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Mineral solubilizing microorganisms (MSM) and their applications in nutrient bioavailability, bioweathering and bioremediation, volume III

Edited by
Muhammad Zahid Mumtaz, Maqshoof Ahmad and Adnan Mustafa

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Mineral solubilizing microorganisms (MSM) and their applications in nutrient bioavailability, bioweathering and bioremediation, volume III

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Editorial: Mineral solubilizing microorganisms (MSM) and their applications in nutrient bioavailability, bioweathering and bioremediation, volume III

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Editorial on the Research Topic
Mineral solubilizing microorganisms (MSM) and their applications in nutrient bioavailability, bioweathering and bioremediation, volume III

1 Introduction

Mineral-solubilizing microorganisms (MSM) are key drivers of mineral transformation in soil, and they play a pivotal role in nutrient cycling, environmental detoxification, and geochemical processes. These diverse microbial communities possess remarkable abilities to solubilize and mobilize essential macro- and micronutrients and improve nutrient availability in soil for plant uptake. They drive essential biogeochemical processes by releasing nutrients from insoluble mineral forms. Additionally, MSM are involved in the natural breakdown of rocks and minerals and offer ecologically friendly remediation of the contaminated environment through metal chelation, acidification, and redox transformation. Vol III of our Research Topic series highlights cutting-edge insights into the mechanisms and ecological significance of MSM, exploring their potential applications in sustainable agriculture, soil fertility enhancement, ecosystem restoration, and remediation technologies.

2 MSM-mediated nutrient bioavailability

Microorganisms are an integral part of soil biogeochemical cycles involved in promoting soil fertility and the transformation of minerals. MSM have emerged as key microbial agents in agricultural ecosystems due to their ability to transform insoluble

minerals into bioavailable forms. They performed extensive functional roles that promote nutrient uptake in plants by catalyzing mineral weathering and the solubilization of minerals. This editorial compiled the research published in Vol III of the Research Topic by discussing various studies that explore diverse interactions between MSM taxa with minerals and plants and drive critical transformations of minerals. The functional role of MSM in enhancing nutrient bioavailability is a rapidly growing area of research in soil microbiology. MSM are involved in the solubilization of silica and silicate minerals, which are gaining attention due to the role of silicon in plant stress tolerance and improving crop productivity. Lei et al. have conducted a bibliometric analysis of global research trends spanning from 1948 to 2024 in the application of phosphate-solubilizing microorganisms, which revealed a rapid growth in this field since 2018. Initially, the research focus was on the application of *Azospirillum brasilense* along with rock phosphate, which shifted toward alleviation of abiotic stresses, especially drought and salt stress, and improvement in crop productivity. This study recommends further exploration of phosphate-solubilizing microorganisms in improving nutrient availability, soil health, and mitigation of abiotic stresses to support sustainable agriculture.

A study by Zhang Y. et al. reports an enhancement in phosphorus-associated *Arthrobacter* sp. M4 and *Sordariomycetes* 2 MS-M4 activity in response to long-term application of swine manure. These microbial species converted available phosphorus into organic phosphorus under high carbon and phosphorus soil conditions through biological immobilization. Meanwhile, they decompose soil organic carbon and promote phosphorus concentration under limited carbon and phosphorus soil conditions by demonstrating their capacity to transform phosphorus. These phosphorus-associated microorganisms significantly promoted phosphorus availability in soil under long-term swine manure with NPK fertilizer. Similarly, Arunachalam et al. demonstrate the potential of plant growth-promoting *Serendipita indica* and vesicular arbuscular mycorrhizae in improving soil fertility, crop yield, and nutrient uptake in onion. They recommend the application of these microorganisms along with recommended chemical fertilizers to promote soil health, onion yield, and bulb quality. Further, Jalal-Ud-Din et al. report zinc solubilization by *Staphylococcus succinus* CLS1, *Priestia aryabhattai* CLS2, and *Priestia megaterium* CLS9 isolated from canola. These strains demonstrate *in vitro* N_2 fixation, production of indole acetic acid, hydrogen cyanide, exopolysaccharides, and siderophores. These strains promoted plant growth and yield attributes and oil contents in canola.

In a study by Maharjan et al., 24 rhizobacterial strains demonstrated silica solubilization and promoted host maize seedling growth. These strains upregulated antioxidant enzymes, including catalase, superoxide dismutase, peroxidase, polyphenol oxidase, and phenylalanine ammonia-lyase. These potent silica-solubilizing strains belonged to *Enterobacter* sp., *Klebsiella* sp., and *Serratia surfactantfaciens* and were recommended as a potential bioinoculation for the development of silicon-based development of biofertilizer. Luqman et al. applied a consortium of *Bacillus megaterium* ZR19, *Paenibacillus polymyxa* IA7, and *Bacillus* sp. IA16, along with recommended NPK and micronutrients in cotton, and reported the increase in antioxidant enzymes, root and shoot growth, and reproductive and yield traits of cotton under arid climate conditions. These microbial consortia demonstrated biocontrol ability against sooty mold and improved post-harvest soil biological and chemical properties. The synergistic potential of microbial consortia in soybeans under field conditions was studied by Rafique et al.. They inoculated a tripartite combination of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54 and *Piriformospora indica,* and reported a significant increase in germination rate, plant height, root nodulation, photosynthetic pigments, and leghemoglobin levels. These microbial consortia promoted nitrogen, phosphorus, and micronutrient accumulation in soybean grains and stover. Gato et al. report increasing grain and oil yields for castor beans through a consortium of *Azospirillum brasiliense*, *Bacillus subtilis*, and *Pseudomonas fluorescens*. This microbial consortium also promoted N uptake in shoots and grains of castor beans. Further, Wang et al. show a shift in microbial and chemical processes involved in weathering dynamics of purple parent rocks. They observe a reduction in pH and an increase in availability of nitrogen and phosphorus by high fertilizer application rate, which impacts weathering through modifying chemical properties and enriching microbial community structure, which ultimately accelerates the breakdown of purple parent rocks.

3 MSM-mediated bioweathering

MSM play a pivotal role in mineral bioweathering processes by transforming stable and geochemically resistant minerals into bioavailable forms for plant uptake. Bioweathering is a biologically driven transformation and dissolution of minerals by MSM, which are capable of mobilizing essential nutrients from insoluble mineral forms. This microbial bioweathering process takes place through microbial acidolysis, chelation, enzymatic degradation, and redox reactions that collectively break down primary and secondary mineral structures. Vol III of our Research Topic publishes work on mineral solubilization by MSM, including phosphate-solubilizing microorganisms (Arunachalam et al., Lei et al., Zhang Y. et al.), zinc-solubilizing rhizobacteria (Jalal-Ud-Din et al.), and silica-solubilizing rhizobacteria (Maharjan et al.), which play a pivotal role in natural rock weathering and soil genesis processes. The application of such MSM offers a sustainable biological tool for ecosystem restoration through mineral weathering in degraded geological substrates of nutrient-poor or extremely weathered landscapes. In bioweathering, the action of MSM is not only limited to nutrient solubilization, but these microorganisms also release the elements from polymineralic substrates. These microbial processes are central to nutrient dynamics in agroecosystems, especially under conditions of nutrient depletion or intensive cropping. The diversity and adaptability of MSM across various edaphic and climatic conditions highlight their value as biological agents for increasing soil nutrient cycling through bioweathering.

4 MSM-mediated bioremediation

Environmental contaminants, especially heavy metal pollution, continue to pose an ecological threat to both agricultural and

mining-impacted lands. Utilization of MSM for bioremediation is a cost-effective and sustainable remediation strategy. MSM indirectly support phytoremediation by extracting, stabilizing, and/or degrading environmental contaminants and promoting plant growth, nutrient acquisition, and stress tolerance. They detoxify soils, improve nutrient availability and plant health, and represent a paradigm shift from traditional remediation to microbe-mediated ecological restoration. Vol III of our Research Topic has published four articles addressing the contamination of lead, cadmium, and arsenic through the application of MSM. A study by Zhang C. et al. showcases insights into the bioremediation of lead and cadmium in phosphate mining wastelands through phosphate-solubilizing *Bacillus cereus* along with biochar. They reported an increase in the phosphorus availability and soil microbial communities, and a decrease in the extractable lead and cadmium concentration. The immobilization of lead and cadmium was caused by the main functional flora of *Janibacter*, *Lysobacter*, *Ornithinimicrobium*, *Bacillus*, and *Salinimicrobium*.

Gul et al. have explored recent advancements in lead remediation strategies through MSM microorganisms. They show the detoxification strategies of lead through biosorption, bioprecipitation, biomineralization, and bioaccumulation, emphasizing how microbes convert toxic lead into non-toxic or less mobile forms. Moreover, advances in genetic engineering have further equipped microbes with resistance traits, enabling their survival and remediation of lead in highly toxic environments. Shahid et al. have extended the cadmium remediation by applying *Klebsiella* strains with jasmonic acid in cauliflower. This biointegrated strategy not only reduces cadmium uptake in cauliflower roots and curds but also enhances plant growth, enzymatic defense mechanisms, and nutrient accumulation. The findings highlight the critical role of plant-microbe synergies in building resilience under metal stress. Similarly, Zaheer et al. explore the co-application of *Azospirillum brasilense* and seaweed extract to combat arsenic toxicity in wheat. This dual treatment improved nutrient availability and plant physiological attributes by reducing arsenic uptake in wheat. Such combinations highlight a promising future in integrating biofertilization and stress mitigation through MSMs and organic stimulants. These studies highlight the possible application of MSM in the alleviation of heavy metal stress and soil restoration. The application of microbial inoculants with soil amendments demonstrated a powerful, environmentally friendly solution to heavy metal contamination. Empowering soils with microbial inoculants is not just a remediation strategy, but it is a sustainable path toward ecological restoration and agricultural sustainability.

Author contributions

MM: Writing – review & editing, Conceptualization, Writing – original draft, Project administration. MA: Writing – review & editing. AM: Writing – review & editing.

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Optimizing plant growth, nutrient uptake, and yield of onion through the application of phosphorus solubilizing bacteria and endophytic fungi

Thangasamy Arunachalam[1]*, Komal Gade[1], Payal Arun Mahadule[1], P. S. Soumia[1], Venkadasamy Govindasamy[2], Suresh Janardhan Gawande[1] and Vijay Mahajan[1]

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Introduction: The application of mineral fertilizers deteriorates soil properties and affects crop yield and nutritional properties. However, plant growth-promoting microorganisms (PGPM- *Serendipita indica*, phosphorus solubilizing bacteria (PSB), and vesicular arbuscular mycorrhizae (VAM)) have great potential to reduce fertilizers and improve soil fertility, crop yield, and nutrient uptake and mitigate the environmental effect of mineral fertilizers.

Material and methods: Hence, a field experiment was conducted involving nine treatments to evaluate the effects of PGPM along with 50% or 100% of the recommended dose of fertilizers on plant growth, soil fertility, nutrient uptake, and onion productivity.

Results and discussion: Results indicated that 100% RDF combined with *S. indica* or PSB led to improved plant growth, and higher nutrient concentrations in both leaves and bulbs of onions compared to RDF alone. Moreover, the application of 100% RDF with *S. indica* increased total dry matter yield by 11.5% and 7.6% in the 2018-2019 and 2019-2020 seasons, respectively, compared to 100% RDF alone. This treatment also resulted in the highest nutrient uptake, with N uptake increasing by 6.9%-29.9%, P by 13.7%-21.7%, K by 20.0%-23.7%, and S by 18.1%-23.4%. Additionally, the combination of 100% RDF with *S. indica* inoculation led to a notable increase in bulb yield, with increments of 16.2% and 13.9% observed in 2018-2019 and 2019-2020, respectively, compared to 100% RDF alone. Similarly, the application of 100% RDF along with PSB inoculation resulted in an increase in bulb yield by 7.2% and 9.4% in the respective years. However, VAM did not exhibit satisfactory performance or improvements in the onion crop.

Conclusion: Overall, the study suggests that combining 100% RDF with *S. indica* or PSB can enhance onion productivity and nutrient use efficiency. The present study may open a new avenue of PGPM application in enhancing onion yield and improving the bulb quality as well as soil health. However, field trials across different regions and soil types are necessary to validate these findings for practical adoption by farmers.

KEYWORDS

onion yield, nutrient use efficiency, soil fertility, *Serendipita indica*, phosphate solubilizing bacteria, vesicular arbuscular mycorrhiza

Introduction

Onion (*Allium cepa* L.) is the 4th economically most important vegetable crop grown worldwide (Torquato-Tavares et al., 2017). India is the leading producer of onions, producing 31.68 million tons from a cultivated area of 1.94 million hectares (FAO, 2024). Despite substantial production, the country faces challenges in onion productivity, with a low yield of 16.32 t ha^{-1} compared to the global average of 18.53 t ha^{-1}. Among the many constraints for low productivity in onions, unbalanced nutrition is the main limiting factor. The indiscriminate use of fertilizers not only harms agricultural sustainability but also pollutes the environment (Jaiswal et al., 2022). Integrated Nutrient Management (INM) offers a comprehensive solution to address these imbalances, promoting soil health and maximizing crop yield by optimizing nutrient sources in an integrated approach (Qiu et al., 2023; Yang et al., 2024).

Historically, a blanket recommendation of 150:50:80:50 kg NPKS and 20 t farm yard manure (FYM) ha^{-1} was suggested to achieve a yield of 40–50 tons of onion bulbs per hectare. However, recent field experiments conducted at ICAR-DOGR demonstrated that applying 110:40:60:30 kg NPKS and 15 t FYM ha^{-1} produced yields comparable to the previous recommendation (Thangasamy and Lawande, 2015). This highlights the potential for improving nutrient management practices to enhance onion productivity.

Notably, nitrogen (N) applications, sourced from both organic and mineral fertilizers, typically range from 175–185 kg ha^{-1}, whereas the crop's actual requirement for optimal yield is around 90–100 kg N ha^{-1} to produce 40–50 tons of onion bulbs (Thangasamy, 2016). Nutrient uptake rates vary with growth stages; N demand is the highest during seedling production and the vegetative growth phase (Padhan et al., 2023; Abou Fayssal et al., 2024). Consequently, excess N applied can lead to leaching, denitrification, and increased vulnerability to pests and diseases (Sekara et al., 2017). Thus, significant scope exists for enhancing nutrient use efficiency in onion cultivation by aligning fertilizer application with crop demand. However, onions, being shallow-rooted and heavy-feeding crops, often face challenges in nutrient uptake, especially when nutrients leach beyond the root zone, rendering them unavailable to the plants (Thangasamy, 2016). To address this issue, applying fertilizer nutrients in reduced amount directly to the root zone or via microbial inoculation has shown promise in enhancing nutrient use efficiency (Shahwar et al., 2023).

The application of plant growth-promoting rhizobacteria, endophytic fungi, or vesicular arbuscular mycorrhiza (VAM) along with mineral fertilizers enhances plant growth and crop yield through phytohormone secretion, nutrient supplementation, and pathogen suppression (Zhang et al., 2021; Yu et al., 2024). Mycorrhizal inoculants such as *Glomus mosseae* have been found to enhance growth and yield, particularly in phosphorus (P)-deficient soil conditions (Chen et al., 2017). Given the shallow root system of onions, inoculation with these endophytic fungi or VAM may enhance P uptake (Augé and Moore, 2005). The hyphae of these microbes serve as extensions of the roots, aiding in the extraction of water and nutrients from beyond root zones as well as from the deeper soil layers, thereby increasing nutrient uptake and crop yield (Bucking et al., 2012). Furthermore, phosphorus-solubilizing bacteria (PSB) play a crucial role in converting fixed or unavailable nutrients into forms that are readily available to plants (Wang et al., 2024; Xu et al., 2024). A recent study by Novello et al. (2021) reported that the inoculation with PSB resulted in increased plant growth and nutrient concentration in onions. Likewise, *Serendipita indica (S. indica)* is a beneficial endosymbiont known for enhancing plant growth, development, induction of stress tolerance, and nutrient acquisition through different modes of action (Gill et al., 2016; Saleem et al., 2022; Roylawar et al., 2023).

To date, several studies have focused on selecting the suitable microorganisms for establishing successful symbiotic relationships in various environmental conditions and farming systems (Bolandnazar, 2009; Albrechtova et al., 2012; Caruso et al., 2018). Furthermore, numerous studies have focused on the implications of these ecological associations on onion plant performance, particularly concerning bulb yield and quality (Shinde and Shinde, 2016; Fredotovic and Puizina, 2019; Petrovic et al., 2020). Despite the potential benefits, *S. indica* has not been widely used as a biofertilizer in onion cultivation, while VAM and PSB have yielded variable results.

Therefore, the following hypotheses were formulated: 1. The application of mineral fertilizers along with PGPM will enhance onion plant growth, yield, and nutrient uptake compared to the use of mineral fertilizers alone, and 2. The combined use of PGPM and mineral fertilizers will result in better post-harvest soil fertility, maintaining or improving soil nutrient levels and health compared to the use of mineral fertilizers alone. To test these hypotheses, a field experiment was conducted with the objective to evaluate the effect of different fertilizer levels and PGPM (such as *Serendipita indica*, *Bacillus megaterium*, *Paenibacillus polymyxa*, *Bacillus* sp., *Glomus fasciculum*, *G. intraradices*, *Aculospora* sp., and *Gigaspora* sp.) inoculations on plant growth parameters, onion yield, nutrient uptake, and post-harvest soil fertility to provide insights for improving onion productivity.

Materials and methods

Experimental site

A two-year field experiment during 2018–2019 and 2019–2020 was conducted at the experimental farm of the Indian Council of Agricultural Research – Directorate of Onion and Garlic Research (ICAR–DOGR) in Pune, Maharashtra, India. The experimental site was situated at coordinates 18.32° N and 73.51° E, at an elevation of 645 meters above mean sea level (MSL). The climatic conditions of the experimental site were characterized by a tropical, dry humid climate with a mean annual precipitation of 820 mm. Throughout the cultivation period, the maximum air temperature ranged from 28.9 to 36.8°C, while the minimum air temperature varied from 9.7 to 17.2°C. The soils in the experimental field were classified as clay loam, and the initial soil analysis conducted indicated low available N and medium soil organic carbon status (Table 1).

Experimental details

The field experiment was designed using a completely randomized block design with nine treatments. The treatments included: T1: Control (without fertilizers), T2: 50% recommended dose of fertilizer (RDF) + PSB consortia (PSB), T3: 50% RDF + VAM consortia (VAM), T4: 50% RDF + *S. indica*, T5: 50% RDF alone, T6: 100% RDF + PSB,

TABLE 1 Values of soil properties recorded pre-planting (mean of two years values with standard error).

