloroplast and thus reduced the photosynthetic efficiency of plants. Further, Cd toxicity altered nutrient absorption by plant roots.	Li et al. (2023)
Copper	10 g L^{-1}	Soil	Barley	Cu toxicity decreased the root and shoot length by affecting stomatal density, conductance and PS II efficiency whereas high Cu reduced the organic matter percentage and microbial population in soil.	Rajput et al. (2018)
Lead	10 mL	Soil	*M. sativa*	Pb toxicity decreased the antioxidant production while increased ROS that reduced the plant growth and physiological functions.	Raklami et al (2021)
Nickle	1000 µM	Soil	Guava	Ni toxicity reduced plant growth and development, photosynthesis and transpiration activities, leaf gas exchanges and K^+ uptake and microbial growth.	Bazihizina et al. (2015)
Nickle	400 µM	soil	Rice	Ni toxicity reduced the fresh and dry weight along with shoot and root length and increased ROS, lipid peroxidation and consequently protein denaturation.	Hassan et al. (2019)
Lead	100 µM	Soil	Wheat	Pb caused stunted growth, chlorosis and blackening of roots that reduced the soil nutrient uptake mechanism.	Tripathi et al. (2016)
Cadmium	4.8 mM	Soil	Wheat	Nutrient availability reduced to plant under high Cd stress that resulted in decreased root length and seedling growth, subsequently less fresh and dry biomass and yield production.	de Souza Guilherme et al. (2015)
Chromium	300 µM	Soil	Wheat	Cr affected the lamellar system of plant and disturbed the photosynthetic machinery, and caused chlorosis, which impaired growth.	Mathur et al. (2016)

plant water relations and are considered to be responsible for the decrease in leaf growth. Heavy metals also negatively affect the photosynthetic machinery however, it depends on the concentration of HMs. Moreover, HMs also negatively affect the light-harvesting, transport of electrons, and RuBisCo activity which in turn reduce the overall plant photosynthetic efficiency (Paunov et al., 2018; Latif et al., 2020). Besides this HMs (Cd) also reduced the photochemical efficiency (Fv/Fm), the effective quantum yield of photosystem II (φPSII), and chlorophyll florescence thereby leading to the inhibition of photosynthesis (Gao et al., 2020; Yotsova et al., 2020).

Generally, HMs (Hg, Cu, Pb, Ni, Cd, and Zn) target the plant chlorophyll in three different ways by increasing the activity of chlorophyllase enzyme, causing oxidation of chlorophyll through increased ROS production, and inhibiting biosynthesis of chlorophyll biosynthesis (Gill et al., 2012; Shahzad et al., 2018; Sharma et al., 2020). HMs not only affect the chlorophyll molecules but also the membranes of the chloroplast and thylakoid cells. For example, swelled thylakoids, degraded chloroplast membranes, and loss of chloroplast membrane were noted in barley plants under Pb stress (Wang et al., 2017). Moreover, HMs also inhibit the light reactions by decreasing the efficiencies of PS-I and PS-II and they also decrease the dark reactions owing to decreased activities of enzymes linked with the Calvin cycle (Souri et al., 2019). Heavy metals also induce overproduction of ROS that damage proteins, DNA, and lipids and lead to the induction of oxidative stress (Foyer and Noctor, 2016). However, plants also activate excellent deference and they also accumulate various osmolytes to counter the toxic effects of HMs. For example, Chowardhara et al. (2020) found that activities of CAT, GST, GR, APX, and POD and accumulation of proline and ascorbic acid were increased in response to Cd toxicity in *B. juncea*. It has been reported that HMs also decrease the uptake of water and nutrient which in turn cause significant growth losses (Rucińska-Sobkowiak, 2016; Wang et al., 2017). For instance, Cd toxicity competes with calcium (Ca), iron (Fe), and magnesium (mg) which caused a significant reduction in growth and biomass production (Raza, 2022).

Under the harmful effects of HMs, nitrogen metabolism is essential for plant growth and development. According to reports, HMs decrease the nitrate and ammonia assimilation enzymes by increasing protease activity. MicroRNAs play an imperative role in HMs toxicity by regulating the plant antioxidant responses, chelations, and auxin and cytokinin signaling (Ding et al., 2020). For instance, Casarrubia et al. (2020) found that mycorrhizal and microRNA played a significant role in Cd tolerance in *Vaccinium myrtillus*. In another study, it was found that MicroRNA expression significantly improved the Cd and Al tolerance in tobacco (Cedillo-Jimenez et al., 2020). Heavy metals also negatively affect the quality of crops and it has been found that Cd toxicity in rice reduced the rice protein contents, and milling degree and increased the kernel chalkiness (Imran et al., 2021).

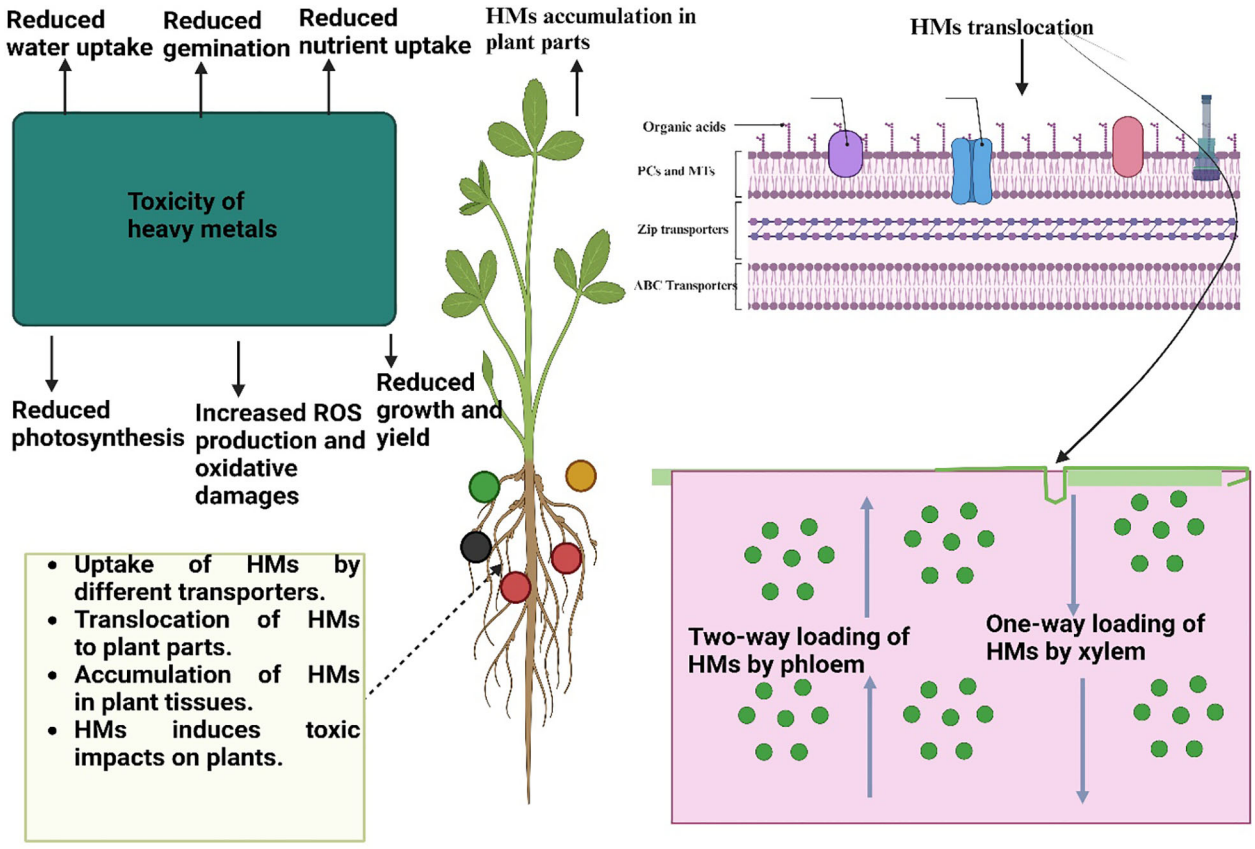

FIGURE 1
Toxic effects of heavy metals on plants. Heavy metals are absorbed by plants roots and then they are moved to above ground plant by different transporters and then accumulated in above ground parts. The accumulation of HMs in plant parts induce ROS production, necrosis, and decrease nutrient and water uptake and caused protein degradation, cell detoxification thus reduce the plant growth and development.