Soil properties	Initial value
Soil pH	8.10 ± 0.11
Electrical conductivity (dS m^{-1})	0.18 ± 0.01
Soil organic carbon (g kg^{-1})	6.78 ± 0.05
Soil available N (mg kg^{-1})	96.1 ± 3.0
Soil available P (mg kg^{-1})	8.99 ± 0.39
Soil available K (mg kg^{-1})	212.7 ± 6.7
Soil available S (mg kg^{-1})	7.40 ± 0.22

T7: 100% RDF + VAM, T8: 100% RDF + *S. indica*, T9: 100% RDF alone. The PSB consortia consisted of *Bacillus megaterium*, *Paenibacillus polymyxa,* and other *Bacillus* sp., while the VAM consortia consisted of *Glomus fasciculum*, *G. intraradices, Acaulospora* sp., and *Gigaspora* sp. Each treatment was replicated three times. Onion cv. Bhima Shakti was sown in the nursery during the second week of October in both years. Simultaneously, the main field was prepared by ploughing using the mold board plough and tilled using the cultivator. Raised beds of 1.2 m in width and 14 m in length were prepared after pulverizing the soil with a rotavator. Organic manures were applied at a rate of 5 t ha^{-1} to all treatments except the control plot. The pre-emergence herbicide oxyfluorfen was applied 7 days before transplanting to control weeds, followed by irrigation. Before transplanting, 100% of the required phosphorus (P), potassium (K), and sulfur (S), along with 20% of nitrogen (N), were applied as basal fertilizer. Mineral fertilizers, including 10:26:26, muriate of potash, and bentonite S, were used to supply N, P, K, and S. Forty-five-day-old seedlings were transplanted at a spacing of 15 cm between rows and 10 cm between plants during the third week of December in both years. The plot size for each treatment was 16.8 m^2. Before transplanting, a slurry of *S. indica*, PSB, and VAM was prepared, and seedling roots were immersed in the slurry for 2 h before transplanting. After treatment, the slurry with microbes was applied to the respective plots. The remaining 80% of N was applied through urea in three equal splits at 15, 30, and 45 days after transplanting (DAT). Irrigation water was applied as required through the drip system. Weeds were manually removed at 45 DAT, and all other intercultural operations and plant protection measures were carried out at timely intervals as per the ICAR-DOGR standard package of practices. Twenty-four plants were labeled and measured for plant growth parameters, including plant height and number of leaves, at 30 and 45 DAT in each plot. Additionally, twenty-four fully matured leaves were collected from each plot to determine the leaf area index. Onion bulbs were harvested in the second week of April after the crop exhibited 50% of the top fall. Three days after field curing, the bulbs were separated, leaving a 2.5 cm neck, and the bulb yield was recorded and expressed in tonnes per hectare (t ha^{-1}).

Soil sampling and analysis

Soil samples were collected from all treatments at a depth of 0–30 cm after harvesting. These soil samples were processed and sieved using a 2.0 mm sieve before being used for soil analysis. Standard protocols were followed to analyze soil pH, electrical conductivity, soil organic carbon, and the concentrations of available N, P, K, and S. A soil water suspension with a ratio of 1:2 was prepared, and soil pH and electrical conductivity were measured using a pH meter and conductivity bridge, respectively. Soil organic carbon (SOC) was determined using the wet-oxidation method described by Walkley and Black (1934). The available soil N was estimated using the alkaline permanganate method, P by using Olsen's method, K by the 1 N ammonium acetate method, and S by the 0.15 M $CaCl_2$ extraction method (Jackson, 1967).

Plant sampling and analysis

Twenty-four plant samples (whole plants) were collected from each treatment at the time of harvest. These samples were thoroughly washed and rinsed with distilled water. The bulbs and leaves were then separated, chopped into pieces, and air-dried. Once air-dried, the bulb and leaf samples were further over-dried in an oven at 58°C until a constant weight was reached. After reaching a constant weight, the dry weight of both the bulbs and leaves was recorded. Subsequently, the leaf and bulb samples were ground, passed through a 2.0 mm sieve, and used for plant nutrient analysis. Total N was analyzed using the micro-Kjeldahl method. To estimate total P, K, and S (S), 0.5 g of plant samples were digested using di-acid. Following digestion, the digest was thoroughly washed with distilled water and filtered through Whatman Number 40 filter paper. Subsequently, the filtrate was used for total P, K, and S analyses. Total P was determined using the ammonium vanado-molybdate method, total K using the flame photometer method, and total S using the turbidimetric method (Jackson, 1967). The nutrient recovery efficiency (%), which represents the quantity of nutrient absorbed per unit of nutrient applied, was computed using the formula: Nutrient recovery efficiency (%) = $(U_n - U_0)/n \times 100$, where U_n represents the nutrient uptake by the crop with the application of N, P, K, and S fertilizers, U_0 signifies the nutrient uptake by the crop without fertilization, and n stands for the quantity of fertilizer applied (Dobermann, 2007).

Statistical analysis

Plant growth parameters, nutrient concentrations, onion yield, dry matter accumulation, nutrient uptake, and nutrient recovery efficiency data were analyzed using the two-way ANOVA in R software version 4.3.3 (R Core Team, 2024). A *post hoc* analysis with the least significant difference was conducted to compare means following the ANOVA. Subsequently, Pearson's correlation coefficient was calculated to assess the associations between various traits. Furthermore, principal component analysis (PCA) and biplot PCA were employed to elucidate the relationships among treatments and parameters. These analyses were used to illustrate the interrelationships among the tested treatments based on different parameters.

Results

Plant growth parameters

The fertilizer treatments and the year of cultivation did not result in a significant increase in the number of leaves (Table 2). However, both the number of leaves and plant height increased with the

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progression of crop age from 30 to 60 DAT. Specifically, the number of leaves and plant height increased by 17.4 to 28.8% and 21.2 to 25.6%, respectively, compared to values recorded at 30 DAT. Notably, during the 2019–2020 season, the fertilizer treatments resulted in significantly higher plant height compared to the control treatment. Although there were no differences with statistical significance among the fertilizer treatments, the combination of 100% RDF with *S. indica* led to the highest plant height compared to other treatments. The increase in plant height ranged from 5.4 to 5.6%, compared to 100% RDF without microbial inoculation. Additionally, across all treatments, the highest values for the number of leaves and plant height were observed in the year 2019–2020 compared to 2018–2019.

All microbial inoculation treatments exhibited a significant increase in leaf area index at both 30 and 60 DAT during the year 2019–2020. Specifically, inoculation with *S. indica* at either 50% or 100% RDF led to a significant increase in leaf area index at both 30 and 60 DAT. The application of 100% RDF, whether with or without microbial inoculation, resulted in a significant increase in leaf area index at 60 DAT, surpassing that of the 50% RDF treatments, with or without microbial inoculation, as well as the control treatment. Although the leaf area index was statistically comparable to that of 100% RDF, the treatment receiving 100% RDF with *S. indica* inoculation exhibited a 7.4% increase compared to 100% RDF alone. Additionally, the roots of onion plants were examined for colonization at 45 DAT using staining techniques, confirming the presence of *S. indica* colonization.

Nutrient concentrations

Leaves generally exhibited higher concentrations of K and lower concentrations of N, P, and S compared to bulbs in both experimental years (Table 3). The fertilizer treatments significantly influenced the concentrations of N, P, K, and S in both leaves and bulbs. The treatment receiving 100% RDF, either alone or in combination with *S. indica* or PSB inoculation, recorded the highest concentrations of N, P, K, and S in both leaves and bulbs. Specifically, the application of 100% RDF along with *S. indica* increased N concentrations in leaves by 7.3–19.5%, P by 42.9–43.8%, K by 1.8–2.8%, and S by 6.7%. In bulbs, it increased N concentrations by 22.9%, P by 8.6%, K by 12.5–12.8%, and S by 8.9–10.6% compared to 100% RDF alone. These values were notably higher than those observed in the treatments receiving 50% RDF alone or in combination with PSB, *S. indica*, or VAM inoculation, as well as the control. In terms of yearly variations, concentrations of N, P, K, and S in bulbs and N and K in leaves were higher in the year 2018–2019 compared to the values recorded in the year 2019–2020. Conversely, P and S concentrations in leaves were higher in 2019–2020 compared to those observed in 2018–2019.

Nutrient uptake

The application of 100% RDF in combination with microbial inoculation significantly increased the total N, P, K, and S uptake compared to treatments involving 50% RDF with or without microbial inoculation and the absolute control in both experimental years (Table 4). The highest uptake of N, P, K, and S was observed in the treatment receiving 100% RDF along with *S. indica* in both years, closely followed by the treatments with 100% RDF along with PSB. The application of 100% RDF with *S. indica* led to the highest N, P, K, and S uptake, showing an increase of 6.9–29.9% for N, 13.7–21.7% for P, 20.0–23.7% for K, and 18.1–23.4% for S compared to the treatment with 100% RDF alone. Similarly, the application of 100% RDF with PSB inoculation increased N, P, K, and S uptake by 4.0–28.1%, 8.3–19.6%, 17.6–22.9%, and 7.0–31.4%, respectively, compared to the treatment with 100% RDF alone. The highest mean total N and K uptake was recorded in the year 2018–2019, whereas the highest mean total P and S uptake was recorded in the year

TABLE 2 Effect of mineral fertilizers and microbial inoculations on plant growth parameters.

Treatment	Number of leaves				Plant height (cm)				Leaf area index	
	2019–20		2020–21		2019–20		2020–21		2020–21	
	30 DAT	60 DAT	30 DAT	60 DAT	30 DAT	60 DAT	30 DAT	60 DAT	30 DAT	60 DAT
Control	6.0 ^{a}	7.7 ^{a}	6.3 ^{a}	7.3 ^{a}	41.3 ^{a}	50.6 ^{a}	38.0 ^{a}	44.6 ^{b}	0.53 ^{c}	1.46 ^{c}
50% RDF + PSB	6.0 ^{a}	7.3 ^{a}	7.0 ^{a}	8.0 ^{a}	42.8 ^{a}	51.8 ^{a}	48.9 ^{a}	56.5 ^{a}	0.64 ^{ab}	1.77 ^{b}
50% RDF + VAM	5.7 ^{a}	7.0 ^{a}	7.0 ^{a}	7.7 ^{a}	43.1 ^{a}	54.5 ^{a}	46.0 ^{a}	54.3 ^{a}	0.62 ^{ab}	1.72 ^{b}
50% RDF + *S. indica*	5.7 ^{a}	7.7 ^{a}	6.7 ^{a}	8.0 ^{a}	44.8 ^{a}	54.3 ^{a}	48.0 ^{a}	56.7 ^{a}	0.66 ^{a}	1.77 ^{b}
50% RDF	5.7 ^{a}	7.3 ^{a}	7.0 ^{a}	8.3 ^{a}	41.3 ^{a}	51.2 ^{a}	47.7 ^{a}	54.3 ^{a}	0.56 ^{bc}	1.70 ^{bc}
100% RDF + PSB	6.0 ^{a}	8.0 ^{a}	7.0 ^{a}	8.3 ^{a}	44.4 ^{a}	55.1 ^{a}	43.9 ^{a}	57.4 ^{a}	0.65 ^{a}	2.12 ^{a}
100% RDF + VAM	6.0 ^{a}	7.7 ^{a}	6.7 ^{a}	8.7 ^{a}	44.2 ^{a}	56.9 ^{a}	45.0 ^{a}	57.4 ^{a}	0.59 ^{abc}	2.05 ^{a}
100% RDF + *S. indica*	6.0 ^{a}	8.0 ^{a}	7.0 ^{a}	8.3 ^{a}	45.0 ^{a}	57.8 ^{a}	47.2 ^{a}	60.2 ^{a}	0.65 ^{a}	2.17 ^{a}
100% RDF	5.7 ^{a}	7.3 ^{a}	7.0 ^{a}	8.3	44.1 ^{a}	58.8 ^{a}	44.8 ^{a}	57.0 ^{a}	0.64 ^{ab}	2.02 ^{a}
Mean	5.9	7.6	6.9	8.1	43.4	54.5	45.5	55.4	0.62	1.87
SEM±	0.2	0.3	0.2	0.4	1.5	2.2	2.1	1.4	0.02	0.05
CV%	6.3	5.8	4.8	7.4	5.9	7.0	7.8	4.4	4.69	4.55
$p = 0.05$	0.70	0.17	0.19	0.28	0.54	0.16	0.06	<0.0001	<0.0001	<0.0001

Values with different letters in the same year indicate significant differences ($p < 0.05$) as assessed using the least significant difference (LSD) test.

TABLE 3 Effect of mineral fertilizer and microbial inoculation on nutrient concentration.

Treatments	Nitrogen concentration (%)				Phosphorus concentration (%)				Potassium concentration (%)				Sulphur concentration (%)			
	2018–19		2019–20		2018–19		2019–20		2018–19		2019–20		2018–19		2019–20	
	Leaves	Bulbs	Leaves	Bulbs	Leaves	Bulbs	Leaves	Bulbs	Leaves	Bulbs	Leaves	Bulbs	Leaves	Bulbs	Leaves	Bulbs
Control	1.02 ^{e}	1.18 ^{d}	0.44 ^{d}	0.72 ^{b}	0.09 ^{a}	0.29 ^{c}	0.34 ^{cd}	0.23 ^{de}	1.55 ^{bc}	1.01 ^{c}	1.30 ^{a}	0.87 ^{a}	0.21 ^{a}	0.44 ^{ab}	0.33 ^{c}	0.32 ^{b}
50% RDF + PSB	1.31 ^{bc}	1.93 ^{ab}	1.12 ^{bc}	1.32 ^{a}	0.09 ^{a}	0.36 ^{ab}	0.35 ^{cd}	0.26 ^{de}	1.60 ^{abc}	1.39 ^{ab}	1.08 ^{b}	0.78 ^{abc}	0.23 ^{a}	0.48 ^{ab}	0.57 ^{a}	0.47 ^{a}
50% RDF + VAM	1.28 ^{c}	1.57 ^{c}	1.29 ^{a}	1.12 ^{ab}	0.07 ^{a}	0.34 ^{ab}	0.44 ^{abc}	0.30 ^{cd}	1.80 ^{a}	1.15 ^{bc}	1.03 ^{b}	0.73 ^{bc}	0.20 ^{a}	0.43 ^{b}	0.48 ^{ab}	0.50 ^{a}
50% RDF + *S. indica*	1.41 ^{ab}	1.83 ^{bc}	1.10 ^{bc}	1.07 ^{ab}	0.09 ^{a}	0.38 ^{ab}	0.43 ^{bc}	0.26 ^{de}	1.45 ^{c}	1.32 ^{ab}	1.03 ^{b}	0.73 ^{bc}	0.20 ^{a}	0.46 ^{ab}	0.53 ^{ab}	0.50 ^{a}
50% RDF	1.12 ^{de}	1.83 ^{bc}	0.98 ^{c}	1.05 ^{ab}	0.09 ^{a}	0.33 ^{bc}	0.26 ^{d}	0.22 ^{e}	1.52 ^{bc}	1.23 ^{abc}	0.99 ^{b}	0.69 ^{c}	0.22 ^{a}	0.41 ^{b}	0.45 ^{ab}	0.47 ^{a}
100% RDF + PSB	1.30 ^{bc}	2.02 ^{ab}	1.06 ^{bc}	1.29 ^{a}	0.06 ^{a}	0.37 ^{ab}	0.32 ^{d}	0.37 ^{abc}	1.62 ^{abc}	1.38 ^{ab}	1.17 ^{ab}	0.87 ^{a}	0.18 ^{a}	0.56 ^{a}	0.44 ^{bc}	0.43 ^{a}
100% RDF + VAM	1.29 ^{bc}	2.05 ^{ab}	1.08 ^{bc}	1.39 ^{a}	0.09 ^{a}	0.36 ^{ab}	0.53 ^{a}	0.43 ^{a}	1.67 ^{ab}	1.37 ^{ab}	1.12 ^{ab}	0.84 ^{ab}	0.12 ^{a}	0.47 ^{ab}	0.50 ^{ab}	0.44 ^{a}
100% RDF + *S. indica*	1.47 ^{a}	2.05 ^{ab}	1.17 ^{ab}	1.34 ^{a}	0.10 ^{a}	0.38 ^{a}	0.46 ^{ab}	0.38 ^{ab}	1.71 ^{ab}	1.44 ^{a}	1.11 ^{ab}	0.88 ^{a}	0.21 ^{a}	0.52 ^{ab}	0.48 ^{ab}	0.49 ^{a}
100% RDF	1.23 ^{cd}	2.20 ^{a}	1.09b ^{c}	1.09 ^{ab}	0.07 ^{a}	0.38 ^{a}	0.32 ^{d}	0.35 ^{bc}	1.68 ^{ab}	1.28 ^{abc}	1.08 ^{b}	0.78 ^{abc}	0.22a	0.47 ^{ab}	0.45 ^{ab}	0.45 ^{a}
Mean	1.27	1.85	1.04	1.16	0.08	0.35	0.38	0.31	1.62	1.29	1.1	0.8	0.2	0.47	0.47	0.45
SEM±	0.03	0.06	0.05	0.08	0.01	0.01	0.02	0.02	0.04	0.06	0.04	0.03	0.03	0.03	0.02	0.02
CV%	3.42	5.78	8.56	12.22	25.06	5.11	9.01	8.1	4.44	7.37	5.97	5.88	21.81	9.17	8.57	6.58
P = 0.05	<0.0001	<0.0001	<0.0001	0.001	0.477	<0.0001	<0.0001	<0.0001	0.001	0.001	0.001	0.001	0.168	0.018	<0.0001	<0.0001

Values with different letters in the same year indicate significant differences ($p < 0.05$) as assessed using the LSD test.

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TABLE 4 Effect of mineral fertilizer application and microbial inoculation on total nutrient uptake by onion.

Treatment	Total N uptake (kg ha^{-1})		Total P uptake (kg ha^{-1})		Total K uptake (kg ha^{-1})		Total S uptake (kg ha^{-1})	
	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20
Control	58.4 ^{c}	37.1 ^{c}	13.17 ^{d}	13.36 ^{d}	54.5 ^{e}	49.6d ^{e}	20.83 ^{c}	17.30 ^{c}
50% RDF + PSB	95.9 ^{b}	118.9 ^{ab}	16.22 ^{cd}	24.33 ^{c}	74.9 ^{bcd}	73.6 ^{bc}	22.80 ^{c}	44.10 ^{ab}
50% RDF + VAM	84.6 ^{b}	85.8 ^{b}	16.47 ^{bcd}	23.92 ^{c}	70.1 ^{d}	57.2 ^{de}	21.87 ^{c}	37.50 ^{b}
50% RDF + *S. indica*	99.9 ^{b}	96.8 ^{ab}	18.82 ^{abc}	24.45 ^{c}	75.8 ^{bcd}	67.9 ^{cd}	23.80 ^{c}	44.93 ^{ab}
50% RDF	93.8 ^{b}	89.1 ^{b}	16.00 ^{cd}	19.40 ^{d}	68.9 ^{de}	61.9 ^{cde}	20.83 ^{c}	40.33 ^{ab}
100% RDF + PSB	126.2 ^{a}	126.3 ^{a}	21.43 ^{a}	36.64 ^{a}	93.4 ^{ab}	89.7 ^{a}	33.47 ^{a}	42.93 ^{ab}
100% RDF + VAM	123.3 ^{a}	116.4 ^{ab}	20.54 ^{ab}	38.06 ^{a}	90.0 ^{abc}	74.4 ^{abc}	26.83 ^{bc}	38.33 ^{b}
100% RDF + *S. indica*	129.8 ^{a}	128.1 ^{a}	22.48 ^{a}	37.28 ^{a}	98.2 ^{a}	87.6 ^{ab}	31.43 ^{ab}	47.40 ^{a}
100% RDF	121.4 ^{a}	98.6 ^{ab}	19.78 ^{abc}	30.64 ^{b}	79.4 ^{bcd}	73.0 ^{bc}	25.47 ^{bc}	40.13 ^{ab}
Mean	103.4	96.7	18.32	27.56	78.3	70.5	25.26	39.22
SEM±	3.4	7.2	0.81	0.96	3.0	3.1	1.32	1.60
CV%	5.7	12.4	7.65	6.02	6.1	7.6	9.01	7.08
P = 0.05	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Values with different letters in the same year indicate significant differences ($p < 0.05$) as assessed using the LSD test.

TABLE 5 Influence of mineral fertilizers and microbial inoculation on nutrient recovery efficiency.

Treatments	N recovery efficiency (%)		P recovery efficiency (%)		P recovery efficiency (%)		S recovery efficiency (%)	
	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20
50% RDF + PSB	68.2 ^{a}	148.6 ^{a}	15.2 ^{b}	55.3 ^{a}	68.2 ^{a}	80.0 ^{a}	13.0 ^{bc}	178.5 ^{ab}
50% RDF + VAM	47.7 ^{b}	88.4b ^{cd}	16.5 ^{b}	53.3 ^{a}	52.2 ^{ab}	25.3 ^{c}	6.8 ^{c}	134.8 ^{c}
50% RDF + *S. indica*	75.4 ^{a}	108.4 ^{b}	28.2 ^{a}	55.9 ^{a}	71.0 ^{a}	61.1 ^{ab}	19.6 ^{abc}	184.4 ^{a}
50% RDF	64.4 ^{ab}	94.5 ^{bc}	14.1 ^{b}	30.7 ^{c}	48.0 ^{ab}	40.0 ^{bc}	−0.3 ^{c}	153.6 ^{bc}
100% RDF + PSB	61.7 ^{ab}	81.0 ^{bcd}	20.6 ^{ab}	58.4 ^{a}	64.8 ^{ab}	66.8 ^{a}	42.0 ^{a}	85.3 ^{de}
100% RDF + VAM	59.0 ^{ab}	72.1 ^{cd}	18.4 ^{ab}	62.0 ^{a}	59.1 ^{ab}	41.3 ^{bc}	19.9 ^{abc}	70.1 ^{e}
100% RDF + *S. indica*	64.9 ^{ab}	82.7 ^{bcd}	23.3 ^{ab}	60.1 ^{a}	72.9 ^{a}	63.3 ^{ab}	35.3 ^{ab}	100.4 ^{d}
100% RDF	57.3 ^{ab}	55.9 ^{d}	16.5 ^{b}	43.6 ^{b}	41.6 ^{b}	38.9 ^{bc}	15.4 ^{bc}	76.1 ^{de}

Values with different letters in the same year indicate significant differences ($p < 0.05$) as assessed using the LSD test.

2019–2020. N, P, K, and S recovery efficiency was notably higher in treatments inoculated with either *S. indica* or PSB compared to those treated with mineral fertilizers alone (Table 5). Furthermore, nutrient uptake efficiencies in these treatments exceeded with those observed in treatments receiving mineral fertilizers alone or in combination with VAM inoculation.

Dry matter yield

Dry matter accumulation in leaves, bulbs, and total dry matter yield responded to both fertilizer treatments and the cultivation year (Table 6). The highest leaf dry matter yield was recorded in treatments receiving *S. indica* inoculation with 100% RDF during the year 2018–2019, which was comparable to treatments receiving 100% RDF with VAM inoculation. In the subsequent year, the leaf dry matter yield was found to be significantly higher in treatments involving PSB inoculation and 100% RDF, which was statistically on par with the treatment receiving 100% RDF alone. For bulb dry matter yield and total plant dry matter yield in both experimental years, the maximum values were observed in treatments receiving 100% RDF with PSB, which was closely followed by treatments receiving 100% RDF with *S. indica* inoculation. The treatment receiving 100% RDF with PSB inoculation increased total dry matter yield by 11.6 and 10.5% in the year 2018–2019 and 2019–2020, respectively, compared to that of 100% RDF alone. Meanwhile, the application of 100% RDF with *S. indica* inoculation increased total dry matter yield by 11.5 and 7.6% in 2018–2019 and 2019–2020, respectively, compared to the treatment receiving 100% RDF alone. Leaf dry matter yield exhibited no significant difference between the two years, while the bulb and total dry matter yield recorded in 2019–2020 were significantly higher than those documented in the previous year 2018–2019.

Total onion yield

The onion bulb yield exhibited a significant increase when inoculated with *S. indica* or PSB in combination with a 100% mineral fertilizer application (Figure 1). This increase was significantly higher in comparison with the onion yield observed in treatments receiving

TABLE 6 Influence of mineral fertilizer and microbial inoculation on dry matter yield.

Treatment	Leaf dry matter yield (kg ha^{-1})		Bulb dry matter yield (kg ha^{-1})		Dry matter yield (kg ha^{-1})	
	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20
Control	647 ^{b}	613 ^{c}	4,398 ^{c}	4,813 ^{d}	5,046 ^{e}	5,426 ^{d}
50% RDF + PSB	913 ^{a}	898 ^{ab}	4,333 ^{c}	8,234 ^{ab}	5,246 ^{de}	9,132 ^{ab}
50% RDF + VAM	926 ^{a}	752 ^{bc}	4,639 ^{bc}	6,778 ^{c}	5,565 ^{cd}	7,530 ^{c}
50% RDF + *S. indica*	875 ^{ab}	814 ^{abc}	4,778 ^{abc}	8,171 ^{ab}	5,653 ^{cd}	8,985 ^{ab}
50% RDF	768 ^{ab}	775 ^{bc}	4,648 ^{bc}	7,783 ^{abc}	5,416 ^{de}	8,558 ^{bc}
100% RDF + PSB	989 ^{a}	1,021 ^{a}	5,630 ^{a}	8,929 ^{a}	6,618 ^{a}	9,950 ^{a}
100% RDF + VAM	938 ^{a}	914 ^{ab}	5,426 ^{ab}	7,680 ^{bc}	6,363 ^{ab}	8,594 ^{bc}
100% RDF + *S. indica*	1,011 ^{a}	960 ^{ab}	5,602 ^{a}	8,727 ^{ab}	6,613 ^{a}	9,687 ^{ab}
100% RDF	942 ^{a}	1,028 ^{a}	4,991 ^{abc}	7,978 ^{abc}	5,933 ^{bc}	9,006 ^{ab}
Mean	890	864	4,938	7,677	5,828	8,541
SEM±	49	48	171	244	171	252
CV%	9.5	9.6	6.1	5.5	5.1	5.1
P = 0.05	0.002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Values with different letters in the same year indicate significant differences ($p < 0.05$) as assessed using the least significant difference (LSD) test.

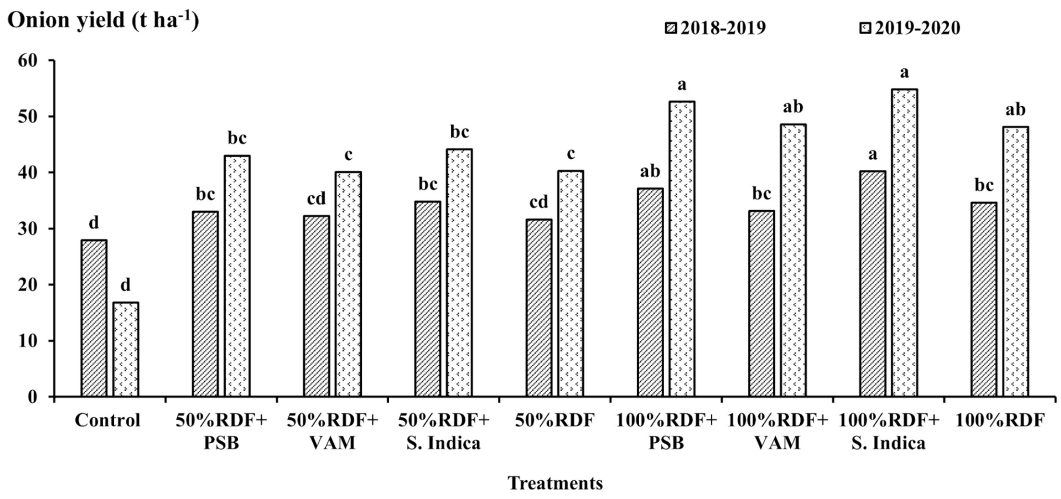


FIGURE 1
Effect of application of mineral fertilizers and microbial inoculation on onion yield (t ha^{-1}). Values with different letters in the same year indicate significant differences ($p < 0.05$) as assessed using the LSD test.

only 50% RDF and the absolute control in both the years 2018–2019 and 2019–2020. Furthermore, the combination of *S. indica* inoculation with 100% RDF fertilizer application produced the highest total bulb yield, which was closely followed by the treatment involving 100% RDF with PSB inoculation during both experimental years. In 2018–2019, the application of 100% RDF along with *S. indica* or PSB significantly increased the total bulb yield compared to 100% RDF alone. However, in 2019–2020, these treatments produced yields that were statistically comparable to 100% RDF alone. The combination of 100% RDF with *S. indica* resulted in yield increases of 16.2% in 2018–2019 and 13.9% in 2019–2020 compared to 100% RDF alone. Similarly, the application of 100% RDF along with PSB inoculation led to an increase in bulb yield of 7.2 and 9.4% in the respective years of the onion crop. Contrarily, treatments involving 50% RDF with microbial inoculation (*S. indica*, PSB, or VAM) and VAM inoculation with 100% RDF did not exhibit a considerable increase in bulb yield compared to the treatments receiving 100% mineral fertilizer in both years. Additionally, there was a significant enhancement in onion yield observed with the progressive increase in fertilizer levels from 50% RDF to 100% RDF. During these experimental years, a higher yield was registered in 2019–2020 when compared to the previous crop year 2018–2019.

Post-harvest soil properties

The application of mineral fertilizers, either alone or in combination with microbial inoculations, did not have a significant

TABLE 7 Influence of application of mineral fertilizers and microbial inoculation on post-harvest soil fertility status.

Treatment	Soil pH		EC (dS m^{-1})		SOC (g kg^{-1})		Soil available nutrients (mg kg^{-1})									
							N		P		K		S			
	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20	2018–19	2019–20		
Control	7.81 ^{a}	7.59 ^{ab}	0.19 ^{a}	0.19 ^{ab}	6.83 ^{a}	6.47 ^{a}	90.6 ^{b}	68.9 ^{a}	9.47 ^{a}	12.78 ^{a}	240.3 ^{a}	289.2 ^{a}	14.32 ^{a}	14.38 ^{a}		
50% RDF + PSB	7.78 ^{a}	7.63 ^{ab}	0.21 ^{a}	0.17 ^{b}	6.80 ^{a}	6.90 ^{a}	99.9 ^{ab}	75.1 ^{a}	10.04 ^{a}	13.90 ^{a}	233.9 ^{ab}	287.8 ^{a}	14.86 ^{a}	16.42 ^{a}		
50% RDF + VAM	7.81 ^{a}	7.63 ^{ab}	0.21 ^{a}	0.19 ^{ab}	6.97 ^{a}	6.37 ^{a}	105.4 ^{a}	69.4 ^{a}	9.57 ^{a}	13.58 ^{a}	223.5 ^{ab}	287.8 ^{a}	14.90 ^{a}	16.16 ^{a}		
50% RDF + *S. indica*	7.94 ^{a}	7.55 ^{ab}	0.20 ^{a}	0.20 ^{ab}	7.03 ^{a}	6.33 ^{a}	99.9 ^{ab}	70.5 ^{a}	9.69 ^{a}	12.59 ^{a}	224.0 ^{ab}	282.5 ^{a}	16.53 ^{a}	18.06 ^{a}		
50% RDF	7.84 ^{a}	7.70 ^{ab}	0.18 ^{a}	0.19 ^{ab}	6.53 ^{a}	6.63 ^{a}	88.8 ^{b}	70.5 ^{a}	10.23 ^{a}	12.07 ^{a}	222.5 ^{a}	290.1 ^{a}	14.58 ^{a}	16.18 ^{a}		
100% RDF + PSB	7.84 ^{a}	7.80 ^{a}	0.20 ^{a}	0.25 ^{b}	6.40 ^{a}	6.57 ^{a}	88.8 ^{b}	73.9 ^{a}	9.54 ^{a}	12.13 ^{a}	218.0 ^{b}	286.0 ^{a}	13.96 ^{a}	18.32 ^{a}		
100% RDF + VAM	7.79 ^{a}	7.50 ^{b}	0.18 ^{a}	0.24 ^{ab}	6.77 ^{a}	7.17 ^{a}	94.3 ^{ab}	73.9 ^{a}	8.18 ^{a}	11.69 ^{a}	221.0 ^{b}	279.2 ^{a}	14.84 ^{a}	17.03 ^{a}		
100% RDF + *S. indica*	7.84 ^{a}	7.51 ^{b}	0.20 ^{a}	0.28 ^{a}	6.70 ^{a}	6.57 ^{a}	94.3 ^{ab}	70.9 ^{a}	10.11 ^{a}	12.72 ^{a}	227.5 ^{ab}	280.7 ^{a}	15.51 ^{a}	16.94 ^{a}		
100% RDF	7.73 ^{a}	7.54 ^{ab}	0.18 ^{a}	0.22 ^{ab}	6.80 ^{a}	6.90 ^{a}	95.4 ^{ab}	72.2 ^{a}	9.69 ^{a}	13.64 ^{a}	226.0 ^{ab}	277.9 ^{a}	17.64 ^{a}	17.14 ^{a}		
Mean	7.82	7.61	0.20	0.20	6.76	6.66	95.3	71.7	9.61	12.79	226.0	284.6	15.24	16.74		
SEM±	0.07	0.06	0.01	0.02	0.25	0.24	2.84	2.87	3.25	1.14	3.42	6.41	0.82	0.47		
CV%	1.55	1.25	6.01	15.29	6.41	6.15	5.16	6.94	7.85	15.38	2.61	3.90	9.35	12.04		

Values with different letters in the same year indicate significant differences ($p < 0.05$) as assessed using the LSD test.

influence on post-harvest soil properties (Table 7). Notably, soil available N concentration was 24.7% higher in 2018–2019 compared to 2019–2020. In contrast, the concentrations of soil available P, K, and S were higher in 2019–2020 than in 2018–2019, with increases of 33.1, 25.9, and 9.9%, respectively, compared to the values recorded in 2018–2019.

Principle component analysis

PCA showed a strong relationship between various parameters and treatments, as illustrated by the principal component biplots (Figure 2). The two-way matrix between treatment and parameter biplots showed that a narrower angle between different parameters in the same direction signified a strong association between those parameters in classifying treatments. Out of nine treatments assessed in the present study, the optimal treatments, specifically 100% RDF with either *S. indica* or PSB, were positioned closer to and aligned along the vector line direction.

Discussion

In a two-year study, the effects of VAM, PSB, and *S. indica* inoculation with 50 and 100% RDF on onion growth, yield, nutrient uptake, and soil fertility were examined.

The application of *S. indica* or PSB with 100% RDF led to notable increases in various plant growth parameters such as plant height, number of leaves, and leaf area index, along with increased biomass production, and onion yield. This increase is potentially attributable to enhanced nutrient concentration and hormonal effects. The positive correlation between these parameters and the uptake of total N, P, K, and S further supports our findings (Table 8).

The mycelia of the endophytic fungus *S. indica* possibly extend onion roots, thereby facilitating root growth, nutrient absorption, stress tolerance, and systemic resistance (Gill et al., 2016). This symbiotic relationship may have increased the root system's surface area, thereby enhancing water and nutrient uptake, including N, P, K, and S (Li et al., 2023) and micronutrients (Padash et al., 2016). The increased levels of N, P, K, and S observed in plots receiving 100% RDF combined with *S. indica* inoculation would have boosted overall photosynthetic activity. This, in turn, led to increased biomass accumulation and onion yield. Gill et al. (2016) also documented that the mechanism contributing to increased plant growth and biomass accumulation could be attributed to the enhanced root growth facilitated by the colonized fungi, thereby promoting nutrient absorption from the root zone. Its colonization not only promoted root growth but also increased nutrient absorption in various crops such as sunflower, rapeseed, and rice (Li et al., 2023). Its colonization has been observed to positively impact the growth and biomass of various crop plants, as evidenced by previous studies on *Allium cepa*, *Oryza sativa*, *Saccharum officinarum*, *Abrus precatorius*, *Zea mays*, *Phaseolus vulgaris*, and *Tridax procumbans* (Varma et al., 2012, 2014; Gill et al., 2016; Roylawar et al., 2023).

Meanwhile, PSB have the ability to convert both organic and inorganic P into soluble forms, thereby enhancing its availability to plants (Kalayu, 2019; Rawat et al., 2021) by secreting extracellular

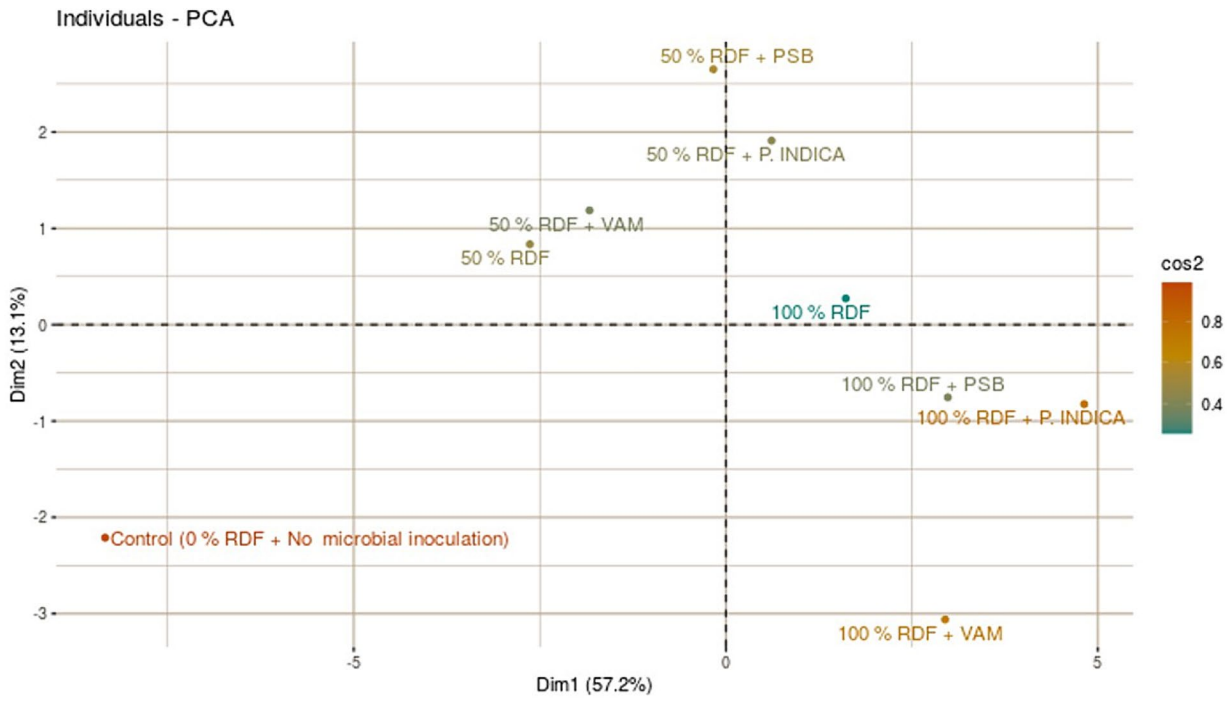


FIGURE 2
Principal component analysis (PCA) showing the effects of mineral fertilizer application and microbial inoculation on various parameters in onion.

enzymes, mineralizing substrates, and producing mineral-dissolving complexes or compounds (Sharma et al., 2013; Silva et al., 2023). Additionally, PSB activity in the rhizosphere may have influenced the production and availability of plant hormones like auxins, cytokinins, and gibberellins (Kenneth et al., 2019; Raza et al., 2019). These hormones are pivotal in governing plant growth and development, including processes such as cell elongation, leaf expansion, and shoot growth. Through the modulation of hormone levels, phosphate-solubilizing bacteria may have indirectly promoted plant height, leaf area expansion, and biomass accumulation (Barazani et al., 2007; Malik et al., 2019). Mangaraj et al. (2022) also documented that increased nutrient availability led to increased conversion of carbohydrates to protein, subsequently enhancing meristematic cellular activity, including cell division and elongation, which manifested morphologically as increased plant growth and ultimately resulted in higher dry matter accumulation and crop yield.