Microorganisms responsible for bioremediation

Heavy metal pollution poses a severe threat to public health by contaminating food supplies and drinking water on a global scale (Huang et al., 2020). Microbial remediation is an imperative approach and it has appreciable potential to improve crop productivity, and human health and restore the ecosystem (Narayanan and Ma, 2023). The microbial-mediated bio-accumulation and bio-magnification are very successful in removing the pollutant to ensure safe and sustainable crop production (Manorma et al., 2023). Different microbes including, algae, bacteria, and fungi are being used to clean up the HMs contaminated soils (Table 3).

Bacteria

The interaction of microbes with HMs occurs through different mechanism which depends on metal and microbe type and surrounding environment. Different factors including temperature, pH, nutrient source, and metal ions play an important role in the mobility and bioavailability of HMs for microbial transformation. Bacteria's small size, rapid growth, and ease of cultivation allow them to thrive in a variety of environmental situations. HMs often connect to functional groups including amino, carboxyl, sulfate, and phosphate groups that are present on the layers of bacterial cell walls (Yue et al., 2015). The potential of bacteria for HMs uptake can vary from 1-500 mg/g. For instance, Hg resistant *pseudomonas aeruginosa* strain absorbed the Hg uptake 180 mg/g (Yin et al., 2016). Likewise, different microbes like *Bacillus* sp. PZ-1 and *Pseudomonas* also absorb the Pb from wastewater (Li et al., 2017a). On the other hand *Arthrobacter viscosus* can absorb the Cr and it also has an excellent capacity to transfer the Cr (VI) into Cr (III) (Hlihor et al., 2017).

Rhodobacter capsulatus also showed a maximum capacity of 164 mg/g to absorb the Zn (II) (Magnin et al., 2014) while *Bacillus ceres* showed a maximum bio-sorption capacity of 31.95 mg/g and 24.01 mg for Cd (II) in dead and living cells (Huang et al., 2013). Extracellular polymeric substances (EPS) protect the microorganism from the toxic effects of HMs by restricting entry of HMs into the cell. It has been discovered that EPS has both anion and cationic functional groups, which help to accumulate HM ions like Cd, Hg, Cu, and cobalt (Fang et al., 2017). After adsorption HMs are converted to diverse ionic states in bacterial cells that reduce their toxicity. *Pseudomonas putida*is an important microbe and it can absorb 100% Hg from the marine environment it also

TABLE 3 Different microbes used to remediate heavy metals polluted soils.

Type of microbe	Microbial species name	Used against heavy metals	References
Bacteria	*Penicillium chrysogenum* A15	Lead	Povedano-Priego et al (2017)
Fungi	*A. fumigatus*	Lead	Khan et al (2019)
Bacteria	Pseudomonas sp.	Chromium	Tirry et al (2021)
Fungi	*Penicillium* sp.	Chromium	Barsainya et al (2016)
Bacteria	Phyllobacterium myrsinacearum	Arsenic	Alves et al (2022)
Bacteria	Acinetobacter	Copper	Ke et al (2021)
Yeast	*Wickerhamomyces anomalus*	Chromium	Joutey et al (2015)
Bacteria	*P. fluorescens*	Cadmium	Abbaszadeh-Dahaji et al (2019)
Algae	*Pelvetia canaliculata*	Chromium	Lytras et al (2017)
Bacteria	*PGPE consortium*	Mercury	Ustiatik et al (2022)
Bacteria	*Sinorhizobium Saheli*	Cadmium	Kang et al (2018)
Aerobic bacteria	*Variovorax paradox*	Nickle	Durand et al (2016)
Bacteria	*Bacillus sp. and Bacillus pumilus*	Cadmium	Narayanan and Ma (2023)
Aerobic Bacteria	*Micrococcus luteus*	Arsenic	Pinter et al (2017)

reduces the Hg(II) into Hg(0) (Sheng et al., 2018). The findings of Zhang et al. (2012) showed that a new microbial strain *Acinetobacter* sp. showed an excellent ability to detoxify the Cr. In another; authors screened 72 *acidothermophilic*autotrophic microbes for their ability to tolerate and bio-absorb the HMs and these authors found that the ATh-14 strain showed an appreciable potential and it showed absorption capacity of 85.82% for solubilization of copper (Umrania, 2006). Bacteria are better bio-sorbents as compared to other microbes due to their size, ubiquity resilience, and ability to grow under a wide range of conditions (Hlihor et al., 2017).

Fungi

Fungi also have an excellent ability to remediate the HMs polluted soils. The presence of chitin, polysaccharides, phosphate, and glucuronic acid in fungal cells is essential for the adsorption of HMs (Purchase et al., 2009). Different functional groups and fungal strains had a significant impact on the adsorption rate of HMs (Iram et al., 2015). In a study, it was found that *Termitomyces clypeatus* detoxified the Cr(VI) by adsorbing Cr on its surface through carboxyl, imidazole, hydroxyl, phosphate, and sulfhydryl groups (Ramrakhiani et al., 2011). Further, Amirnia et al. (2015) found that *Saccharomyces cerevisiae* eliminated the Cu(II) from water sources, while Talukdar et al. (2020) found that *Aspergillus flavus* fungal species removed the Cr by more than 70%. Moreover, *Aspergillus fumigates* also showed an appreciable potential to remove the Cd, Cr, Cu, Ni, and Zn from the contaminated soils (Shazia et al., 2013). In another investigation, three different fungal species including *Penicillium citrinum*, *Trichoderma viride*, and *Penicillium* showed a significant potential (250 mg/L) to adsorb the Cr(VI) (Zapana-Huarache et al., 2020).

Algae

Algae have also shown a good potential to remediate HM-polluted sites owing to the fact algae produce various peptides that help the accumulation of HMs and defend against the HMs (Bilal et al., 2018). For instance, *Fucus vesiculosus* showed a tremendous potential to adsorb the Pb(II) (Demey et al., 2018), likewise, *Cladophora fascicularis* also showed a significant potential to remediate the Pb(II) from wastewater. Similarly, Sargassum marine algae also showed a significant potential to detoxify the Cu (II) from the aqueous solution (Barquilha et al., 2017). In another study Christoforidis et al. (2015) tested the absorption capacity of *Cystoseira crinitophylla* for copper and found that this algae showed a maximum capacity of 160 mg/g to adsorb Cu (Christoforidis et al., 2015). On the other hand, authors noted that *Saccharina fusiforme* and *Saccharina japonica* substantially detoxify the Zn(II), Cd(II), and Cu(II) (Poo et al., 2018) while *Desmodesmus* also showed an appreciable potential to remove the Cu(II) and Ni(II) from the wastewaters (Rugnini et al., 2018). The study findings of Aslam et al. (2019) showed that microalgae showed promising results in the accumulation Mn, Cu, and Zn (Freitas et al., 2011). Moreover, the findings of Freitas et al. (2011) showed that algal biomass showed an appreciable potential for HMs like Fe.

Factors affecting the bioremediation process

Different factors including metal concentration, valance state, metals bioavailability, redox potential, soil temperature, and pH affect the bioremediation process (Bandowe et al., 2014). The pH of the soil has an impact on bacterial enzymatic activity as well as microbial bio-sorption (Morton-Bermea et al., 2002). Soil pH also changes the surface charge of microbes by affecting the ability of microbes to absorb the HM ions (Galiulin and Galiulina, 2008). Soil pH substantially affects both the transportation and hydration of HM ions in soil (Dermont et al., 2008) and it has been documented that the rate of HMs removal is increased with increasing pH over a certain rate and after this, the rate of removal starts declining (Wierzba, 2015). The ideal pH range for most bacteria is 5.5-6.5

(Wang et al., 2001), however, some bacteria like *Bacillus jeotgali* can thrive at a pH of 7 (Rodríguez-Tirado et al., 2012). Another significant component that influences the absorption of HMs is temperature; which influences the development and proliferation of microorganisms (Fang et al., 2011). Different bacteria require different temperatures to carry out their functions (Acar and Malkoc, 2004). However, HM ions, soil additives, and soil type all have an impact on microbial activity. It is challenging to achieve microbial adsorption due to the low mobility of HM ions caused by soil adsorption and retention of HM ions (Hu et al., 2010).