However, inoculation with *S. indica* or PSB in treatments where only 50% RDF was applied did not result in a significant increase in leaf area or plant growth. This suggested that the reduced fertilizer doses may have contributed to a smaller leaf area due to inadequate nutrient availability and uptake, thus limiting the plant's capacity to capture sunlight and perform photosynthesis effectively (Zhang et al., 2021; Rawat et al., 2022), which has ultimately limited biomass accumulation (Tan et al., 2005). The reduction in biomass production and nutrient uptake may have led to a decrease in bulb yield in plots that received 50% RDF, with or without *S. indica* or PSB inoculation, compared to plots that received 100% RDF, regardless of *S. indica* or PSB inoculation.

The root system was also evaluated for colonization by VAM fungi; however, VAM colonization was not observed in onion roots. The application of mineral fertilizers could have affected the efficacy of VAM fungi in onions. The soils used in this experiment had high concentrations of P, K, S, copper, manganese, and adequate levels of zinc, iron, and boron. The presence of high nutrient levels in the soil may have inhibited the colonization of VAM fungi in onions. Many researchers have documented reduced colonization of arbuscular mycorrhizal fungi in plots treated with mineral fertilizer (Trejo et al., 2021; Fall et al., 2023). However, further investigation is required to understand the reasons for the poor or non-infection of onion roots by VAM fungi, as they are known to be one of the best sources for improving plant growth, development, and crop yield not only in perennial trees but also in various field and vegetable crops.

Additionally, onion yield exhibited significant variation from year to year, with a lower yield recorded in 2018–2019 compared to 2019–2020. The reduced yield in the first year could potentially be attributed to temperature increases at maturity during the harvesting stage (Parthasarathi et al., 2022). The plant's allocation of energy toward enhancing its antioxidant enzyme system, as part of its defense mechanism against temperature stress, could have potentially contributed to the reduction in onion crop yield. These findings are consistent with the conclusions drawn by Warland et al. (2006) in Brassicaceae, Shamloo et al. (2017) in wheat, and Thangasamy et al. (2021) in garlic, which suggest a notable decline in the yield of cool-season crops in response to even slight temperature increases.

Conclusion

The comprehensive findings suggest that the application of 100% RDF additionally inoculated with *S. indica* or PSB significantly enhanced plant growth, nutrient uptake, and onion yield compared to both the control and treatments with 50% RDF, with or without

TABLE 8 Correlation coefficient matrix.

	LN	BN	LP	BP	LK	BK	LS	BS	TDM	TNU	TPU	TKU	TSU	NL60	PH60	Yield	LAI60
NL	1																
NB	0.69***	1															
PL	−0.40**	−0.69***	1														
PB	0.56***	0.59***	−0.16	1													
KL	0.38**	0.73***	−0.85***	0.37**	1												
KB	0.54***	0.86***	−0.81***	0.50***	0.88***	1											
SL	−0.32*	−0.67***	0.88***	−0.31*	−0.90***	−0.85***	1										
SB	0.63***	0.40**	−0.13	0.21	0.06	0.25	−0.05	1									
TDM	−0.09	−0.43**	0.75***	−0.05	−0.77***	−0.67***	0.81***	0.18	1								
TNU	0.59***	0.60***	−0.01	0.55***	0.05	0.26	0.05	0.54***	0.45***	1							
TPU	0.08	−0.18	0.68***	0.46***	−0.54***	−0.41**	0.60***	0.15	0.84***	0.57***	1						
TKU	0.57***	0.62***	−0.21	0.63***	0.33*	0.54***	−0.23	0.49***	0.24	0.83***	0.43**	1					
TSU	0.06	−0.33*	0.68***	−0.04	−0.72***	−0.59***	0.77***	0.45***	0.95***	0.51***	0.77***	0.28*	1				
NL60	−0.01	−0.18	0.42**	0.03	−0.45***	−0.33*	0.52***	0.22	0.57***	0.33*	0.52***	0.19	0.57***	1			
PH60	0.49***	0.30*	0.16	0.43**	−0.11	0.03	0.23	0.49***	0.48***	0.71***	0.56***	0.55***	0.54***	0.40**	1		
Yield	0.25	−0.13	0.59***	0.31*	−0.57***	−0.42**	0.65***	0.33*	0.88***	0.63***	0.90***	0.42**	0.87***	0.56***	0.64***	1	
LAI60	0.53**	0.62***	0.28	0.79***	−0.1	0.33	0.29	0.35	0.74***	0.73***	0.91***	0.82***	0.62***	0.50**	0.74***	0.85***	1

LN, Leaf nitrogen (N) concentration; BN, Bulb N concentration; LP, Leaf phosphorus (P) concentration; BP, Bulb P concentration; LK, Leaf potassium (K) concentration; BK, Bulb K concentration; LS, Leaf sulfur (S) concentration; BS, Bulb S concentration; TDM, total dry matter yield; TNU, Total N uptake; TPU, Total P uptake; TKU, Total K uptake; TSU, Total S uptake; NL60, Number of leaves at 60 Days after transplanting (DAT); PH60, Plant height at 60 DAT; LAI, Leaf area index at 60 DAT. *Significant at $p = 0.05$, **significant at $p = 0.01$, and ***significant at $p < 0.0001$.

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microbial inoculation. Conducting field trials across diverse regions and soil types could further validate the efficacy of microbial inoculation and fertilizer dosages under varying environmental conditions and provide more reliable data for practical adoption by farmers. However, it's noteworthy that VAM did not exhibit satisfactory performance in onion cultivation, as observed in other crops. Additional investigation is necessary to understand the underlying reasons for the poor and inadequate plant colonization or non-infection of onion roots by VAM fungi.

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

TA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. KG: Formal analysis, Investigation, Methodology, Writing – original draft. PM: Formal analysis, Investigation, Methodology, Writing – original draft. PS: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. VG: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. SG: Investigation, Methodology, Resources, Writing – original draft. VM: Funding acquisition, Project administration, Resources, Writing – original draft.

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Conflict of interest

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

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Supplementary material

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Enhancing cauliflower growth under cadmium stress: synergistic effects of Cd-tolerant *Klebsiella* strains and jasmonic acid foliar application

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The pollution of heavy metals (HMs) is a major environmental concern for agricultural farming communities due to water scarcity, which forces farmers to use wastewater for irrigation purposes in Pakistan. Vegetables grown around the cities are irrigated with domestic and industrial wastewater from areas near mining, paint, and ceramic industries that pollute edible parts of crops with various HMs. Cadmium (Cd) is an extremely toxic metal in arable soil that enters the food chain and damages the native biota, ultimately causing a reduction in plant growth and development. However, the use of microbes and growth regulators enhances plant growth and development as well as HM immobilization into the cell wall and hinders their entry into the food chain. Thus, the integrated use of bacterial consortium along with exogenously applied jasmonic acid (JA) mitigates the adverse effect of metal stress, ultimately reducing the metal mobility into roots by soil. Therefore, the current study was conducted to check the impact of Cd-tolerant bacteria and JA on the growth, nutrient status, and uptake of Cd in the cauliflower (*Brassica oleracea*). Our results demonstrated that increasing concentrations of Cd negatively affect growth, physiological, and biochemical attributes, while the use of a bacterial consortium (SS7 + SS8) with JA (40 μmol L^{-1}) significantly improved chlorophyll contents, stem fresh and dry biomass (19.7, 12.7, and 17.3%), root length and root fresh and dry weights (28.8, 15.2, and 23.0%), and curd fresh and dry weights and curd diameter (18.7, 12.6, and 15.1%). However, the maximum reduction in soil Cd, roots, and curd uptake was observed by 8, 11, and 9.3%, respectively, under integrated treatment as compared to the control. Moreover, integrating bacterial consortium and JA improves superoxide dismutase (SOD) (16.79%), peroxidase dismutase (POD) (26.96%), peroxidase (POX) (26.13%), and catalase (CAT) (26.86%). The plant nitrogen, phosphorus, and potassium contents were significantly increased in soil, roots, and curd up to 8, 11, and 9.3%, respectively. Hence, a consortium of *Klebsiella* strains in combination with JA is a potential phytostabilizer and it reduces the uptake of Cd from soil to roots to alleviate the adverse impact on cauliflower's growth and productivity.

KEYWORDS

Cd toxicity, bacterial consortium, jasmonic acid, antioxidants, microbial interaction, immobilization, translocation factor, bioconcentration factor

1 Introduction

Heavy metals (HMs) pose a serious threat to the native biota due to natural and anthropogenic activities all around the globe (Behera et al., 2023). Among abiotic stresses, HMs are one of the biggest threats to arable soils in recent times, which disturb the environment, crop sustainability, and food security (Altaf et al., 2022a; Ghuge et al., 2023). Rapid industrialization and anthropogenic activities, such as the use of inorganic fertilizer, pesticides, and wastewater irrigation for vegetables, are major factors responsible for HM pollution in soil (Meena et al., 2020; Haider et al., 2021). Cadmium (Cd) is a highly toxic metal among all HMs due to its high solubility in water, which can lead to complete crop failure (Sanita di Toppi and Gabbrielli, 1999). Major sources of Cd are mining, electroplating, ceramic, and paint industries, which disturb the environment and whole ecosystem (Villen-Guzman et al., 2021). Moreover, the use of synthetic and phosphatic fertilizers increases the concentration of Cd in soil, air, and water, ultimately increasing the accumulation of Cd in soil (Annar, 2022). However, Cd enters soil by various mechanisms, such as leaching, industrial process, and waste, which are adsorbed by the soil particles. Subsequently, Cd uptake by plants leads to its entry into vital parts, which ultimately destroy plant growth and development (Liu et al., 2021; Khanna et al., 2022). Moreover, the higher amounts of Cd in plants also disturb the CO_2 absorption rate, photosynthetic rate, and chlorophyll contents (Melki et al., 2022). Cadmium enters the food chain when Cd-contaminated food is consumed by humans, which may cause damage to the human reproductive system, nephrotoxicity, teratogenicity, and endocrine toxicity (Altaf et al., 2022b; Ayub et al., 2023). To remediate these harmful impacts of Cd and all other HMs in the ecosystem, a unique, cheaper, and eco-friendly approach needs to be explored that can reduce Cd uptake from the soil to the root system and enhance the plant tolerance against toxic levels of Cd. Among all recent approaches, bioremediation is the most sustainable and environment-friendly approach to remediate HMs and hinder their entry into the food chain by forming metal complexes and secreting plant growth-promoting substances in the rhizosphere (Kalkan, 2022; Shabaan et al., 2022; Yaashikaa et al., 2022). Moreover, using microbes positively enhances the fertility status of soil and reduces the uptake of Cd in plants (Idris and Yuliar, 2022). The Cd-tolerant bacterial strains sequester Cd in soil through immobilization by secreting polymeric substances, phytohormones, and siderophores. Plant growth-promoting bacteria and phytohormones may form stable complexes with Cd, thus stabilizing it in soil. However, the negative charge of roots may bind with Cd and reduce its uptake in the upper part of the plant. The Cd-tolerant bacterial strains isolated from wastewater include different genera such as *Klebsiella*, *Pseudomonas*, *Enterobacter*, and *Bacillus*, which possess plant growth-promoting abilities and can reduce Cd toxicity (Kamaruzzaman et al., 2020). Thus, the utilization of microbes to ameliorate Cd toxicity for improving environmental quality can be a tool to develop biofertilizers for growing vegetables, fruits, and field crops in contaminated soils.

In addition, exogenous application of hormones, signaling molecules, and osmolytes also gains attention to reduce abiotic stresses (Rizwan et al., 2016a). JA, a signaling molecule and plant growth regulator, acts as a protective agent to boost plant growth and antioxidants during abiotic stress (Mir et al., 2018). The exogenously applied JA binds the various sugars, hydroxylated derivatives, and carbohydrates that accumulate in plant cells in response to various stresses (Ali et al., 2023). The previous study depicted that foliar application of JA decreases the concentration of Cd in rice by reducing the Cd translocation from roots to grain (Li et al., 2022). When the concentration of HMs exceeds in plants, it produces reactive oxygen species (ROS) and causes oxidative stress in response to HM toxicity. However, JA scavenges the overproduction of ROS and reduces the oxidative damage caused by HMs (Foyer and Noctor, 2011). Moreover, the previous studies also depicted that JA can significantly increase the activities of antioxidant enzymes to boost the defense mechanism against HM stress in plants (Kamran et al., 2021). Manzoor et al. (2022) stated that the exogenously applied JA enhanced the growth of *Pisum sativum* L. cultivars by enhancing photosynthetic pigments and antioxidant efficiency. The individual application of Cd-tolerant bacteria and JA has been reported in the literature to alleviate Cd toxicity under normal and stressed conditions (Ali et al., 2018; Nazli et al., 2020), but no study is available on the combined effect of both stress alleviator JA and metal-tolerant bacteria. Our previous study demonstrated that the combined application of a Cd-tolerant bacterial consortium along with JA foliar application significantly improved cauliflower growth and immobilized the soil Cd in plant roots (Shahid et al., 2023). Here, we hypothesized that a Cd-tolerant bacterial consortium in combination with JA subsequently alleviates the Cd-induced adverse impact on the cauliflower crop. Moreover, co-application of the Cd-tolerant bacterial consortium with JA could be more efficient as compared to sole application to alleviate the Cd stress by regulating the antioxidant defense system in cauliflower. Thus, the current study has been planned to address the synergistic effects of Cd-tolerant *Klebsiella* strains and JA foliar application to enhance the growth of cauliflower plants with the following objectives: (i) to examine the role of synergistic effects of Cd-tolerant *Klebsiella* strains and JA in mitigating the adverse impact of Cd on the growth and development of the cauliflower crop and (ii) to evaluate the potential use of bacterial strains and JA in reducing the entry of Cd into plants.

2 Materials and methods

2.1 Collection of bacterial strains and inoculation of cauliflower seeds

A pot trial was conducted to check the efficacy of plant growth-promoting Cd-tolerant bacterial strains in combination with JA to

ameliorate Cd toxicity, promote growth under Cd toxicity, regulate the antioxidant defense system, and improve nutrient efficiency. Pre-isolated and pre-characterized Cd-tolerant bacterial strains *Klebsiella* sp. (SS7) and *Klebsiella pneumoniae* (SS8) with accession nos. MW829780 and MW829781, respectively, were used in this study and screened for plant growth-promoting characteristics in our earlier study (Shahid et al., 2023). The experiment was performed in the Department of Soil Science, Institute of Soil and Water Resources warehouse, the Islamia University of Bahawalpur. Cauliflower seeds of the latest variety (HS-65), widely accepted in the farmer's community, were obtained from the Oilseeds Research Station, Bahawalpur. The seeds were disinfected with a 2% sodium hypochlorite solution. The inoculum was prepared by growing bacterial strains SS7 and SS8 separately in Dworkin and Foster (DF) minimal salt media, which is composed of sucrose (10 g), K_2HPO_4 (2.5 g), KH_2PO_4 (2.5 g), $(NH_4)_2HPO_4$ (1.0 g), $MgSO_4.7H_2O$ (0.2 g), $FeSO_4.7H_2O$ (0.01 g), $MnSO_4.7H_2O$ (0.007 g), and agar powder (15 g). The media was incubated at 30 ± 1°C in a shaking incubator at 100 rpm for 48 h (Model SI9R-2, Shellab, Riverside, OH, USA). After incubation, the bacterial cells were harvested by centrifugation at 22°C for 20 min at 9000 rpm (Model: UNIVERSAL 320R, Hettich, Germany). Furthermore, the pallets were dissolved in sterilized distilled water after repeated washing, and the final culture was prepared by taking equal proportions (v/v) from both strains in sterile flasks and vortexed for 1 min for consortium application.

2.1.1 Soil sampling and pot trial

The soil sample was collected from the research area of the Department of Soil Science at the Islamia University of Bahawalpur and analyzed for various physicochemical properties of soil following a standard protocol by Ryan et al. (2001), as given in Table 1. Moreover, the soil was analyzed for HM contamination by using the atomic absorption spectrophotometer (AAS) model AAS-240FS, Agilent, Santa Clara, CA, USA, following the standard protocol of Hseu et al. (2002). The pot experiment was conducted in the wire house by carefully managing the natural growth conditions, ambient light, and 10–12°C temperature. For soil contamination, $CdCl_2$ salt at 150 mg kg^{-1} was used and mixed in soil 2 weeks before the experiment.

TABLE 1 Physiochemical properties of soil used in the pot experiment.

Physiochemical properties	Values
Soil texture	Sandy clay loam
ECe	1.64 dSm^{-1}
pH	7.9
Saturation percentage	33%
Organic matter	0.49%
Nitrogen	0.05%
Phosphorus	5.69 mg kg^{-1}
Potassium	87 mg kg^{-1}
Cadmium	1.11 mg kg^{-1}
Lead	0.42 mg kg^{-1}
Nickel	ND
Iron	35 mg kg^{-1}
Zinc	38 mg kg^{-1}

Approximately 10 kg of air-dried soil was filled in each pot with three replications, and 10 inoculated and un-inoculated cauliflower seeds were sown in each pot. Surface sterilized cauliflower seeds were inoculated by dipping them in a broth mixture for half an hour before sowing. The pots were arranged as a completely randomized design (CRD) with factorial arrangements. Ten days after germination, the extra plants were removed by thinning, and a uniform population was maintained. The plant nutrients requirement was fulfilled by applying the recommended doses of P and K (90 and 60 kg ha^{-1}) by using sulfate of potash (SOP) and diammonium phosphate (DAP) at the time of sowing, while a recommended dose of nitrogen (N) (120 kg ha^{-1}) using urea was applied in three splits after 20 days of germination. After 3 weeks of sowing, only one plant of the cauliflower crop was maintained in the pot and a foliar application of JA was applied. A solution of JA was prepared by dissolving it in 100 µL of absolute ethanol (Thaler et al., 1999). A stock solution of 1 mM JA was prepared in a 1-L volumetric flask, and the working solution of JA (40 µmol L^{-1}) was prepared by diluting the stock solution as described by Bano et al. (2021). After 3 weeks of sowing, 40 µmol L^{-1} JA was sprayed with a nozzle sprayer (5 mL per plant) to the respective treatments and repeated every week.

2.2 Plant parameters

2.2.1 Cauliflower growth and yield attributes

At physiological maturity, plants were analyzed for physiological attributes such as chlorophyll content (SPAD value). Furthermore, at the time of harvesting after 35 days of sowing, the length of roots and stems and the fresh weight of roots and stems of cauliflower plants were measured using an electrical balance and meter rod. The growth parameters were recorded for each plant in every treatment, and the mean values were determined in triplicate. After that, the roots of cauliflower plants were washed using distilled water and oven-dried at 70°C for 48 h to check dry weight (Rizwan et al., 2019).

2.3 Antioxidant activity

The fresh leaf sample (0.5 g) was taken in a pre-cooled mortar on ice. The sample was dissolved in 4 mL of pre-cooled phosphate buffer solution [$Na_2HPO.12H_2O$ (16.385 g) + $NaH_2PO_4.2H_2O$ (0.663 g)], and the solution was made up to 1,000 mL by adding distilled water and maintained at a pH of 7.8. After homogenization of the sample on ice, phosphate buffer solution (4 mL) was added and centrifuged for 20 min at 4°C at 10,000 rpm, and the supernatant was collected to determine the different antioxidants such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POX). For the determination of SOD and POD, a modified nitro blue tetrazolium (NBT) indicator was used, as described by Beauchamp and Fridovich (1971). In addition, the decomposition of H_2O_2 at 240 nm was observed to measure the CAT activity, Whereas the POX activity was measured by mixing phosphate buffer and guaiacol with enzymes extract and reading was taken at 470 nm an ultraviolet–visible (UV–VIS) spectrophotometer (Model; Carry 60; Agilent, Santa Clara, CA, USA) (Chance and Maehly, 1995).

Proline contents in the curd of cauliflower were determined by using a 0.5-g fresh leaf sample. Sulphosalicylic acid (3%) was used to

process the sample in flasks according to the following procedure by Bates et al. (1973). The contents were filtered by using a Whatman No. 2 after homogenization and adding glacial acetic acid and ninhydrin. The flask was cooled, and the contents were extracted using toluene. The absorbance was measured by using a spectrophotometer at 520 nm, and a standard curve was prepared for the proline concentration (Bates et al., 1973).

2.4 Mineral analysis

For mineral analyses, a dry plant sample of 0.2 g with 6 mL of concentrated H_2SO_4 was mixed and left overnight. In addition, 1 mL of H_2O_2 was also used, and the solution was heated at 300°C on a hot plate for 1 h until the color of the sample was transparent. The samples were analyzed for total macronutrients (NPK) analysis by using the standard protocol, as N was determined by Kjeldahl distillation and titration with 0.01 N H_2SO_4, P was determined by the yellow method on a spectrophotometer, and K was determined using a flame photometer (Ryan et al., 2001). Moreover, for the determination of Fe and Zn, the method of extraction by diethylenetriaminepentaacetic acid (DTPA) was used. The reading was determined by using an atomic absorption spectrophotometer (model AAS-240 FS, Agilent, USA). The Fe and Zn in soil were calculated by a standard calibration curve (Lindsay et al., 1978).

2.5 Determination of Cd concentration

For Cd determination, 1 g of air-dried soil was weighed, and digestion proceeded at 300°C, as followed by Allen and Rae (1986). After a clear solution, the samples were filtered and diluted with distilled water. The Cd concentration in plants and soil was measured by using an atomic absorption spectrophotometer (AAS) model AAS-240 FS, Agilent, USA.

2.6 Statistical analysis

The data were evaluated for significance by using the CRD with factorial arrangement to construct a two-way analysis of variance (ANOVA) of the results (Steel et al., 1997), and the means were compared by using the least significant difference test (LSD). Excel (MS Office) was used to calculate the standard error, mean, and graphs.

3 Results

3.1 Agronomic parameters

A pot trial was conducted to check the synergistic effects of Cd-tolerant *Klebsiella* strains and JA foliar application under Cd stress. The use of integrating bacterial consortium and JA significantly enhanced the growth and yield attributes of the cauliflower crop under Cd stress. A significant decrease in physiological and growth parameters of the cauliflower crop under Cd stress without amendment of Cd-tolerant bacterial strains and JA was observed. Furthermore, the application of Cd-tolerant bacterial strains in combination with JA significantly enhanced the growth attributes under Cd stress. The combined application of Cd-tolerant bacterial consortium and JA was significantly efficient as compared to individual applications under Cd toxicity. The bacterial consortium (SS7 + SS8) in combination with JA significantly improved cauliflower leaf chlorophyll content by 19.8% as compared to the control treatment. The same treatment also significantly improved ($p < 0.05$) the stem fresh weight (12.7%) and stem dry weight (17.3%) of cauliflower as compared to the control treatment (Table 2). Moreover, the cauliflower root length (28.8%), root fresh weight (15.2%), and root dry weight (23.0%) were also significantly enhanced by 28.8, 15.2, and 23.0%, respectively, as compared to the control treatment (Table 3). The yield parameters of cauliflower were also significantly enhanced by the application of consortium with JA under Cd stress, and the maximum increase in fresh weight of curd (8.68%), dry weight of curd (12.6%), and curd diameter (15.2%) was 8.68, 12.6, and 15.2%, respectively, as compared to the control treatments ($p = 0.0316$) (Table 4). However, individual application of the Cd-tolerant bacterial consortium significantly improved stem dry weight by 12.7%, root dry weight by 17.3%, plant height by 8%, and curd dry weight by 8.5% as compared to the control treatment. Moreover, the foliar application of JA significantly improved chlorophyll contents by 15.1% as compared to other treatments under Cd stress. However, a bacterial consortium in combination with JA improved chlorophyll content by 29.7%, stem fresh weight by 14.3%, stem dry weight by 19.9%, root fresh weight by 19.2%, root dry weight by 22.1%, curd fresh weight by 29.6%, and curd dry weight by 12.6% under normal soil in the absence of Cd stress as compared to the sole application of treatments ($p < 0.05$).

TABLE 2 Effect of Cd-tolerant bacterial consortium in combination with JA on chlorophyll content, stem fresh weight, and stem dry weight of cauliflower in a pot trial.

Treatment	Chlorophyll		Stem fresh weight (g)		Stem dry weight (g)	
	0 mg Cd kg^{-1}	150 mg Cd kg^{-1}	0 mg Cd kg^{-1}	150 mg Cd kg^{-1}	0 mg Cd kg^{-1}	150 mg Cd kg^{-1}
Control	37 ± 0.6^{d}	29 ± 0.3^{f}	42 ± 0^{c}	37 ± 0.3^{e}	8.8 ± 0.2^{c}	6.1 ± 0.3^{e}
SS7 + SS8	41 ± 0.7^{c}	31 ± 0.3^{f}	45 ± 0.3^{b}	38 ± 0.3^{d}	9.6 ± 0.1^{b}	6.3 ± 0.2^{de}
JA	44 ± 0.6^{b}	33 ± 0.1^{f}	46 ± 0.6^{b}	40 ± 0.3^{d}	9.6 ± 0.1^{b}	6.7 ± 0.3^{de}
SS7 + SS8 + JA	48 ± 1.5^{a}	34 ± 0.3^{e}	47 ± 0.6^{a}	41 ± 0.3^{c}	10.5 ± 0.3^{a}	7.2 ± 0.2^{d}
LSD ($p \leq 0.05$)	**2.14**		**1.46**		**0.76**	

The superscript letter also tell us about the significance of the treatments as treatments with different letters are significantly differ from each other.

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TABLE 3 Effect of Cd-tolerant bacterial consortium in combination with JA on root fresh weight, root dry weight, and root length of cauliflower in a pot trial.

Treatment	Root fresh weight (g)		Root dry weight(g)		Root length (cm)	
	0 mg Cd kg^{-1}	150 mg Cd kg^{-1}	0 mg Cd kg^{-1}	150 mg Cd kg^{-1}	0 mg Cd kg^{-1}	150 mg Cd kg^{-1}
Control	32 ± 0.2^{c}	25 ± 0.3^{f}	7.2 ± 0.2^{bc}	5.5 ± 0.3^{d}	12 ± 0.2^{c}	8.6 ± 0.3^{f}
SS7 + SS8	35 ± 0.7^{b}	26 ± 0.2^{ef}	7.5 ± 0.3^{b}	5.9 ± 0.4^{d}	12 ± 0.4^{bc}	10.5 ± 0.3^{de}
JA	35 ± 0.3^{b}	26 ± 0.3^{e}	7.9 ± 0.1^{b}	5.8 ± 0.2^{d}	13 ± 0.1^{b}	10.1 ± 0.2^{e}
SS7 + SS8 + JA	38 ± 0.3^{a}	28 ± 0.3^{d}	8.8 ± 0.2^{a}	6.8 ± 0.1^{c}	14 ± 0.3^{a}	11.1 ± 0.2^{d}
LSD ($p \leq 0.05$)	**1.21**		**0.67**		**0.87**	

The superscript letter also tell us about the significance of the treatments as treatments with different letters are significantly differ from each other.

TABLE 4 Effect of Cd-tolerant bacterial consortium in combination with JA on curd fresh weight, curd dry weight, and curd diameter of cauliflower plants in a pot trial.

Treatment	Curd fresh weight (g)		Curd dry weight (g)		Curd diameter (mm)	
	0 mg Cd kg^{-1}	150 mg Cd kg^{-1}	0 mg Cd kg^{-1}	150 mg Cd kg^{-1}	0 mg Cd kg^{-1}	150 mg Cd kg^{-1}
Control	215 ± 3.5^{c}	175 ± 2.1^{e}	56 ± 0.3^{c}	49 ± 0.2^{f}	14 ± 0.3^{b}	11 ± 0.6^{d}
SS7 + SS8	253 ± 11.8^{b}	187 ± 1.5^{de}	59 ± 0.3^{b}	52 ± 0.3^{de}	15 ± 0.3^{ab}	12 ± 0.7^{cd}
JA	257 ± 1.8^{b}	192 ± 3.6^{d}	58 ± 0.1^{b}	51 ± 0.6^{e}	15 ± 0.6^{ab}	12 ± 0.4^{cd}
SS7 + SS8 + JA	276 ± 3.1^{a}	196 ± 3.0^{d}	63 ± 1.2^{a}	54 ± 0.3^{d}	16 ± 0.6^{a}	13 ± 0.3^{c}
LSD ($p \leq 0.05$)	**14.1**		**1.7**		**1.6**	

The superscript letter also tell us about the significance of the treatments as treatments with different letters are significantly differ from each other.

3.2 Antioxidants

The data regarding the antioxidant enzyme activities of cauliflower plants showed that SOD, POD, CAT, and POX activities were significantly increased under Cd stress. The inoculation of the bacterial consortium (SS7 + SS8) resulted in a greater increase in SOD by 11.5%, POD by 12.5%, CAT by 13.8%, and POX by 15.9% under Cd stress as compared to the control treatment ($p < 0.05$). In addition, individual application of JA significantly enhanced SOD (10.8%), POD (19.1%), POX (21.6%), and CAT (30.2%) activities as compared to the control treatment ($p < 0.05$) (Figure 1). Contrastingly, the combined application of bacterial consortium (SS7 + SS8) and foliar application of JA stimulated the increased antioxidant activity to minimize ROS-induced damage under Cd toxicity, as SOD, POD, POX, and CAT activities were improved by 16.8, 26.9, 26.1, and 26.9%, respectively ($p < 0.05$) (Figure 1).

3.3 Efficacy of bacterial consortium and JA on the mineral concentration in cauliflower

The combined application of bacterial consortium and JA improved the macronutrients in soil and cauliflower plant in the current study. The Cd-tolerant bacterial consortium in combination with JA led to a substantial production of N, P, and K contents under Cd stress as compared to the control treatments (Figure 2). Moreover, a bacterial consortium in combination with JA significantly improved N, P, and K in soil by 14.9, 11.7, and 11.7%, respectively, under Cd stress as compared to the control treatment. However, in the absence of Cd toxicity, bacterial consortium in combination with JA increased N, P, and K in soil by 16.8, 15.6, and 10.9%, respectively, as compared to the control treatment (Figure 2). Additionally, a significant increase in mineral content was also observed in the roots of cauliflower plants, where N, P, and K contents were significantly improved by 7.6, 17.0, and 10.4%, respectively, under Cd stress ($p < 0.05$) (Figure 3). Moreover, individual application of Cd-tolerant bacteria improved N, P, and K in roots by 5, 10, and 8.5%, respectively, as compared to the control treatments.

In addition, under Cd stress, a maximum concentration of N, P, and K was also observed in the curd of cauliflower, where N, P, and K contents were increased by 9.5, 10.0, and 6.27%, respectively, as compared to the un-inoculated control treatment. In addition, individual application of Cd-tolerant bacterial consortium significantly improved N by 6.6%, P by 5.8%, and K by 5.2%, respectively, in curd as compared to the control treatment under Cd stress. However, the application of bacterial consortium with JA significantly increased N, P, and K contents of curd by 10.7, 10.0, and 7.2%, respectively, compared to the control treatment ($p < 0.05$) (Figure 4).

3.4 Cd concentration in soil and cauliflower plant

The bacterial consortium in combination with JA application resulted in a remarkable reduction in the concentration of Cd in soil and different parts of the cauliflower plant (Figure 5). The maximum reduction in Cd concentration was measured in soil by 8%, roots by 11.5%, and curd by 9.3% as compared to the individual application of amendments ($p < 0.05$). However, individual application of Cd-tolerant bacterial consortium also reduced Cd concentration by 6.2, 5.8, and 8.5% in soil, roots, and curd, respectively ($p < 0.05$). Moreover, in the absence of Cd stress, the concentration of Cd in cauliflower plants was not detected. Simultaneously, as compared to

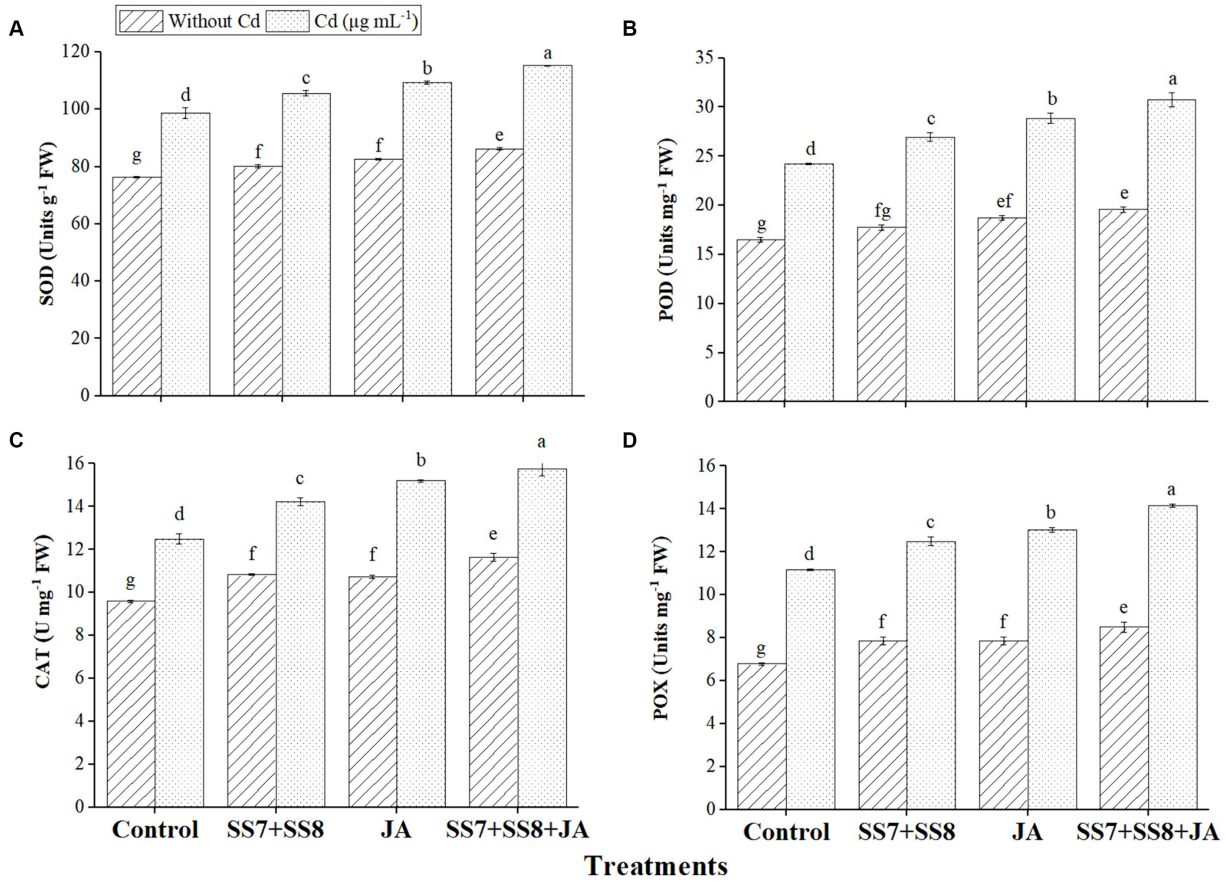


FIGURE 1
Effect of integrating bacterial consortium with jasmonic acid on **(A)** SOD ($p = 0.0021$); **(B)** POD ($p = 0.7621$); **(C)** CAT ($p = 0.0001$); and **(D)** POX ($p = 0.0012$) activity of a cauliflower plant under Cd stress in a pot trial.

other treatments, Cd-tolerant bacterial strains in combination with JA inhibit Cd translocation from soil to plant parts by 7.5%. Bioconcentration factors were also reduced by the immobilization of Cd by Cd-tolerant bacterial consortium under Cd stress (Figure 6).

3.5 Yield parameter of curd

The application of bacterial consortium and JA significantly improved yield parameters and Zn, Fe, proline, and total sugar content in the curd of cauliflower and resulted in higher micronutrient efficiencies (Figure 7). The maximum biofortification of Zn in cauliflower curd was observed under treatment with the bacterial consortium and JA, which was 12.9% higher under Cd stress compared to the control treatment. Similarly, a significant increase was also observed for Fe (15.1%), proline (12.1%), and total sugar content (10.1%) by integrating the bacterial consortium with JA under Cd stress as compared to the control treatment. However, individual application of bacterial consortium also increased the fortification and osmolyte concentrations in cauliflower curd, as Zn contents were increased by 6.82%, Fe contents by 5.28%, proline contents by 6.75%, and sugar contents by 5.78% as compared to the un-inoculated control treatment. However, foliar application of JA also increased Zn, Fe, proline, and sugar contents by 5.84, 5.01, 5.23, and 4.98%, respectively, as compared to the control treatments.

3.6 Pearson's correlation

Pearson's correlation described the relation between different parameters of the study. The results presented in Figure 8 depict an inverse relationship between the plant stress indicator (proline) and the growth and yield parameters and a direct relationship with Cd concentration in soil, root, and curd. This indicates that the uptake of cadmium increased the proline contents in plant as a stress indicator. Similar tendencies for antioxidants with growth and yield attributes and Cd concentration were found. Hence, the integration of bacterial consortium and JA application regulated the physiological stress indicators and antioxidants to cope with the Cd stress and sustain the growth and development of cauliflower.

4 Discussion

HM stress has interfered with the productivity and sustainability of agriculture around the world. However, global resources have not

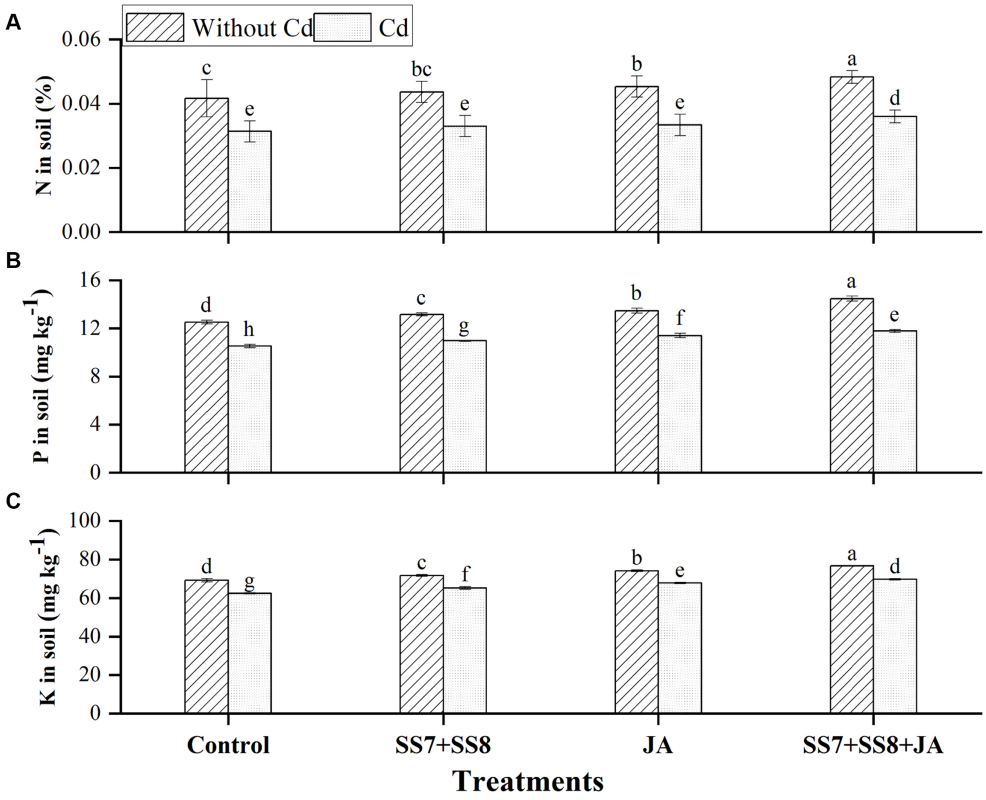


FIGURE 2
Effect of integrating bacterial consortium with jasmonic acid on **(A)** N in soil ($p = 0.0001$); **(B)** P in soil ($p = 0.2568$); and **(C)** K in soil ($p = 1.2413$) under Cd stress in a pot trial.