Soil pH is an important factor that affects microbial growth. For instance, unfavorable pH affects enzyme activity which lowers the rate of microbial metabolism and it also affects the binding capacity between HMs and adsorbants (Bandowe et al., 2014). The changes in pH also affect the mobility and hydration of metals (Bandowe et al., 2014). For instance, the adsorption capacity of Zn and Pb was increased with increasing pH, and an increase in soil pH above 5.5 decreased the removal of Pb and Zn (Wierzba, 2015). Other authors also documented that soil acidification increased the mobility of metals in the following order Cd>Zn>Pb. These authors also document that soil pH affects mobility, causes metal ions to become more or less active, and increases or decreases their environmental risk (Kicińska et al., 2022). Temperature is also a factor that affects microbial growth (Fang et al., 2011). The increase in temperature affects the diffusion of metals and increases the bioavailability of metals. However, optimum degradation temperature can vary according to metal types, for instance, Cd bio-degradation by Bacillus jeotgali was maximum at 35°C while bio-degradation by the same bacteria was higher at 30°C (Chanmugathas and Bollag, 1988). The adsorption efficiency is also affected by soil organic matter, for instance, organic matter tends to fix the metals in soil which reduces the availability to metals (Wang et al., 2022). A short-term study investigates the response of different temperatures (5, 15, and 25oC) Cd, Cu, Pb, and Zn removal by *Carex pseudocyperus*, *C. riparia*, and *Phalaris arundinacea*. Low temperatures reduce the removal capacity of all the metals and an increase in temperature increases the removal capacity of all the metals (Schück and Greger, 2023). Climate change also induces a significant impact on soil microbial activities. For instance, climate-induced variation in soil temperature, and humidity affect the decomposition of SOM and nutrient cycling (Burns et al., 2013), and it partially or fully depends on microbial activity. The change in soil temperature and moisture can change the growth, structure, function, composition, and interaction among microbes for the degradation of pollutants in soils (Alkorta et al., 2017).

Bioremediation is generally limited to bio-degradable compounds, and it is also susceptible to rapid degradation which more toxic compounds. Besides it, bio-remediation also needs extensive monitoring and it has major drawbacks in terms of environmental growth conditions, nutrient requirement, temperature, and pH conditions. Therefore, it is essential to find ways to identify the microbes having a wider adaptability under a wide range of temperature and pH conditions for an efficient remediation process. On a long-term basis, microbial mediation remediation is a simple, cheap, and environmental method and it can improve the overall soil fertility, ecosystem health, and safer and sustainable food production. Nonetheless, implantation of bioremediation needs a comprehensive understanding of soil microbial communities, properties of contaminants, and environmental conditions as these factors play a critical role in getting effective results.

Microbial mediated remediation of heavy metals polluted soils

The use of microbes is considered as an effective way to treat the HMs in polluted soils, as these microbes absorb HMs and also convert them into less toxic forms (Gupta et al., 2016). Microorganisms play a critical role in remediating HMs polluted soils owing to the fact they can with stand metal toxicity. Numerous HMs have been reported to be precipitated, undergo oxidation state changes, and be sequestered by microbes (Figure 2; Kang et al., 2016).

Microbial mediated remediation of antimony contaminated soils

Microorganisms are crucial for remediating Sb polluted soils and they reduce the toxicity of Sb through different ways including, bio-reduction and bio-oxidation (Jeyasundar et al., 2021). Many bacteria have been identified that can be used to remediate the Sb-polluted soils (He et al., 2019). For instance, two bacteria *Shinella and Ensifer* discovered from Sb-contaminated soils showed a tremendous potential to oxidase Sb (Choi et al., 2017) while the bacterial *Bacillales* strain also showed marked results to change the Sb-V into Sb-III (Lai et al., 2018). Similarly, fungi have been also used to remediate the Sb polluted soils, and study findings of Xi et al. (2022) showed that AMF increased plant antioxidant activities by reducing the retention of Sb in plant parts. The findings of Liu et al. (2013) showed that the bacterial strain *Pseudomonas* substantially increased the plant growth, and microbial activity and decreased Sb availability (Liu et al., 2013). In another study Zhang et al. (2012) found that microbes isolated from the rice field contributed significantly towards the oxidation of Sb-III likewise, Li and Yu (2015) also found that *Agrobacterium tumefaciens* contributed towards the oxidation of Sb-III.

Some environmental microorganisms, particularly those that thrive in anaerobic environments, are capable of converting Sb(V) to Sb(III). For instance, Hockmann et al. (2014) noted that microbes converted the SB-V to Sb-III with the help of lactate as an electron donor. Similarly, Kulp et al. (2014) found that microbes in Sb-polluted mines reduced Sb-V to Sb-III. In the case of flooded mine pit soils group of researchers from China found that autotrophic bacteria reduced the Sb-V and generated Sb2O3 by using hydrogen gas (H2) as an electron donor (Lai et al., 2016). Additionally, Huang et al. (2022) found that after 60 days of injection of the *B. cereus* solution into plant roots; the concentration of As and Sb in soil was significantly reduced as compared to soil without bacteria solution

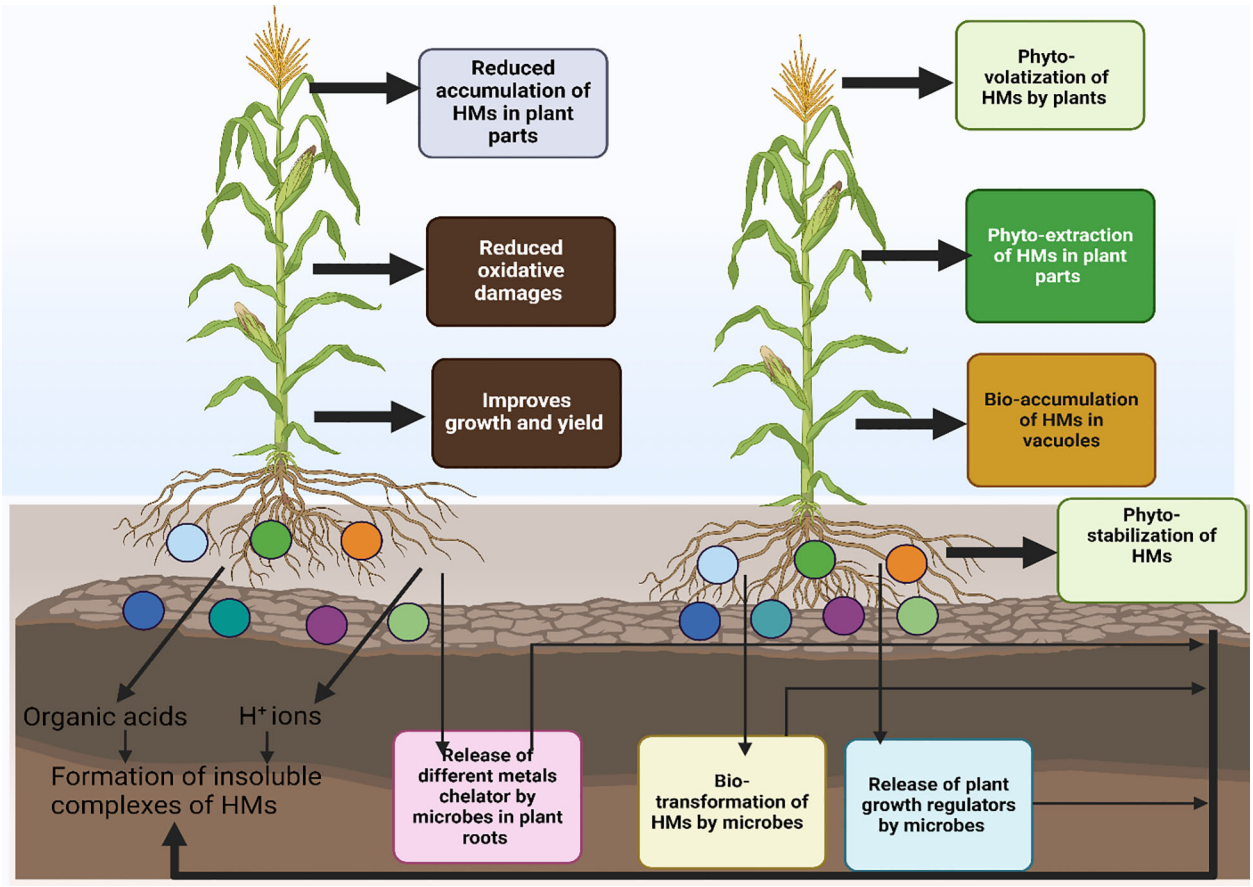

FIGURE 2
Different mechanism used by microbes to induce heavy metals toxicity in plants. Microbes use different mechanisms bio-sorption, bio-mineralization, bio-accumulation, bio-leaching and bio-transformation to remediate polluted soils. They also increase the availability of nutrients by increasing production of IAA and ACC deaminase, and siderophores thus resulting in better growth under polluted soils.

this indicates that this strain promoted the absorption of As and Sb from soil (Huang et al., 2022).

Microbial mediated remediation of arsenic contaminated soils

Arsenic occurs in the environment in different inorganic forms including As-0, As-III, and As-V, and organic forms like dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), trimethylarsine oxide (TMAO) and arsenobetaine. It has been found that bacteria, algae, and fungi can methylate As-III into methylated species (Yang and Rosen, 2016; De Francisco et al., 2021). Different fungal species like *Aspergillus*, *Candida*, *Scopulariopsis*, and *Penicillium* can also cause a change in the methylate inorganic As to the organic As species (Bentley and Chasteen, 2002). It is important to keep in mind is that the ability of certain microorganisms to methylate and volatilize depends on soil organic matter (SOM), soil chemistry, and As concentration (Mestrot et al., 2011). Spagnoletti et al. (2016) tested the impact of AMF (R. *intraradices*) on soybean plants under As stress and found a marked improvement in plant biomass and a reduction in As accumulation. Likewise, Chan et al. (2013) also found that AFM (*Geosporum*) enhanced the phosphorus uptake and reduced the As concentration in rice grains.

Transgenic microbes are also an effective way to treat As toxicity. For instance, transgenic microbes with expressed arsM showed an ability of 2.2-4.5% to remove As from soil while the same microbe showed an ability of 10-fold in nutrient solution (Liu et al., 2011). In another study, Huang et al. (2015) found that thermophilic strain *Bacillus subtilis* 168 was unable to do methylation and volatilization of As. They genetically modified this bacteria with CmarsM gene and found that genetically modified bacteria caused methylation and volatilization of As it occurred within 48 hours in As-contaminated organic compost. Moreover, Villadangos et al. (2014) also modified the As-resistant bacteria named *Corynebacterium glutamicum* by ArsC1 and ArsC2. These authors found that As(V) was significantly increased after the introduction of genetically modified bacteria. Additionally, Preetha et al. (2023) also prepared the mutant *C. glutamicum* strain and found that this strain showed an ability of 15 folds and 30 folds more to accumulate As-III and As-V as compared to the control treatment.

Microbial mediated remediation of cadmium contaminated soils

Cadmium is a very toxic HM posing a serious threat to human health and the environment. The application of microbes is an effective and promising technique to treat Cd-polluted soils. In a study, Ma et al. (2020) discovered the Cd immobilization PGPR (TZ5) and found that this bacteria significantly increased ryegrass weight by 77.78% and decreased the concentration of Cd in ryegrass by 48.49%. Further, the application of this bacteria also increased the soil enzymatic activities and microbial growth which indicates that this bacterial strain (TZ5) can provide a practical approach to remediate Cd-polluted soils (Ma et al., 2020). Limited studies are conducted to determine the impacts of single and co-inoculation of *Bacillus mycoides* and *Micrococcus roseus* on growth and nutrient uptake of maize grown under Cd stress (100 and 200 mg kg-1). These authors found that all bacterial treatments appreciably improved the plant growth and biomass and the combination of both bacteria reduced the root and shoot Cd uptake and transfer and translocation as compared to control (Malekzadeh et al., 2012).

In Cd-contaminated soils, Cd-tolerant bacteria play an important role (Bravo, 2022). The microbes use various mechanisms including biosorption and intra-cellular accumulation to mitigate the adverse impacts of Cd stress (Ghosh et al., 2022). Recently, genetically modified organisms also played an important role in remediating Cd-polluted soils (Abbas et al., 2018). Different genetically modified organisms (CdtB Enterobacter and Klebsiella variicola) showed an appreciable potential to remediate Cd polluted soils (Feria-Cáceres et al., 2022; Quiroga-Mateus et al., 2022). Similarly, Arce-Inga et al. (2022) found that the application of *Theobroma cacao* (CCN51) significantly decreased the uptake of Cd, and its translocation to plant parts. Feng et al. (2023) studied the impact of *mixotrophic acidophiles* under Cd-contaminated soils. These authors also found that soil solution pH and reduction level of glucose affected the abundance of *Acidithiobacillus* which contributes significantly towards removal of Cd (Feng et al., 2023). On the other hand, the fungal strain belonging to *Purpureocillium lilacinum* tolerated the Cd stress up to 12000 mg/L. The SEM analysis indicated Cd can be accumulated on the mycelial surface generating plenty of metal precipitation particles. Further, these authors also found that in pot experiments this fungal strain also reduced the soil Cd concentration in soil by 12.56% and promoted plant growth this indicates that this fungal could be an important candidate to remediate Cd polluted soils (Deng et al., 2021).

Microbial mediated remediation of chromium contaminated soils

Chromium is released into the environment as a result of human and anthropogenic activities which pose a serious threat to living organisms. Microbial remediation is an effective approach to treatment the Cr polluted soils. For instance, in a study authors tested the impact of *Nostoc linckia* to remediate Cr polluted soils. They found that this microbe showed an appreciable potential to accumulate Cr and suggested that this bacteria could be an effective candidate to remediate Cr-polluted soils (Cepoi et al., 2021). In another study, edaphic cyanobacteria were tested for Cr remediation and it was found that these bacteria produce polysaccharides, glycoproteins, lipopolysaccharides, and ionic functional groups that can coordinate with Cr and reduce its availability (Cheung and Gu, 2007). Moreover, different Cr-tolerant bacteria including *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Streptomyces*have been identified and they can remove the Cr by 50-90% (Ramesh and Winkler, 2010; Bansal et al., 2019; Elahi and Rehman, 2019; Murthy et al., 2022). The study findings of Wen et al. (2023) showed that the addition of SR-2, PA-1, and LB-5 improved the plant fresh weight by 10.3%, 13.5%, and 14.2% and increased the soil enzymatic (catalase and sucrose) activities and significantly decreased the shoot Cr concentration by 19.2-83.6%.

Chen et al. (2021) studied the impact of *B. cereus* WHX-1 on mitigating Cr toxicity. They found that this microbial species improved the soil physicochemical properties, soil bulk density and decreased the redox potential. They also found that this microbial species transferred the Cr-IV by 94.225 into Cr-III increasing the residual fraction of Cr by 63.38%. Further, these authors also found that the application of *B. cereus* improved the growth and biomass production of ryegrass. In another study, Ahmed (2018) studied the impact of chromium-tolerant auxin-producing rhizobacteria on growth characteristics of *Lens culinaris* growing under different Cr concentrations (0, 50, 100, 200, 400, and 500 μgml−1). The results of their study findings showed that *Bacillus* species mitigated the deleterious impacts of Cr reduced the Cr accumulation in soil and reduced Cr availability to plants.

Microbial mediated remediation of lead contaminated soils

Bioremediation with microbes is considered an effective approach is a promising technique to remediate the Pb-contaminated soils. For instance, a pot study conducted on wheat showed that *R. sphaeroides*reduce the Pb concentration in root and lead by 14.78% and 24.01% (Li et al., 2016). On the other hand, Rhee et al. (2012) found that two fungal species *Paecilomyces javanicus* and *Metarhizium anisopliae* isolated from mining produced organic acids that resulted in precipitation of Pb. In another research study Sun et al. (2017) found that soil inoculation with *M. circinelloides* significantly increased the Pb removal by *S. nigrum* L. These authors also found that soil fertility was also increased after inoculating the soil with *S. nigrum* (Sun et al., 2017). Likewise, Zhou et al. (2016) added WH16-1 strain in Pb2+ contaminated paddy soil and found that this bacterial strain decreased the exchangeable and carbonate-bound Pb in the paddy soil 14.04 and 10.69% (Zhou et al., 2016).