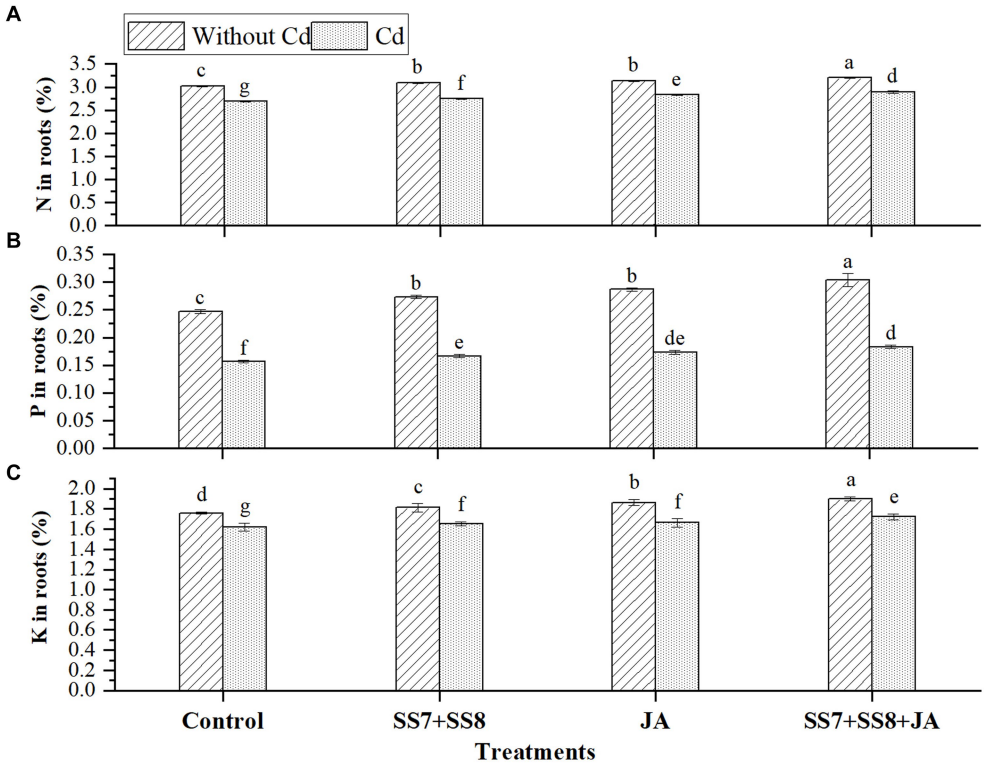


FIGURE 3
Effect of integrating bacterial consortium with jasmonic acid on **(A)** N in roots ($p = 0.0444$); **(B)** P in roots ($p = 0.0166$); and **(C)** K in roots ($p = 0.0308$) of cauliflower plant under Cd stress in a pot trial.

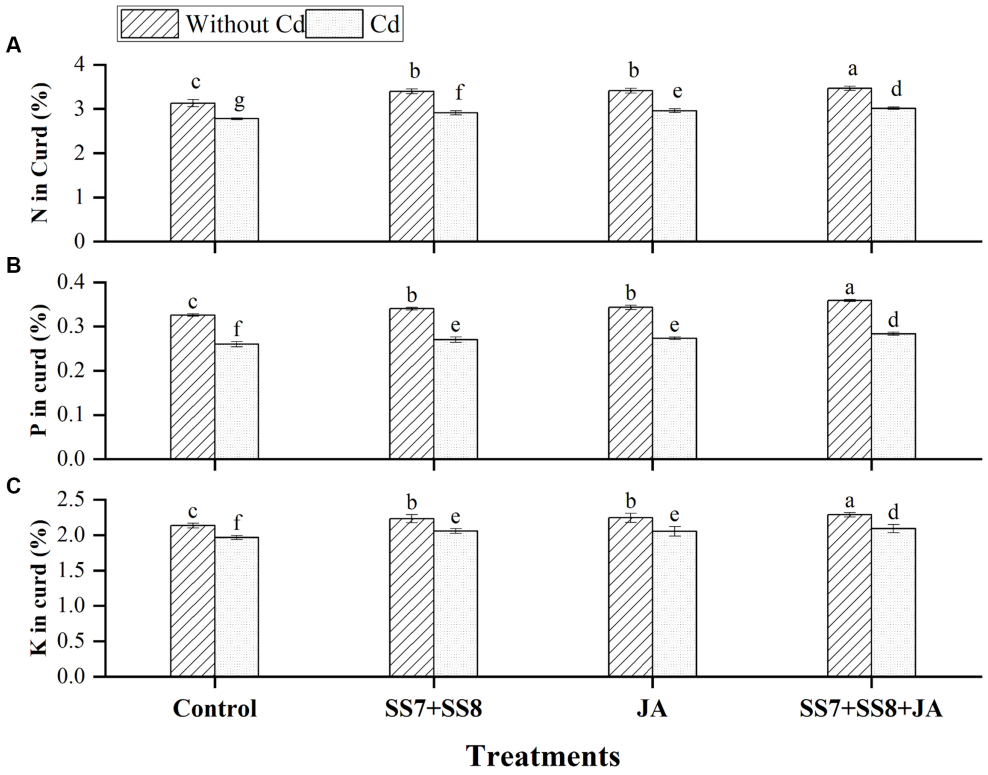


FIGURE 4
Effect of integrating bacterial consortium with jasmonic acid on **(A)** N in curd ($p = 0.0435$); **(B)** P in curd ($p = 0.0167$); and **(C)** K in curd ($p = 0.0331$) of cauliflower plant under Cd stress in a pot trial.

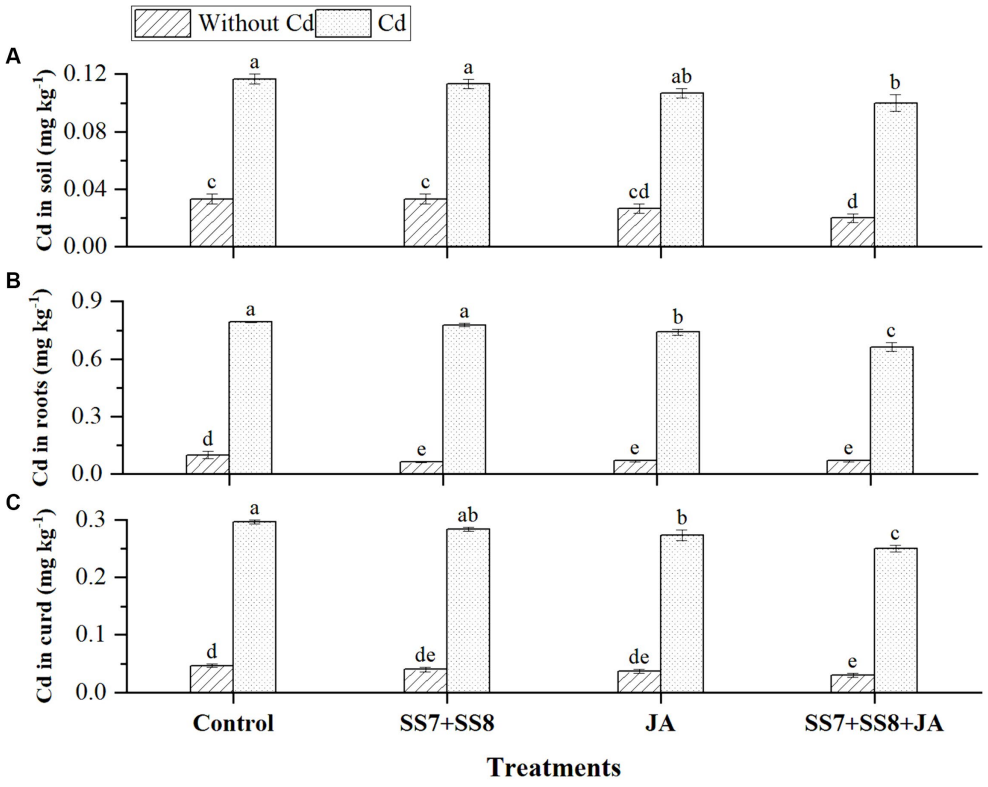


FIGURE 5
Effect of integrating bacterial consortium with jasmonic acid decreases **(A)** Cd in soil ($p = 0.0112$); **(B)** Cd in roots ($p = 0.0244$); and **(C)** Cd in curd ($p = 0.0138$) of cauliflower plant under Cd stress in a pot trial.

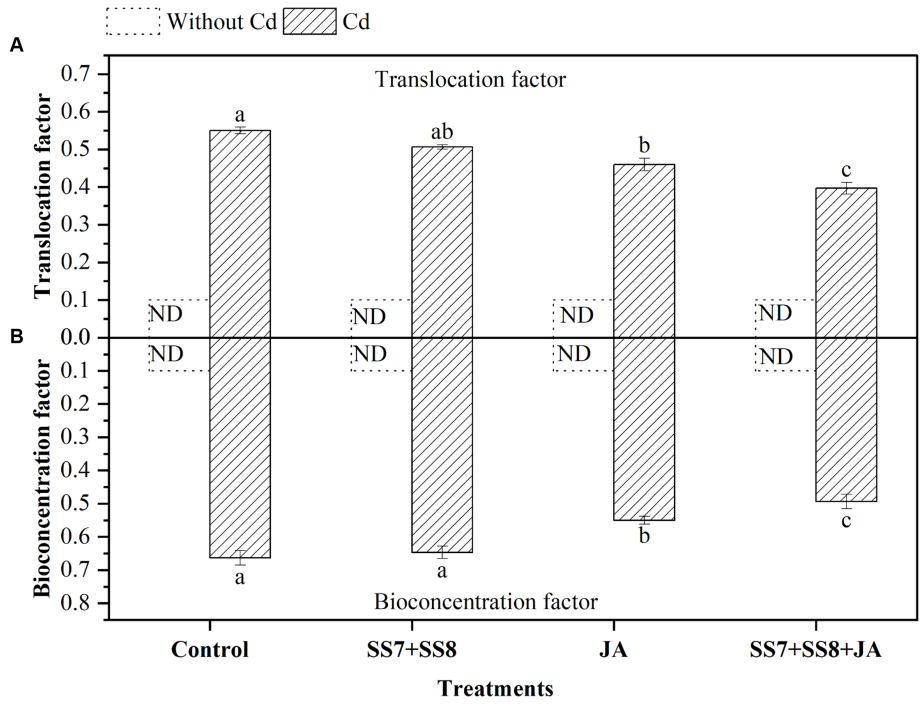


FIGURE 6
Effect of integrating bacterial consortium with jasmonic acid on **(A)** translocation factor ($p = 0.0001$) and **(B)** bioconcentration factor ($p = 0.0009$) of Cd under Cd stress in a pot trial.

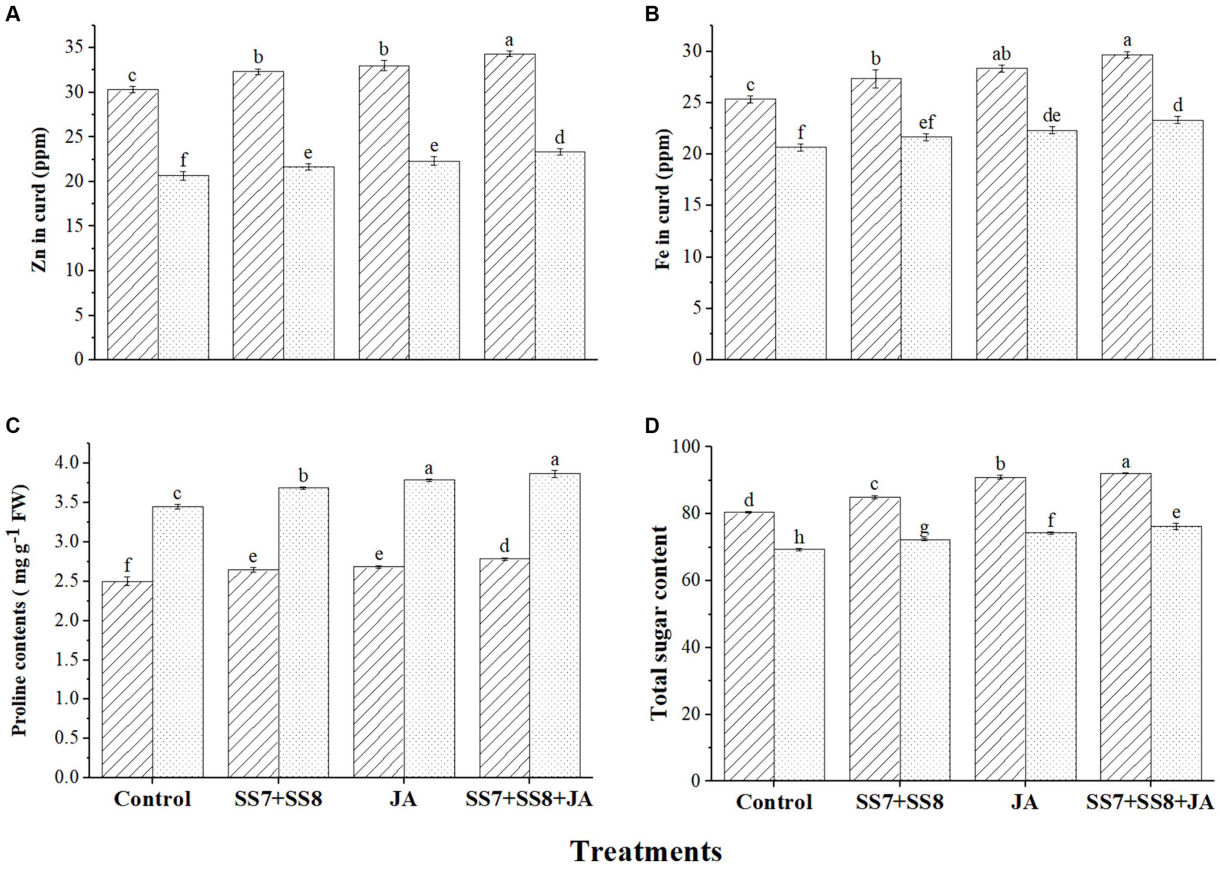


FIGURE 7
Effect of integrating bacterial consortium with jasmonic acid on **(A)** Zn in curd ($p = 0.0021$); **(B)** Fe in curd ($p = 0.0001$); **(C)** proline contents ($p = 0.0974$); and **(D)** total sugar content ($p = 0.1224$) of cauliflower plant under Cd stress in a pot trial.

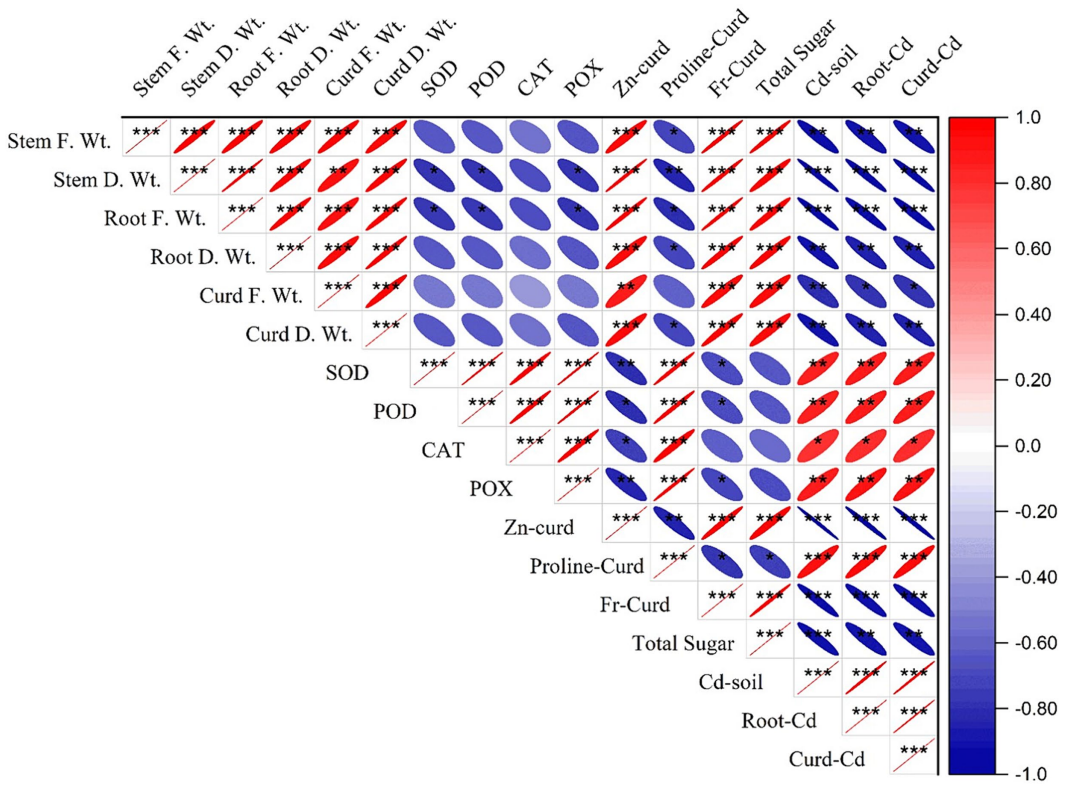


FIGURE 8
Correlation of Cd concentration in plants, with its different growth and physiological parameters. *p<0.05, **p<0.01, ***p<0.001.

maintained the same pace as the population (Grant, 2018). Moreover, the use of synthetic fertilizers, paints, mining, and ceramic industries has increased the concentration of Cd to exploit the whole ecosystem and environment (Hayat et al., 2019). The Cd concentration in Pakistani soil is more than 184 mg kg^{-1} and increasing day by day due to anthropogenic sources and this is an alarming situation as the threshold level is approximately 100 mg kg^{-1} for arable soils in Pakistan (Waseem et al., 2014; Asgher et al., 2015). However, the resources are insufficient to upgrade and meet the demands of the population. To combat all the nutritional and sustainable requirements of the global population, alternate and eco-friendly strategies need to be explored to immobilize and phytostabilize the metal in soil (Haider et al., 2021). In this regard, to mitigate the adverse effects of HMs, plant-growth-promoting metal-tolerant bacterial strains and plant growth regulators play an important role in mitigation strategies due to their environmental-friendly and cost-effective nature in the environment. These metal-tolerant bacterial strains utilize carbon in their niche as a food source, ultimately improving plant growth. These PGPRs bind the various functional groups on their surface, ultimately sequestering and immobilizing metal to reduce toxicity (Shabaan et al., 2022). Moreover, exogenously applied growth regulators play a significant role in crop physiology and stress defense in plants, ultimately mitigating the abiotic stresses (Rizwan et al., 2016b). Hence, the current study examined the individual and combined application of a Cd-tolerant integrating bacterial consortium in combination with JA on growth and yield attributes, production of antioxidants, mineral analysis, and Cd content in cauliflower under Cd stress.