The study findings of Puyen et al. (2012) showed that *Micrococcus luteus* marked decreased Pb concentration in soil, likewise, findings of Kalita and Joshi (2017) showed that *Pseudomonas aeruginosa* application to Pb-polluted soil

appreciably reduction the concentration of Pb with 40 mg g-1 sorption capacity (Kalita and Joshi, 2017). Shanab et al. (2012) tested the potential of different algae to remediate Pb-polluted soils and they found that different algae isolates like *Phormidium ambiguum*, *Pseudochlorococcum typicum*, and *Scenedesmus significantly* reduced the Pb toxicity. Fungi is also an effective candidate for reducing Pb toxicity (Fawzy et al., 2017) application of AMF under Pb stress effectively increased the sunflower biomass and mitigated the toxic effects of Pb (Hassan et al., 2013). In addition to producing various organic acids, polyphosphates, peptides, and sulfur compounds, fungi also do cell wall binding, and make chelate, and precipitate that decreases Pb toxicity (Bellion et al., 2006).

Microbial mediated remediation of mercury contaminated soils

Mercury microbial remediation needs the microbial species to withstand and remove the Hg over extended periods. Various authors noted that microbes effectively remediate the Hg-contaminated soils. For instance, *Vigna unguiculata* inoculated with *Photobacterium* and grown on Hg-contaminated soil (27 mg/kg) showed increased root growth (11%), seed production (33%), leaf numbers (50%), Hg uptake in roots (25%) and decreased Hg concentration in aerial plant organs (55%) as compared to un-inoculated control (Mathew et al., 2015). Similarly, two bacterial strains like *Brevundimonas diminuta* and *Alcaligenes faecalis* applied to Hg and Pb-contaminated soil increased the phyto-accumulation of Pb and Hg by roots and shoots (Hamzah et al., 2015). In another study Hg resistant microbials including *Enterobacter ludwigii* and *Klebsiella pneumoniae*, promoted plant growth and decreased proline concentration, MDA concentration and electrolyte leakage in wheat seedlings growing under Hg stress 75 µM; (Gontia-Mishra et al., 2016).

In another study, bacteria inoculation significantly improved maize growth and reduced the Hg uptake by maize plants growing under Hg (Mariano et al., 2020). Fungi have also shown an appreciable potential to remediate Hg-contaminated soils and it has been found that AMF inoculation increased the plant growth, P uptake, and reduced Hg uptake as well as translocation in *Lactuca sativa* growing under Hg stress under (10 mg/kg) (Cozzolino et al., 2016). Moreover, commercial AMF like *Glomus*, *Entrophospora* and *Scutellospora genera*, appreciably improved the seedling growth and root elongation of rice plants growing under Hg toxicity (Vargas Aguirre et al., 2018). Another group of authors also found that commercial AMF also promoted plant growth and stimulated the uptake of Hg in *Lolium perenne* and rice plants growing under Hg toxicity (Leudo et al., 2020). Likewise, Pietro-Souza et al. (2020) found that compared with *Chrysopogon zizanioides* plants growing with AMF under Hg stress showed a marked improvement in plant growth, root and shoot biomass, chlorophyll concentration and showed a reduction in Hg accumulation. Moreover, *Aspergillus* and *Curvularia geniculata* also appreciably increased the maize root growth, root dry weight, shoot dry weight, chlorophyll, and Hg accumulation by 40% and 34% respectively (Pietro-Souza et al., 2020).

Microbial mediated remediation of nickel contaminated soils

Microorganisms are extremely important for the bioremediation of Ni-polluted soils owing to the fact this method is very economically effective against Ni toxicity (Hassan et al., 2019). Various bacterial strains including *Bacillus thuringiensis* and *Bacillus cereus* have shown promising results in treating the Ni contaminated soils (Zhu et al., 2016). Cabello-Conejo et al (2014) recorded that *Arthrobacter nicotinovorans* appreciably improved plant growth and increased the phyto-extraction of Ni from polluted soil. Zaidi et al. (2006) documented that *Bacillus subtilis* decreased the toxicity of nickel while noticeably boosting mustard growth and nickel phyto-extraction. Other authors also found that inoculation with *Trichoderma atroviride*and *Glomus intraradices* improved the Ni phyto-extraction and reduced the Ni toxicity in linseed and mustard (Cao et al., 2008).

In another study, Alboghobeish et al. (2014) tested the potential of bacterial strain (*Klebsiella oxytoca*) and found that this strain showed a Ni tolerance of 24 mM. Likewise, *Enterobacter asburiae* from industrial water depicted the Ni tolerance to a 15 mM concentration and it removed the 75% Ni by bio-accumulation (Paul and Mukherjee, 2016). Heidari and his colleagues found that a *Microbacterium oxydans* strain showed a Ni removal efficiency of 83-91% (Heidari et al., 2020) while Das et al. (2014) reported that *Bacillus thuringiensis* found that removed the Ni by 82% through bio-sorption process. According to Costa and Tavares (2017), Alternaria and Penicillium species have respective Ni biosorption potentials of 11.3 and 13.1 mg g-1. Trichoderma and Aspergillus inoculation also considerably increased the effectiveness of Ni's phytoextraction (Jiang et al., 2008) and Stenotrophomonas from industrial waste also showed an appreciable potential to remove the Ni (Aslam et al., 2020). However, the biosorption of Ni by microbes significantly affects microbial strain, pH, temperature, and initial Ni concentration (Heidari et al., 2020).

Microbial resistance to heavy metals and their mechanisms

During HMs stress microbes either die owing to toxicity developed by HMs or they thrive in this condition through different resistance mechanisms against HMs (Table 4). Microbes develop different mechanisms including, extra and inter-cellular sequestration, and extracellular barriers, and they actively transport the metal ions to tolerate HMs toxicity. On the surface of bacteria, there are several barriers such as cell walls, plasma membranes, and other structures like EPS that prevent HMs from entering bacterial cells (Bhati et al., 2019). The research findings of Kumar et al. (2014) indicated that bacteria and fungi cause the bio-sorption of

TABLE 4 Microbial remediation of heavy metals contaminated soils and different mechanism used by microbes to remediate heavy metals contaminated soils.

Type of microbe	Microbial species name	Used against heavy metals	Potential mechanism	References
Bacteria	*Bacillus* sp. KL1	Nickel	Biosorption	Taran et al. (2019)
Algae	*Spirulina sp.*	Chromium	Biosorption	Rezaei (2016)
Bacteria	*Bacillus thuringiensis*	Nickel	Immobilization of Ni	Zhu et al (2016)
Algae	*Spirulina platensis*	Chromium	Biosorption	Kwak et al (2015)
Bacteria	*Streptomyces sp. NRC21696*	Arsenic	Chelation	AL-Huqail and El-Bondkly (2022)
Bacteria	*Sphingomonas paucimobilis*	Chromium	Enzymatic transformation	Ibarrolaza et al (2011)
Yeast	*Candida tropicalis*	Chromium	Biosorption	Bahafid et al. (2013)
Bacteria	*Acidithiobacillus*	Nickel	Bioleaching	Wu et al. (2020)
Bacteria	*Aspergillus spp.*	Nickel	Oxidation and reduction	Bisht and Harsh (2014)
Yeast	*Cyberlindnera fabianii*	Chromium	Biosorption	Fernández et al. (2018)
Bacteria	*Bacillus sp. E1S2*	Cadmium	IAA production and ACC deaminase synthesis	Ma et al. (2015)
Fungus	*Ganoderma lucidum*	Lead	Biosorption	Chang et al. (2020)
Filamentous fungi	*Aspergillus niger*	Chromium	Biotransformation	Gu et al (2015) Singh et al. (2021)
Bacteria	*Aspergillus niger*	Nickel	Biosorption	Oyewole et al (2019)
Fungi	*Phanerochaete chrysosporium BKM-F-1767*	Lead	biosorption and bioaccumulation	Huang et al (2017)
Bacteria	*Bacillus amyloliquefaciens*	Chromium	Biosorption/Bioreduction	Fernández et al (2018)

ACC, 1-Aminocyclopropane-1-carboxylate; IAA, indole-3-acetic acid.

metals like Cu, Pb, and Cr. Microbial biofilms contain polymers that accumulate HM ions and protect the inside bacterial cells and the presence of biofilm on *Pseudomonas aeruginosa* showed tolerance against, Cu, Pb, and Zn (Teitzel and Parsek, 2003). Further, the presence of biofilms also increased the elimination efficiency of HMs (Grujic et al., 2017). Additionally, cell walls and EPS also work as an excellent barrier and they substantially adsorb the metal ions like Pb and Cr (Kushwaha et al., 2017).

The cellular membranes of microorganisms contain additional proteins and metabolic products that interact with HMs to decrease their availability. Microbes also develop extracellular sequestration which involves the complexation of metal ions as insoluble compounds and this mechanism is an important way to reduce the HMs toxicity (Thelwell et al., 1998). Microbes also develop intra-cellular sequestration in the metal ions form complexes with distinct compounds in the cell cytoplasm and this is a very common mechanism used by microbes to withstand the toxicity of HMs. Microbes with the aid of low molecular proteins like cysteine accumulation HMs like Cu, Cd and Zn intra-cellularly (Higham et al., 1986) and other microbes like *Rhizobium leguminosarum* use glutathione to accumulate HMs (Cd) intra-cellularly (Lima et al., 2006). The cell wall of fungi is made of lipids, chitin, polysaccharide, polyphosphates, and proteins which help them to accumulate HMs both intracellularly and extra-cellularly (Remenar et al., 2018).

Numerous metal exporting proteins, including ABC transporters, P-type efflux ATPase, cation diffusion facilitator, and proton-cation anti-porters, are found in microorganisms and assist in the efflux of harmful metals (Soto et al., 2019). ABC transporters also help microorganisms tolerate the stress brought on by HM by facilitating the ions' transfer across membranes (Lerebours et al., 2016; Zammit et al., 2016). The microbial resistance to HM is also contributed by enzymes that transfer the HMs ions from hazardous to less toxic forms (Giovanella et al., 2016; Liu et al., 2017). Microbes use different mechanisms including, biotransformation, extrusion, EPS production, and proteins to survive the toxicity of metals (Wu et al., 2009). They also produce different proteins like metallothioneins that bind heavy metals thereby reducing HMs toxicity (Wu et al., 2010). Further, EPS produced by microbes is a mixture of proteins, nucleic acid, and polysaccharides that find metals and reduce their concentration in the surrounding environment. Different mechanisms including electrostatic interaction, ion exchange, precipitation, redox process, and surface complexation are involved in processes (Yang et al., 2015). The enzymes transfer metals into less toxic forms in cells through oxidation, reduction, complexation, sequestration, methylation, and de-methylation. Different enzymes like arsenite oxidase, mercuric reductase, chromate reductase, and nickel-coenzyme m reductase have been identified to convert the metals Lerebours et al., 2016; Zammit et al., 2016).

Microbial mechanism used clean up HMs polluted soils

Different mechanisms were used by microbes to clean up the HM-polluted soils. Microbes play a critical role in the oxidation of metals, for instance, *Thiobacillus ferrooxidans*) can promote the oxidation of metal sulfides to enhance the release of HMs. Microbes mediate the transformation of metal sulfides by sulfur oxidation. In this process, microbes oxidize sulfide ores into metal ions by the process of biological leaching (Kaksonen et al., 2020). Microbes also cause the reduction of metals to reduce their toxicity. The removal capacity of HM-nFeS against Cr-VI was 12-20% lowest as compared to DM-nFeS which was linked with the capacity of both HM-nFeS and DM-nFeS to reduce the Cr (Du et al., 2016). The details of various mechanisms used by microbes to remediate the HMs polluted soils are discussed below.

Bioaccumulation and biosorption

Bioaccumulation and biosorption are the most common mechanisms used by microbes to remediate polluted soils and in both mechanisms, microbes bound the HMs from the surrounding environment (Joutey et al., 2015). In bio-sorption microbes use cellular structure to capture the HM ions and then absorb these HMs on the binding sites of cell walls (Malik, 2004). Microbes also used adsorption mechanisms as bioremediation of HM. Different microbes including *Magnetospirillum gryphiswaldense*, *Bacillus subtilis*, microalgae, *Chaetomorphalinum*, *Rhizopus arrhizus*, and *Saccharomyces cerevisiae* produce biosorbents for remediation of HM (Zhou et al., 2012). In comparison to other microbes, bacteria are thought to be superior bio-sorbents because of their larger surface-to-volume ratio and variety of chemosorption sites in their cell walls, including teichoic acid (Beveridge, 1989). Dead bacterial strains also have good biosorbent properties and it has been found that dead *Bacillus sphaericus* showed 13-20% more bio-sorption capacity for Cr as compared to living cell of the same strain (Velásquez and Dussan, 2009). On the other hand, bio-accumulation depends on an import storage mechanism. This process is known as active bio-accumulation and it involves the movement of HM ions across the lipid bilayer of the cell membrane and into the cytoplasm or intracellular regions. The bioaccumulation of HM in bacterial membranes is mediated via a variety of ionic channels, carrier-mediated transports, permeation, and lipid permeation (Shahpiri and Mohammadzadeh, 2018). In literature, it has been well documented that microbes cause bioaccumulation of Pb, Ni, Hg, Cd, and Cr (Rani and Goel, 2009; Sher and Rehman, 2019; Naskar et al., 2020). Different researchers also identified the micro-bacterium that shows resistance to HMs. For instance, Henson et al. (2015) reported that Microbacterium sp. (Cr-K29) reduced the Cr-IV uptake by 88% while Pattanapipitpaisal et al. (2001) found that Microbacterium liquefaciens eliminated the Cr by 90-95%. These microbes use heavy metal ions in order to facilitate their metabolic activities or they also use enzymes produced by bacterial cells to detoxify ions of HMs (Kubrak et al., 2010).

Bioleaching

Bioleaching is another important mechanism used by a wide range of microbes to remediate polluted soils. For instance, in a research study, authors found that *Acidophiles* and *chemolithotrophs* oxidized the Fe-II to Fe-III and reduced sulfur to sulfuric acid. The production of sulfuric acid leads to the synthesis of ferric ions as well as protons which helps to extract metals through solubilizing oxides and sulfides of metal (Srichandan et al., 2014). Microbes are utilized in bioleaching as reduction agents, but they can also be used to extract and recover HMs (Wang and Zhao, 2009). Bio-remediation has been offered as an excellent tool to recover raw materials from effluents (Gadd, 2010). Using an Annona squamosa-based absorbent with 0.1 M HCl, Isaac and Sivakumar (2013) achieved Cd recovery efficiency of 98.7%. Contrarily, matrix-immobilized P. putida cells demonstrated 100% recovery for Cu while Pseudomonas aeruginosa biomass demonstrated 82% recovery efficiency for Cd (Hammaini et al., 2007). In another study, co-application *Pseudomonas aeruginosa* biomass, and hydrochloric acid (0.1 M HCl) achieved the Cd recovery rate by 82% (Dickerhof et al., 2019), while*P. putida* achieved a Cu recovery rate of 100%. Further, autochthonous variant *Enterobacter* brought an exceeding recovery of >90% for Cu and Pb (Bayramoglu and Arica, 2011). Acidphiles produce different acids through their metabolic process which aids in the dissolution of metal ores, thereby reducing the availability of metals. On the other hand, chemolithotrophs cause oxidation and reduction of sulfur compounds which provide energy to them and also increase the production of acids subsequently increasing solubilization of metals. The bio-leaching carried by both acidophiles and chemolithotrophs is eco-friendly and it can be carried at lower temperatures along with additional benefits of energy saving (Adetunji et al., 2023).