In the current study, a reduction in growth attributes was observed due to Cd stress, which might have decreased the potential use of nutrients and water (Ahmad et al., 2015). A significant decline was observed in chlorophyll content due to the uptake of Cd in the cauliflower plant due to higher stress-generating ethylene levels, which might be a reason for the significant reduction in growth and chlorophyll content. Glick et al. (1999) reported that ethylene production under stress conditions induced a negative and harmful impact on crops' productivity. Matile et al. (1997) also reported the mechanism by which, under stress conditions, ethylene accumulates in plants and promptly decomposes lipids in the cell wall. When lipids degrade in plants, ethylene comes into contact with chlorophyllase genes and activates the chloroplast, which ultimately degrades chlorophyll, resulting in poor photosynthesis and chlorophyll content. The chlorophyll content was significantly improved with the application of bacterial consortium and plant growth regulator due to less accumulation of ethylene by the treatments applied (Danish et al., 2020). These PGPRs secrete IAA and ACC deaminases that cleave ethylene into ammonia and are utilized as nitrogen sources by PGPRs (Zafar-Ul-Hye et al., 2020). The findings were also supported by Dutta et al. (2018), who reported a reduction in root and shoot lengths due to Cd stress in *Brassica juncea* L. Moreover, we observed that Cd reduced the length and dry weight of shoots and roots due to a reduction in photosynthesis activity and restricted water and mineral uptake by roots (Hasan et al., 2020). However, the literature also supported and demonstrated that under Cd stress, the root and shoot length of *Vigna mungo* reduced due to the uptake of Cd (Rizvi and Khan, 2018). The reason for the Cd-induced decline in plant growth

is attributed to photosynthetic activity and restricted water and mineral uptake by the roots (Sharma et al., 2020). We observed a synergistic effect of the dual combination of *Klebsiella* strains with the foliar application of JA, which significantly improves plant growth by enhancing photosynthetic activity and root proliferation. Moreover, due to the synergistic effect, a Cd-tolerant bacterial consortium in combination with JA binds the Cd by immobilizing its mobility into plants via roots and restricting entry to the aerial part of the plant. Naveed et al. (2020) and Bali et al. (2018) reported that the Cd-tolerant bacterial strains and foliar application of JA mitigate Cd, Cu, and Pb stress in *Arabidopsis thaliana* and tomato plants. The effective use of Cd-tolerant bacterial strains also improves the growth and development of plants, as described in previous studies (Shahid et al., 2023).

Under HM stress, the overproduction of ROS and H_2O_2 damages the plant cell and membrane structure; however, the production of antioxidants in response to abiotic stress is a significant indicator of stress tolerance (Kusvuran, 2021; Rolon-Cardenas et al., 2022). Our results demonstrated that the combined application of bacterial consortium and JA increased the antioxidant status in cauliflower plant leaves compared to the control treatments under Cd toxicity. Mir et al. (2018) also reported that the use of bacterial consortium and foliar application of JA enhanced the higher production of antioxidant enzymes such as SOD, POD, CAT, and POX under Cd stress. Several studies have shown that foliar application of JA could regulate the redox potential of plants. Kamran et al. (2021) also reported that under Cd and Cr stress, the level of antioxidant status increased under different vegetable crops with the application of JA. The study is also in line with Pramanik et al. (2017), who reported the positive effect of inoculation on antioxidant status by using *Klebsiella pneumoniae* strain K5 under Cd stress.

The rate of Cd accumulation in plant parts from soil varies and is transported from soil to roots depending on plant species and the available soil fraction. In the current study, the bacterial consortium reduced the uptake of Cd from soil to roots and decreased the Cd accumulation by foliar application of JA on the surface of leaves during growth stages. A similar study was also reported by Zeng et al. (2022) that Cd-tolerant bacterial strains reduced the Cd uptake by immobilization in the soil, thus enhancing the sequestration of Cd in soil by producing polymeric substances, phytohormones, and siderophores.

Moreover, the reduction in Cd concentration in cauliflower was associated with the ability of bacterial consortium and foliar application of JA. The use of bacterial consortium binds the Cd by chelating agents in soil and makes it less available for plant uptake. The translocation factor indicates the ability of Cd transportation from one tissue to the next, while the bioconcentration factor indicates the ability of Cd accumulation by cauliflower. In the current study, the Cd-tolerant bacterial consortium inhibits Cd uptake from soil to roots. In contrast, foliar application of JA inhibits Cd uptake from the roots to the curd of cauliflower.

Under stress conditions, plants use different adjustments to overcome the negative impact of environmental stresses in which proline and other metabolites accumulate in plants and act as protective agents (Nadeem et al., 2015; Tanveer et al., 2022). Irfan et al. (2014) and Ahmad et al. (2019) reported higher production of proline levels under Cd stress by different plants. When proline and other metabolites are produced in plants, they boost metabolic energy by adjusting intracellular osmotic potential and regulating the metabolic process, thus protecting the plant against abiotic stresses (Rai et al., 2003; Silveira et al., 2003). Our study revealed that Cd-tolerant bacterial strains with the potential for EPS production enhance the level of proline due to a reduction in the negative effects of Cd, which is also supported by Khan and Bano (2019). The cauliflower curd was also evaluated for mineral content, and the results revealed that the combined application of bacterial consortium and JA significantly improved Fe, Zn, and total sugar content in the curd as compared to the control treatment. The results are also in line with the studies by Hussain et al. (2011) and Anwar et al. (2022), who reported a significant increase in nutrient contents in soil, roots, and curd of cauliflower plant. In the current study, pre-identified Cd-tolerant bacterial strains *Klebsiella* sp. (SS7) and *Klebsiella pneumoniae* (SS8) and foliar application of JA were examined under pot conditions to check their efficacy for the productivity of the cauliflower crop. However, the dual functionality of integrating the bacterial consortium in combination with JA significantly improved overall results as compared to the un-inoculated control treatment. Thus, bacterial consortium along with JA can be used as a potential biofertilizer to promote productivity on a sustainable basis.

5 Conclusion

HM pollution caused by urbanization and industrialization poses a serious threat to vegetables, oilseed crops, and fodder. It also causes detrimental effects to humans when HMs enter the food chain. In the current study, the dual application of a Cd-tolerant integrating bacterial consortium and JA seemed to be a viable option to improve plant growth and antioxidants and reduce the uptake of Cd under Cd stress. Moreover, it also plays a significant role in detoxifying the metal from soil and improving plant growth in metal-stress conditions. Hence, the use of bacterial consortiums and growth regulators could be further enhanced, and it is needed to evaluate their efficacy to reduce the toxicity of other metals in various vegetable crops for sustainable crop production.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, accession numbers: MW829780 and MW829781.

Author contributions

SS: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing, Software. AD: Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Writing – original draft, Writing – review & editing, Visualization. AH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. IK: Investigation, Writing – review & editing, Software. ML: Supervision, Validation, Writing – review & editing. HA: Validation, Writing – review & editing, Data curation, Visualization. TM:

Validation, Writing – review & editing, Data curation, Visualization. SA: Funding acquisition, Validation, Writing – review & editing.

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Conflict of interest

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

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Cultivating resilience in wheat: mitigating arsenic toxicity with seaweed extract and *Azospirillum brasilense*

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Arsenic (As) toxicity is a serious hazard to agricultural land due to growing industrialization, which has a negative effect on wheat crop yields. To address this issue, using seaweed extract and *Azospirillum brasilense* has emerged as an effective strategy for improving yield under stress conditions. However, the combined application of *A. brasilense* and seaweed extract in wheat crops under As toxicity has not been fully explored. The effectiveness of combining *A. brasilense* and seaweed extract in reducing As toxicity in wheat production was examined in this study through a 2-year pot experiment with nine treatments. These treatments included a control with no additives and two As concentrations (50 and 70 μM). At 50 and 70 μM, As was tested alone, with seaweed extract, with *A. brasilense*, and both. Significant results were achieved in reducing As toxicity in wheat crops. Arsenic at 70 μM proved more harmful than at 50 μM. The application of *A. brasilense* and seaweed extract was more effective in improving crop growth rates, chlorophyll levels, and stomatal conductance. The combined application notably decreased As concentration in wheat plants. It was concluded that applying *A. brasilense* and seaweed extract not only improves wheat growth but can also improve soil parameters under As toxicity conditions by increasing organic matter contents, boosting nutrient availability, and increasing the production of antioxidant enzymes.

KEYWORDS

arsenic toxicity, *Azospirillum brasilense*, seaweed extract, soil organic matter, wheat growth

1 Introduction

Arsenic is a chemical element that occurs naturally and is known for being hazardous for crops. It is a metalloid widely distributed in soil, water, and the environment, occurring in various forms within the Earth's crust (Fatoki and Badmus, 2022). Although arsenic has various industrial uses, its toxicity to living things, such as people and plants, makes its presence in soil and water a serious problem (Ng et al., 2003; Du et al., 2023). Arsenic is harmful to soil because plants and microbes can easily absorb and store it. Arsenic interferes with crucial plant metabolic pathways, causing toxicity (Stazi et al., 2015). Arsenic interferes with the growth and development of plants by inhibiting essential enzymes involved in photosynthesis, respiration, and nutrient intake (Sharma et al., 2020). This disruption may cause plants to develop more slowly, produce fewer crops, and have fewer vigorous plants. Arsenic can also indirectly impact the microbial communities crucial for soil fertility and nutrient cycling (Bose et al., 2022).

The toxic effects of soil-borne arsenic are particularly harmful to wheat crops, which are staple crops in many world regions. Arsenic contamination in wheat-growing areas can have various detrimental effects on wheat yield (Zhang et al., 2009; Huang et al., 2019). Arsenic-contaminated soil can prevent wheat seeds from germinating, resulting in poor plant establishment (Tong et al., 2014). After germination, arsenic-exposed wheat plants could develop stunts due to poor photosynthesis and nutrient uptake, leading to shorter plants with fewer tillers (Luo et al., 2023). Arsenic buildup in wheat grains can affect crop productivity and quality, providing health dangers to consumers, including both people and animals. Arsenic can also prevent wheat plants from absorbing important nutrients like phosphorus and iron, resulting in nutrient imbalances and deficiencies (Saeed et al., 2021). Arsenic can change the physical and chemical properties of soil, causing soil deterioration and decreasing the agricultural output in impacted areas (Saeed et al., 2021; Koley et al., 2023). Arsenic contamination in agricultural soils significantly threatens crop safety and human health. In wheat, arsenic concentrations exceeding 0.1 mg/kg are considered potentially toxic and can lead to reduced growth and yield (Rasheed et al., 2018). The toxicity of arsenic also extends to soil microbes, where concentrations above 10 mg/kg can disrupt microbial communities and affect soil health (Zecchin et al., 2021). Addressing arsenic toxicity is crucial for maintaining sustainable agricultural practices and safeguarding food quality. Recent studies have highlighted various mitigation strategies, including the use of seaweed extracts and beneficial microorganisms such as *Azospirillum brasilense*, which have shown promise in alleviating arsenic stress in crops (Zhang et al., 2009; Vezza et al., 2020; Znad et al., 2022).

Seaweed extract, made from different marine algae, can boost wheat development. Seaweed extract can be used as a biostimulant or added as fertilizer to benefit wheat crops in a number of ways. Seaweed extract also contains a range of micronutrients, including many trace elements that can be a great source of essential nutrients for plant growth (Panda et al., 2022). Adding these minerals to wheat crops via seaweed extract promotes healthier plant growth by increasing wheat biomass and grain yield (Wedad et al., 2015). Natural plant growth regulators, including auxins, cytokinins, and gibberellins, are present in seaweed extract. The ability of these substances to protect wheat plants from environmental stresses, including drought, salt, and high temperatures, is vital. Seaweed extract can potentially promote wheat plant root growth by making more effective nutrient and water uptake available (Castlehouse et al., 2003).

Azospirillum brasilense is a plant growth-promoting rhizobacteria (PGPR) that positively impacts soil fertility and wheat growth. PGPR has the ability to form symbiotic relationships with many plants through the root system (Helman et al., 2012). *A. brasilense* can boost the nitrogen content of the plant and make crucial nutrients for plant growth available. *A. brasilense* plays a vital role in enhancing soil fertility (Zaheer et al., 2019a; Li et al., 2023). It can increase nitrogen availability, which directly helps to increase biomass and grain output (He et al., 2023; Yi et al., 2022). *A. brasilense* can produce different compounds, such as auxins and cytokinins, that can improve plant growth through various physiological processes. PGPR can lead to a wider and more effective root system in wheat crops by uptake of more nutrients and water (Zaheer et al., 2022; Wang et al., 2024). *A. brasilense* has demonstrated potential for reducing the harmful effects of heavy metals on plant growth and physiology (Vezza et al., 2022). Heavy metal contamination of soil can poison crops like wheat, decreasing growth and output. Certain heavy metals in the soil can be mobilized by *A. brasilense*, which reduces their availability for plant absorption (Helman et al., 2012; Vezza et al., 2020). *A. brasilense* is a useful tool for sustainable agriculture, especially in regions with heavy metal-contaminated soils. *A. brasilense* can enhance wheat growth and nitrogen availability, fostering root growth and improving overall plant health (Zaheer et al., 2019b).

Arsenic toxicity significantly threatens wheat crops, adversely affecting yield and quality. While prior research has demonstrated the individual benefits of *Azospirillum brasilense* and seaweed extract in mitigating various stressors, their combined effect on arsenic-affected wheat has not been extensively investigated. This study aims to fill this gap by exploring how the synergistic application of these two agents can alleviate arsenic-induced stress in wheat. We aim to elucidate the mechanisms through which *A. brasilense* and seaweed extract interact to improve wheat resilience under arsenic stress. By advancing our understanding of these interactions, this research seeks to contribute valuable insights that could inform more effective agricultural practices for managing arsenic contamination in wheat farming. We hypothesize that leveraging the combined effects of *A. brasilense* and seaweed extract will enhance the sustainability and productivity of wheat crops affected by arsenic.

2 Material and methods

2.1 Experimental layout and crop growth conditions

Pot experiments were conducted at the Khwaja Fareed University of Engineering and Information Technology (KFUEIT), Rahim Yar Khan, Pakistan, to understand the mitigation of arsenic toxicity with seaweed extract and *Azospirillum brasilense* in wheat crops. This is the 2nd experiment of our previous research reported by Zaheer et al. (2022), in which we observed that the arsenic

toxicity can be reduced with the application of *A. brasilense* and *trans-zeatin riboside* in the wheat crop, but *trans-zeatin riboside* is a chemical. Instead of using chemicals, we want to set an organic way to control arsenic toxicity, so we use seaweed extract with *A. brasilense* to control arsenic toxicity in wheat. This was a 2-year experiment with three replications in 2021–2022 and 2022–2023. An approved wheat variety from Punjab Seed Corporation, "Galaxy-2013," was used for the experiment. *A. brasilense* was obtained from the government college (GC) university, and Lahore and seaweed (*Sargassum denticulatum*) extract was collected from the coastal areas of Pakistan. Seaweed (*Sargassum denticulatum*) was air-dried in the shadow for 10 days, and after 10 days, it dried in the oven at 60°C for 48 h. Dried seaweed was crushed in the grinder to make powder. However, 500 ml of sterilized water was added to 500 g of the seaweed powder and stored at room temperature for use as a foliar spray (Ali et al., 2022). A 2-year experiment was conducted with a complete randomized design (CRD) having nine treatments (T_0 = control, T_1 = As 50 μM, T_2 = As 50 μM + seaweed extract, T_3 = As 50 μM + *A. brasilense*, T_4 = As 50 μM + seaweed extract + *A. brasilense*, T_5 = As 70 μM, T_6 = As 70 μM + seaweed extract, T_7 = As 70 μM + *A. brasilense*, T_8 = As 70 μM + seaweed extract + *A. brasilense*). Ten kilogram of sandy loam soil was used in each pot, and this soil was mixed with different concentrations of arsenic ($Na_3AsO_4.12H_2O$) solution as reported by Maghsoudi et al. (2020). *A. brasilense* was inoculated with wheat seeds as reported by Fukami et al. (2016) as per treatments. A total of 10 wheat seeds were sown in each pot on 20th November of each year (2021 and 2022). Seaweed extract was applied after every 20 days from the days of sowing. Irrigation water was applied at critical growth stages, and fertilizers (120–80–60 NPK kg ha^{-1}) were applied uniformly after calculation for a 1-foot sq pot as per recommendation in every treatment pot. The properties of the soil used in this experiment are given in Table 1.

2.2 Measured parameters

2.2.1 Growth-related parameters

All crop growth-related parameters, such as plant height, spike length, spikelets per spike, grains per spike, 1,000 grain weight, and grain yield per plant, were manually calculated using a meter rod and weight balance. Crop growth rate (CGR) was observed using the following formula, as reported by Watson (1958).

$$CGR\ (g/m^2/day) = (W_2 - W_1) / (t_2 - t_1)$$

where W_2, final biomass (grams) at time t_2 (usually measured in days); W_1: initial biomass (grams) at time t_1; t_2: final time (days); t_1: initial time (days).

Relative growth rate (RGR) and net assimilation rate (NAR) were observed with the formulas reported by Willians (1946),

$$RGR\ (g\ g^{-1}day^{-1}) = (\log_e W_2 - \log_e W_1) / (t_2 - t_1),$$

where $\log_e$ is the natural logarithm.

$$NAR = \{(W_2 - W_1) / (t_2 - t_1)\} \times \{\log_e L_2 - log\ L_1 / (L_2 - L_1)\},$$

where L_1 and L_2 are the leaf areas at t_1 and t_2, respectively.

2.2.2 Physiological parameters

Stomatal conductance (SC) and chlorophyll contents (CC) were observed with the use of an infrared gas analyzer (Cl-340 Handheld Photosynthesis System, USA) and chlorophyll meter (CL-1: Hansatech Instruments Ltd., UK), respectively. Relative water contents (RWC) were observed using the Barrs and Weatherley (1962) equation.

$$RWC\ (\%) = [(FW - DW) / (TW - DW)] \times 100,$$

where FW is the fresh weight, DW is the dry weight, and TW is the turgid weight.

To calculate the electrolyte leakage (EL), samples of the leaves were placed in the test tube and incubated with distilled water at 23°C for 24 h. After shaking the test tube, electrical conductivity (EC) was noted using an EC meter. Samples were autoclaved at 60°C for 15 min, and after that, samples were cooled at 25°C and measured the EC again. EL was observed with the procedure reported by Sullivan and Ross (1979) by following the formula.

EL Initial electrical conductivity = Final electrical conductivity.

2.2.3 Arsenic concentration in leaves, grains, and root

Plant samples were collected to observe arsenic (As) concentration, and any surface contaminants were removed by carefully cleaning them. Samples of leaves, grains, and roots were oven-dried at 60–70°C for 72 h and dissolved (0.25 g) in a tri-acid mixture until a clear solution as concentration was quantified with the use of ICP-AES (IRIS/AP, TJA-USA) as reported by Maghsoudi et al. (2020).

2.3 Soil-related parameters

Soil samples were collected at crop harvesting to analyze available N, P, K, and organic matter. Organic matter was measured using the Walkley and Black (1934) method. Soil extraction for available P was performed using sodium bicarbonate, as per Subbaiah and Asija (1956). For available K, extraction with ammonium acetate followed the method reported by Nelson and Heidel (1952). The Kjeldahl method was used to determine available N, involving digestion and subsequent titration to measure total N content. Phosphorus content was determined using spectrophotometric techniques at a wavelength of 882 nm, while potassium content was measured using flame photometry. Standard procedures were applied for calculating available N, P, and K. Soil organic carbon (SOC) was measured following the standard procedure by Sparks et al. (1996), and dissolved organic nitrogen (DON) and carbon (DOC) were determined using the method by Smolander and Kitunen (2002).