Biotransformation

In biotransformation, microbes converted the toxic metal ions to less hazardous forms (Pervaiz et al., 2013). To adapt to environmental changes, bacteria have developed bio-transformation mechanisms. Production of carbon bonds, isomerization, functional groups, oxidation, reduction, condensation, hydrolysis, methylation, and demethylation help the microbes to transform the HMs. These are all processes that can be used to alter HM in microbes. Microbes cause the transformation of HM and Nagvenkar and Ramaiah (2010) noted that *Micrococcus*and *Acinetobacter* caused the oxidation of As-III into less soluble and non-toxic form. Moreover, Thatoi et al. (2014) documented that Cr (VI) tolerant *Bacillus*species cause the biotransformation of Cr (VI) and changed it into a less hazardous form of Cr (III). Both Micrococcus and Acinetobacter reduce the toxicity of metals by causing oxidation, reduction, biological

chelation, and inducing the metabolic transformation and bio-film formations (Adetunji et al., 2023).

Bio-volatilization

Bio-volatilization is a process where microbes convert the HMs into volatile compounds enzymatically. This process significantly reduced the availability and toxicity of metals in soil and water. Bio-volatilization uses enzymatic reduction and methylation to convert toxic metals into less toxic forms. Different enzymes like Arsenic methyltransferase, Mercury reductase, and Antimony methyltransferase are involved in the bio-volatilization of As, Hg, and Sb. This method is considered to be suitable for HMs like Hg, As, and Sb, and in this process, these HMs are converted into non-toxic compounds by bio-volatilization (Boriová et al., 2014). Bacterial enzymes like *methyltransferases* transfer the As(V) into the mono, di, and tri-methylated As species which is then transferred into the atmosphere owing to its volatile nature. In another study, enzymes like reductase (MerA) and mercurial lyase present in archaea and eubacteria caused bio-volatilization (Freedman et al., 2012). Similarly, *Scopulariopsis brevicaulis*, also showed promising results to convert the As(V) and Hg(II) to their nontoxic states (Urík et al., 2007; Boriová et al., 2014).

Bio-mineralization

In the bio-mineralization process, microbes activate the synthesis of minerals and microbes to tackle with HMs. Different bacteria cause immobilization of Pb and Cr by carbon mineralization (He et al., 2019). Similarly, another bacterial strain *Sporosarcina ginsengisoli* caused immobilization of different HM calcite, aragonite, and vaterite biomineralization (Achal et al., 2012; Cheng and Holman, 2012). Fungal species also showed promising results for bio-mineralization, for example, *Penicillium chrysogenum* causes mineralization of Pb and Cr (Qian et al., 2017). Likewise, *Penicillium chrysogenum* effectively causes bio-mineralization of Pb (Povedano-Priego et al., 2017) additionally, due to the synthesis of PO43- that is released during the breakdown of Pb, Bacillus subtilis triggered bio-mineralization of Pb (Lin et al., 2016). Moreover, other authors reported that *Pseudomonas putida* forms the carbonate and phosphate minerals which speed up Cd precipitation (Li et al., 2016). Microbes play a critical role in the bio-mineralization process as this process involves the production of mineral deposits to immobilize HMs. The microbes produce EPS, specific metabolites, and organic acids which promote the formation of mineral deposits thereby leading to the immobilization of HMs (Qian et al., 2017). The siderophores and polysaccharides produced by microbes bind the HMs by forming complexes with metals thereby reducing uptake and accumulation of metals by plants. Besides this, they also facilitate the sequestration of metals in soil thereby reducing toxicity of metals on plants.

Modern approaches used to remediate HMs contaminated soils

Different techniques are being applied globally to clean up HM-polluted soils. The role of modern approaches to remediate HM-contaminated soils is discussed below.

Phyto-microbial system for remediation of polluted soils

The application of plants and microbes has emerged as an excellent tool to remediate HM-polluted soils. The use of PGPR has been tested as an effective, and environmentally friendly way to eliminate HMs (Sati et al., 2023). Different microbes like bacteria and fungi can help the plants absorb the HMs (Bojórquez et al., 2016). For instance, Joner and Leyval (1997) noted that fungal inoculated plants uptake more Cd by 90, 127, and 131% growing under different Cd levels (1, 10, and 100 mg/kg) as compared to un-inoculated plants. Similarly, fungal inoculation improves the plant's ability to absorb Cu, Cd, and Zn (Sati et al., 2023). Different PGPB also produce polysaccharides which increase the transformation, immobilization, and chelation of HM thus reducing their availability. PGPB decreases soil pH by increasing the production of organic acids which helps to remove the HM ions, further, these PGPB also provide nutrients to plants thus reducing the negative effects of HM on plants. Siderophore is also an important microbe and it has shown an appreciable ability to form complexes with different metals like Al, Cd, Cu, Zn, and Pb (Rajkumar et al., 2010). When bio-augmentation and phytoremediation are used together, they produce noticeable results and can also get around some of the challenges that arise with using them alone. The plant also showed significant results to remediate polluted soils and according to Wang et al. (2021), planting Salix in soils with Cd contamination increased the diversity of helpful fungi and microorganisms and contributed to impressive bioremediation outcomes. Plant growth-promoting rhizobacteria (PGPR) interact with plants to increase their ability to absorb HMs through a different mechanism like the production of chelators, increased nutrient uptake, volatilization, transformation, and phytostabilization. This technique is considered sustainable and eco-friendly which can help to mitigate the HMs pollution in agricultural settings.

Genetically engineered microbes: key player to remediated HM polluted soils

The recent advancements in genetic engineering and the production of genetically modified microbes have shown promising results for the remediation of polluted soils. Molecular biology involves understanding and changing the genes to improve the bio-remediation process. It has been documented that different microbes possess resistance mechanisms against HMs (Jaiswal et al., 2019). This includes genes that encode different metal proteins,

transporters, and enzymes involved the detoxification (Malla et al., 2018). Thus, engineering these genes can allow for an increase in the microbial ability to effectively carry the microbial remediation process. The recent advance in CRISPR-Cas9 also ensured the editing of microbial genes and the introduction of new genes resulting in improved performance of microbes against HMs (Lee and Lee, 2021). The microbial metabolic pathways can also be modified which can enhance the microbe's ability, while fine-tuning genes is also leading to better remediation capabilities. Moreover, omics and microbial consortia engineering also provided insights into the response of microbes to HMs (Peña-Castro et al., 2023). This can help to identify different genes, regulatory pathways, and elements to improve the remediation process (Peña-Castro et al., 2023).

The literature shows that genetically modified microbes have a better capacity to remove the HMs (Bhatt et al., 2022). The editing of a single gene and changing the sequence of the gene are important practices used to produce genetically modified microbes (Diep et al., 2018). Different HMs like Cd, Cu, Hg, Ni, and Fe are eliminated by engineered bacteria (Azad et al., 2014) however, the degradation rate largely depends on enzymes present in bacterial cells (Kang, 2014). Moreover, the use of recombinant DNA technology and the introduction of foreign genes has also allowed to develop the genetically modified microbes. For instance, the use of genetically modified *Pseudomonas putida* and *Escherichia coli*e effectively removed the Hg from polluted soils (Deckwer et al., 2004), similarly, the addition of mer operon from *Escherichia coli* to bacterium *Deinococcus geothemalis* also reduce the Hg pollution even at higher temperature (Dixit et al., 2015).

Cupriavidus metallidurans modified genetically with pTP6 plasmid also significantly reduced the Hg from polluted soils (Dixit et al., 2015). The use pMR68 plasmid to introduce novel genes into Pseudomonas also led to the development of Hg resistance (Sone et al., 2013). To enhance the bioremediation of HM, microbial membrane transporters can also be genetically engineered and in this context, transporters and binding mechanisms play a critical role to remediate polluted soils (Manoj et al., 2020). When HMs enter the cell, several phytochelatins, metallothioneins, and polyphosphates collaborate to sequester the HM and alter the HM key storage system, enhancing their ability to take HMs from soil and water (Diep et al., 2018).

The use of genetically modified microbes (GEMs) has speeded up the remediation process. For the successful implementation of implementation of GEMs bacteria must be capable of tolerating the antagonism induced by other native bacterial species (Dixit et al., 2015). Therefore, more novel approaches to screening as well as isolation of microbes for remediation of polluted soils must be used. Recently, different approaches like genomics, metagenomics, metabolomics, proteomics transcriptomics, and computational biology have been used to develop the GEMs for the remediation of HMs (Raza et al., 2024). The recent advancement in high throughput techniques has allowed us to identify the genes involved in the bio-remediation of diverse metals. Further, recent techniques like CRISPR-Cas also made it possible to create GEMs containing genes that can break down the HMs. Besides this, it also made it easy to transfer the desired set of information into microbial genomes to develop the microbes with better ability (Miglani, 2017).

CRISPR-Cas9 techniques have also allowed to development of microbes with appreciable precision, high efficiency, and targeting multiple metals. Genetically modified microbes can provide better results to remediate polluted soils. For instance, genetically modified microbes enhance metal uptake capacity, and they have better metal tolerance and resistance with minimal environmental impacts. They also have appreciable sequestration, transformation, detoxification, and uptake abilities which make them effective tools to mitigate metals toxicity. However, many potential ethical and environmental implications must be considered when using genetically modified microbes for the remediation of polluted soils. For instance, it includes proper regulation and monitoring of genetically modified organisms to balance the benefits of reduction in contamination along with potential risks. Other concerns could be human health, environmental quality, and the negative effects of farming practices. Many environmental considerations must be used while using genetically modified microbes. These microbes should not disrupt biodiversity, food webs, and ecosystem health.

Use of nano-technology for microbial remediation of HM polluted soils

Nano-materials have documented appreciable results in remediating polluted soils owing to their higher surface area, reactivity, and surface chemistry (Khati et al., 2017; Baragaño et al., 2020. Different types of nano-materials including zero-valent metals, metal oxide nanoparticles, carbon-based nano-materials, nano-composites, and nano-biosensors are used around the globe to remediate polluted soils (Aliyari et al., 2023). The nano-materials serve as electron donors in the microbial reduction process and they promote the reduction of toxic metals. On the other hand, nano-particles serve as absorbents and they also favor HMs degradation (Dhanapal et al., 2024). Further, carbon-based produces also enhance the transfer of electrons among metal ions and microbes which in turn increases the efficiency of bio-remediation. Recently, nano-biosensors have also shown appreciable results in detecting HMS which has allowed the monitoring of the remediation process (Dhanapal et al., 2024). Nano-biosorbents can be employed as a substitute for conventional bio-sorbents (Alviz-Gazitua et al., 2019). There are various functional groups found in NPs, including NH2, -COOH, and -OH, and customizing the right functional groups by activating them physically or chemically or by altering their surfaces has produced promising results for the elimination of HMs. Additionally, bacterial strains produce the NPs that can aid in the bio-remediation of the HMs (Arshad et al., 2019). It has been shown that using NPs in conjunction with microorganisms boosted the reduction of HMs, producing more beneficial benefits than using them alone. Nano-particles have a higher surface area, ion exchange, reduction and stabilization capacity, mobility, and delivery which enhance the remediation efficacy. The interaction between NPs and microorganisms is, however, influenced by a variety of factors,

including NPs' chemical properties, size and shape, coating qualities, crystalline phase, level of contamination, and resistance to hazardous elements (Tan et al., 2018). Their tailored properties and enhanced adsorption capacities make them promising candidates for sustainable and efficient remediation strategies, provided that environmental and safety considerations are carefully addressed in their application. However, nano-sorbents must be tested for their environmental impacts in terms of stability and NPs release into the environment. Microbes trapped with nano-materials produce the nano-composite, a combination of *Halomonas*and iron oxide NPs substantially eliminated the Cd-II and Pb-II (Cao et al., 2020). Since, separation and recovery of HMs from nano-materials is laborious and time-consuming thus magnetic NPs gained significant attention in recent times, wherein surface amendment, coating of diverse materials, and encapsulation focused on simple separation of HMs.

Conclusion and future research directions

Heavy metals pollution is a serious issue across the globe and it is considered the biggest challenge of this century. Heavy metal pollution has drastic effects on soil quality, soil fertility, microbial activities, and diversity, and it also impost deleterious impacts on human health by entering the food chain. Globally, different physiochemical strategies are used to remediate the HMs polluted soils. However, these strategies are very expensive, difficult to application, inefficient in certain conditions and they can also alter the soil quality. Therefore, new biological methods have been developed to remediate polluted soils. Among biological methods, the use of microbes is considered as an effective, economical, and eco-feasible measure to remediate polluted soils. The microbes use different mechanisms to remediate polluted soils and recently engineered microbes provided excellent results for bioremediation which makes them an effective measure to be used on polluted soils.

The use of a single strategy could be both noneffective and inefficient in reclaiming polluted soils. Therefore, a combination of microbes and plants, nano-particles, and additives could also be an important approach to remediate polluted soil. Moreover, a combination of microbes with other strategies including organic and carbon-based materials must also be tested. Additionally, to create the HM tolerance in microbes more focus must be done to understand the physiochemical, biological, and molecular characteristics of microorganisms in soil and water habitats where HMs are prevalent. In the literature, no studies are available about the long-term effects of altering soil pH, temperature, and redox conditions for bioremediation efforts on soil health, microbial diversity, and the persistence of heavy metals over a long period. Therefore, efforts must be made to study the long-term impacts of soil pH, temperature, and redox conditions on on soil health, microbial diversity, and the persistence of heavy metals. Besides this, there is also a lack of information about the interactions between metal concentration, pH, redox potential, and temperature affecting the efficiency and effectiveness of microbial bioremediation processes in contaminated soils. Thus, it is interesting to study the interactions between metal concentration, pH, redox potential, and temperature affecting the effectiveness of the remediation process.

To identify prospective metal resistance and detoxification genes that can be regulated in other species to improve their particular performance, meta-genomic techniques, and microbial metabolic studies are required. Additionally, genetic study is required to comprehend the routes and mechanisms that plants and microorganisms use to tolerate and detoxify heavy metals. The recent advance in omics-based approach can also help to develop the strains tolerant against the prevalent environmental conditions. Recently, yeast has been modified and it showed promising hyper-accumulation capacity, therefore, other bacteria can also be developed in the same way to clean the polluted soils. Future research should pay more attention to the usage of algae since it may be a promising strategy for the sorption of heavy metals. The application of nanotechnology in combination with microbes can also promote microbial use and their efficiency on polluted soil.

Author contributions

HT: Writing – original draft. GX: Writing – original draft, Writing – review & editing. WX: Writing – review & editing. ZY: Writing – review & editing. BZ: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported and funded by National Natural Science Foundation of China (32060318); Hunan Provincial Natural Science Foundation of China (2023JJ50474, 2023JJ50475); 2023 Industry-University Collaborative Education Project of the Higher Education Department of the Ministry of Education (230801720230445). 2023 Hunan University Students Innovation and Entrepreneurship Training Program (S202310553008). 2024 Ministry of Education Supply and Demand docking Employment Education Project: Research on Employment-oriented Practical Teaching Model Reform of Plant Production Courses (2024011153277).

Conflict of interest

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

Publisher's note

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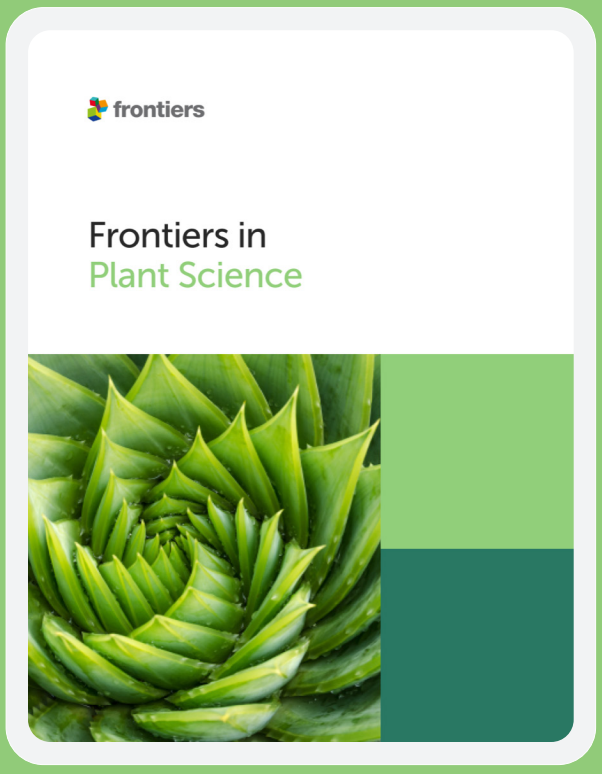

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