2.4 Statistical analysis

Data from the three replications were statistically analyzed at a 95% probability level using computer software Statistix 8.1 software and got results for two-way ANOVA. MS Excel was also used to put data into the software and to make figures.

2.5 Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript the author(s) used Chat GPT and Grammarly in order to improve the technical and English language of the paper. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

3 Results

Plant height significantly affects the biological yield of the wheat crop. The highest plant height (101.33 and 100.32 cm) was observed in T_0 under control treatment when no arsenic (As) stress was applied, followed by T_4 (99.34 and 98.12 cm) when As stress was at the level of 50 μM and *Azospirillum brasilense* and seaweed extract were applied in the combined form, and the lowest plant height was observed in T_5 (87.21 and 84.23 cm) when As stress was observed at the level of 70 μM with no soil amendment. The application of seaweed extract and *A. brasilense* under As stress also significantly affected spike length. The highest spike length (12.34 and 11.34 cm) was observed in T_0 (11.33 and 10.21 cm), followed by T_4, and the lowest spike length was observed in T_5 (06.23 and 05.11 cm). Spikelets per spike were also negatively affected by the As toxicity, but the combined application of seaweed extract and *A. brasilense* significantly improved the growth-related parameters of wheat crops. The highest spikelets (28.32 and 26.34) were observed in T_0, followed by T4 (26.33 and 25.12), and the lowest results were observed in T_5 (15.12 and 15.33) in both years (Table 2).

Different letters indicate significant differences between treatments. Data regarding the grains per spike were significantly affected by the application of seaweed extract and *A. brasilense* (Table 2). The highest grains per spike were observed in T_0 (49.33 and 48.12) under control treatment when there was no As toxicity and no soil amendment, followed by T_4 (47.32 and 46.32) when As toxicity was at the level of 50 μM having the seaweed extract and *A. brasilense* application, and the lowest grains per spike were observed in T_5 when As toxicity was at the level of 70 μM without any soil amendment in both years. Moreover, the 1,000-grain weight was also severely affected by the seaweed extract and *A. brasilense*. The highest 1,000-grain weight (39.12 and 36.12 g) was observed in T_0, followed by T_4 (36.43 and 34.12 g), and the lowest 1,000-grain weight was observed in T_5 (25.12 and 23.12 g). It was also observed that the *A. brasilense* is more effective than the seaweed extract in grain yield under As toxicity, but the combined application of both amendments is more effective. The highest grain yield per plant was observed in T_0 (1.54 and 1.41 g), followed by T_4 (1.43 and 1.32 g), and the lowest grain yield was observed in T_5 (0.78 and 0.52 g) in both years (Table 3).

Crop growth rate (CGR), relative growth rate (RGR), and net assimilation rate (NAR) were negatively affected under As toxicity, but the application of seaweed extract and *A. brasilense* significantly improved the CGR, RGR, and NAR. The highest CGR (9.87 and 9.75 g m^{-2} day^{-1}), RGR (4.547 and 4.423 g g^{-1} day^{-1}), and NAR

TABLE 1 Properties of the soil used in the experiment.

Parameters	2021–2022	2022–2023
Organic matter (%)	0.78	0.80
pH	7.48	7.49
EC (μS/cm)	227	228
T.S.S. (%)	0.51	0.53
Available-P (ppm)	5.15	5.18
Available-K (ppm)	112	113
Saturation percentage	35	37
Soil separates		
Sand (%)	37	38
Silt (%)	38	40
Clay (%)	25	22
Textural	Sandy loam	Sandy loam

TABLE 2 Synergistic effect of seaweed extract and *Azospirillum brasilense* on plant height, spike length, and number of spikelets per wheat spike under arsenic soil toxicity.

Treatments	Plant height (cm)		Spike length (cm)		Spikelets per spike	
	2021–2022	2022–2023	2021–2022	2022–2023	2021–2022	2022–2023
T_0 = control	101.33 a (±0.602)	100.32 a (±0.050)	12.34 a (±0.210)	11.34 a (±0. 063)	28.32 a (±0. 063)	26.34 a (±0.086)
T_1 = As 50 μM	94.23 de (±0.476)	93.12 e (±0.057)	09.23 d (±0.203)	08.11 d (±0.061)	20.12 e (±0. 798)	20.11 e (±0.093)
T_2 = As 50 μM + seaweed extract	95.54 d (±0.573)	94.23 d (±0.045)	09.45 d (±0.211)	08.33 d (±0.078)	22.54 d (±0. 657)	22.12 d (±0.078)
T_3 = As 50 μM + *A. brasilense*	97.23 c (±0.575)	96.34 c (±0.035)	10.23 c (±0.224)	09.34 c (±0.061)	24.11 c (±0. 532)	23.34 c (±0.086)
T_4 = As 50 μM + Seaweed extract + *A. brasilense*	99.34 b (±0.601)	98.12 b (±0.036)	11.33 b (±0.212)	10.21 b (±0.072)	26.33 b (±0. 336)	25.12 b (±0.078)
T_5 = As 70 μM	87.21 h (±0.441)	84.23 i (±0.046)	06.23 h (±0.212)	05.11 h (±0.072)	15.12 i (±0. 424)	15.33 i (±0.062)
T_6 = As 70 μM + seaweed extract	88.34 g (±0.447)	86.11 h (±0.062)	06.54 g (±0.232)	05.32 g (±0.064)	16.32 h (±0. 524)	17.12 h (±0.092)
T_7 = As 70 μM + *A. brasilense*	90.43 f (±0.551)	89.34 g (±0.037)	07.23 f (±0.243)	06.34 f (±0.062)	17.34 g (±0. 462)	18.34 g (±0.063)
T_8 = As 70 μM + seaweed extract + *A. brasilense*	92.12 e (±0.57)	91.34 f (±0.033)	08.21 e (±0.209)	07.54 e (±0.056)	19.12 f (±0. 063)	19.12 f (±0.078)

Different letters indicate significant differences between treatments.

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TABLE 3 Synergistic effect of seaweed extract and *Azospirillum brasilense* on grains per spike, 1,000 grain weight, and grain yield per wheat plant under arsenic soil toxicity.

Treatments	Grains per spike		1,000 grain weight (g)		Grain yield per plant (g)	
	2021–2022	2022–2023	2021–2022	2022–2023	2021–2022	2022–2023
T_0 = control	49.32 a (±1.028)	48.12 a (±1.321)	39.12 a (±0.644)	36.12 a (±0.598)	1.54 a (±0.066)	1.41 a (±0.0.45)
T_1 = As 50 µM	41.22 e (±1.035)	40.12 e (±1.243)	30.33 d (±0.634)	28.23 e (±0.543)	1.12 e (±0.054)	1.02 e (±0.047)
T_2 = As 50 µM + Seaweed extract	43.12 d (±1.087)	42.52 d (±1.245)	32.12 c (±0.624)	30.33 d (±0.532)	1.16 d (±0.056)	1.13 d (±0.052)
T_3 = As 50 µM + *A. brasilense*	45.45 c (±0.984)	44.12 c (±1.265)	34.11 bc (±0.578)	32.44 c (±0.562)	1.26 c (±0.057)	1.24 c (±0.051)
T_4 = As 50 µM + Seaweed extract + *A. brasilense*	47.32 b (±1.103)	46.32 b (±1.301)	36.43 b (±0.543)	34.12 b (±0.523)	1.43 b (±0.059)	1.32 b (±0.055)
T_5 = As 70 µM	33.12 i (±1.034)	32.12 i (±1.244)	25.12 h (±0.562)	23.12 i (±0.532)	0.78 i (±0.052)	0.52 i (±0.062)
T_6 = As 70 µM + Seaweed extract	35.21 h (±1.045)	34.66 h (±1.307)	26.45 g (±0.582)	24.65 h (±0.533)	0.83 h (±0.053)	0.58 h (±0.054)
T_7 = As 70 µM + *A. brasilense*	37.56 g (±1.043)	36.23 g (±1.306)	27.33 f (±0.532)	25.55 g (±0.542)	0.93 g (±0.055)	0.63 g (±0.057)
T_8 = As 70 µM + Seaweed extract + *A. brasilense*	39.23 f (±1.034)	38.12 f (±1.256)	28.64 e (±0.544)	26.54 f (±0.544)	1.00 f (±0.052)	0.76 f (±0.058)

Different letters indicate significant differences between treatments.

TABLE 4 Synergistic effect of seaweed extract and *Azospirillum brasilense* on crop-growth rate (CGR), relative growth rate (RGR), and net assimilation rate (NAR) of wheat at flag leaf stage under arsenic soil toxicity.

Treatments	CGR (g $m^{-2}day^{-1}$)		RGR (g g^{-1} day^{-1})		NAR (g $m^{-2}day^{-1}$)	
	2021–2022	2022–2023	2021–2022	2022–2023	2021–2022	2022–2023
T_0 = control	9.87 a (±0.050)	9.75 a (±0.062)	4.547 a (±0.043)	4.423 a (±0.051)	4.722 a (±0.025)	4.643 a (±0.033)
T_1 = As 50 µM	8.21 e (±0.052)	8.15 e (±0.067)	3.453 e (±0.045)	3.212 e (±0.052)	3.643 e (±0.027)	3.563 e (±0.032)
T_2 = As 50 µM + seaweed extract	8.34 d (±0.050)	8.25 d (±0.062)	3.634 d (±0.047)	3.445 d (±0.049)	3.823 d (±0.025)	3.765 d (±0.036)
T_3 = As 50 µM + *A. brasilense*	8.48 c (±0.056)	8.35 c (±0.068)	3.823 c (±0.045)	3.621 c (±0.051)	4.021 c (±0.025)	4.011 c (±0.038)
T_4 = As 50 µM + seaweed extract + *A. brasilense*	8.62 b (±0.062)	8.56 b (±0.059)	3.921 b (±0.042)	3.867 b (±0.053)	4.233 b (±0.026)	4.133 b (±0.038)
T_5 = As 70 µM	07.13 i (±0.064)	07.04 i (±0.061)	2.533 i (±0.042)	2.312 i (±0.056)	2.633 i (±0.031)	2.523 i (±0.031)
T_6 = As 70 µM + seaweed extract	07.24 h (±0.054)	07.16 h (±0.057)	2.732 h (±0.046)	2.572 h (±0.056)	2.832 h (±0.027)	2.642 h (±0.032)
T_7 = As 70 µM + *A. brasilense*	07.36 g (±0.052)	07.23 g (±0.059)	2.942 g (±0.045)	2.733 g (±0.048)	2.912 g (±0.028)	2.865 g (±0.034)
T_8 = As 70 µM + seaweed extract + *A. brasilense*	07.56 f (±0.058)	07.57 f (±0.061)	3.193 f (±0.042)	2.932 f (±0.047)	3.215 f (±0.021)	3.143 f (±0.034)

Different letters indicate significant differences between treatments.

(4.722 and 4.643 g m^{-2} day^{-1}) were observed in T_0 when there was no As toxicity and no soil amendment, followed by T_4 (CGR: 8.62 and 8.56 g $m^{-2}day^{-1}$, RGR: 3.921 and 3.867 g g^{-1} day^{-1}, NAR: 4.233 and 4.133 g $m^{-2}day^{-1}$) when As toxicity was at the level of 50 µM with seaweed extract and *A. brasilense*. The lowest CGR (7.13 and 7.04 g $m^{-2}day^{-1}$), RGR (2.732 and 2.572 g $g^{-1}day^{-1}$) and NAR (2.633 and 2.523 g m^{-2} day^{-1}) were observed in T_5 when As toxicity was at the level of 70 µM in both years (Table 4).

Stomatal conductance (SC) was significantly affected by the application of seaweed extract and A. brasilense under arsenic (As) toxicity. Maximum SC (323 and 321 mmol m^{-2} s^{-1}) was observed under control treatment (T0) when there was no As toxicity and no application of seaweed extract and *A. brasilense*, followed by T_4 (313 and 310 mmol m^{-2} s^{-1}) when As toxicity was at the level of 50 µM having the application of seaweed extract and *A. brasilense*, and the lowest SC (231 and 310 mmol m^{-2} s^{-1}) was observed in T_5 when the As toxicity was 70 µM having no application. Relative water content (RWC) and chlorophyll content (CC) were also decreased under As toxicity and improved with the application of seaweed extract and *A. brasilense*. The highest RWC (70.32 and 69.23%) and CC (52.23 and 51.23%) were observed in T_0, followed by T_4 (RWC: 68.88 and 67.45%, CC: 50.56 and 49.33%), and the lowest results (RWC: 54.33 and 53.12%, CC: 40.23 and 38.12%) were observed in T_5 in both years (Table 5).

Electrolyte leakage (EL) was significantly enhanced under As toxicity and controlled with the application of seaweed extract and *A. brasilense* (Figure 1) in both years (2021–2022 and 2022–2023). Maximum EL (70.34 and 68.45%) was observed in T_5 when As toxicity was at the level of 70 µM having no application of seaweed extract and *A. brasilense*, followed by T_6 (65.12and 62.34%) when there was As toxicity at the level of 70 µM having the application of seaweed, and the lowest EL (19.25 and 18.34%) was observed in T0 when there was no As toxicity and no application of anything. Arsenic concentration in root, leaf, and grains also significantly increased under As toxicity, and the application of seaweed extract and *A. brasilense* was very effective in controlling their negative

TABLE 5 Synergistic effect of seaweed extract and *Azospirillum brasilense* on stomatal conductance (SC), relative water contents (RWC), and chlorophyll contents (CC) of wheat at flag leaf stage under arsenic soil toxicity.

Treatments	SC (mmol $m^{-2}s^{-1}$)		RWC (%)		CC (%)	
	2021–2022	2022–2023	2021–2022	2022–2023	2021–2022	2022–2023
T_0 = control	323 a (±1.452)	321 a (±1.323)	70.32 a (±0.906)	69.23 a (±0.734)	52.23 a (±0.753)	51.23 a (±0.634)
T_1 = As 50 µM	284 e (±1.444)	281 e (±1.342)	65.32 e (±0.912)	64.33 e (±0.745)	47.34 e (±0.764)	46.32 e (±0.732)
T_2 = As 50 µM + seaweed extract	292 d (±1.305)	289 d (±0.1.343)	66.46 d (±0.914)	65.12 d (±0.765)	48.33 d (±0.723)	47.35 d (±0.612)
T_3 = As 50 µM + *A. brasilense*	299 c (±1.245)	297 c (±1.367)	67.34 c (±0.908)	66.13 c (±0.788)	49.34 c (±0.712)	48.35 c (±0.633)
T_4 = As 50 µM + seaweed extract + *A. brasilense*	313 b (±1.255)	310 b (±1.355)	68.88 b (±0.908)	67.45 b (±0.789)	50.56 b (±0.721)	49.33 b (±0.644)
T_5 = As 70 µM	231 i (±1.254)	228 i (±1.344)	54.33 i (±0.903)	53.12 i (±0.799)	39.32 i (±0.713)	37.23 i (±0.643)
T_6 = As 70 µM + seaweed extract	236 h (±1.232)	234 h (±1.387)	55.32 h (±0.903)	54.33 h (±0.766)	40.23 h (±0.721)	38.12 h (±0.621)
T_7 = As 70 µM + *A. brasilense*	245 g (±1.342)	242 g (±1.377)	56.34 g (±0.911)	55.56 g (±0.734)	41.34 g (±0.745)	39.34 g (±0.632)
T_8 = As 70 µM + seaweed extract + *A. brasilense*	249 f (±1.234)	246 f (±1.379)	57.33 f (±0.921)	56.65 f (±0.732)	42.45 f (±0.745)	40.23 f (±0.641)

Different letters indicate significant differences between treatments.

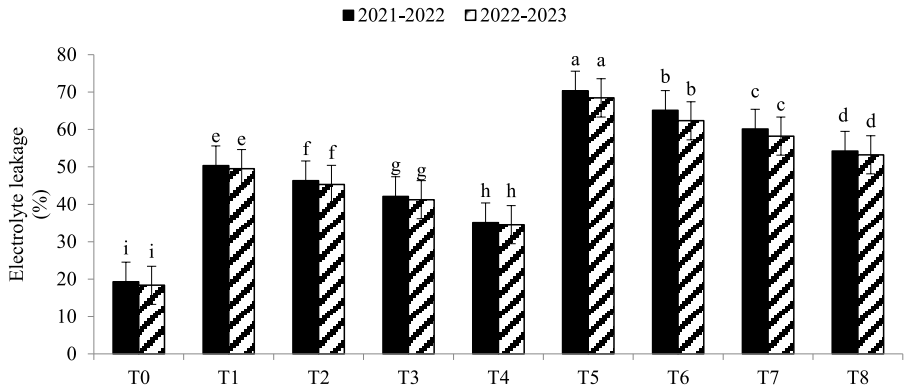


FIGURE 1
Synergistic effect of seaweed extract (SwE) and *Azospirillum brasilense* (B) on electrolyte leakage (EL) of wheat at flag leaf stage under arsenic (As) soil toxicity.

effect (Figures 2–4). The highest As concentration in the root (26.34 and 25.34 mg kg^{-1}), leaf (12.98 and 12.89 mg kg^{-1}), and grains (6.88 and 6.81 mg kg^{-1}) were observed in T_5, followed by T_6 (As in root: 24.32 and 23.12 mg kg^{-1}, As in leaf: 11.57 and 11.34 mg kg^{-1}, As in grains: 5.99 and 5.92 mg kg^{-1}) and the lowest (As in root: 6.32 and 6.27 mg kg^{-1}, As in leaf: 2.98 and 2.87 mg kg^{-1}, As in grains: 0.32 and 0.30 mg kg^{-1}) was observed in T_0 in both years.

Available N, P, and K in the soil were significantly affected by all studied treatments (Table 6). The highest available N (33.45 and 33.32 mg kg^{-1}), P (16.34 and 16.25 mg kg^{-1}), and K (61.34 and 58.23 mg kg^{-1}) were observed under control treatment (T_0) when there was no application of seaweed extract and *A. brasilense,* followed by T_4 (available N: 32.33 and 31.21 mg kg^{-1}, available P: 15.21 and 14.65 mg kg^{-1}, available K: 57.33 and 56.43 mg kg^{-1}) when there were As toxicity at the level of 50 µM having the seaweed extract and *A. brasilense*. The lowest N, P, and K were observed in T_5 (available N: 24.56 and 23.65 mg kg^{-1}, available P: 09.34 and 09.21 mg kg^{-1}, and available K: 49.32 and 50.23 mg kg^{-1}) when there was As toxicity at the level of 70 µM having no application of seaweed extract and *A. brasilense*. Soil organic carbon (SOC), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON) are also negatively affected under As toxicity. The combined application of seaweed extract and *A. brasilense* is very effective in enhancing SOC, DOC, and DON under As toxicity (Table 7). The highest SOC (1.64 and 1.62 g kg^{-1}), DOC (152.32 and 150.34 µg g^{-1}), and DON (23.87 and 21.23 µg g^{-1}) were observed in T_0, followed by T_4 (SOC: 1.45 and 1.42 g kg^{-1}, DOC: 147.54 and 144.56 µg g^{-1}, DON: 20.34 and 17.33 µg g^{-1}), and the lowest results (SOC: 0.72 and 0.71 g kg^{-1}, DOC: 123.54 and 121.23 µg g^{-1}, DON: 13.45 and 12.34 µg g^{-1}) were observed in T_5 in both years.

4 Discussion

Seaweed extract and *Azospirillum brasilense* can have noticeable effects on plant height, spike length, and spikelets per spike, especially under arsenic (As) toxicity. These procedures present in

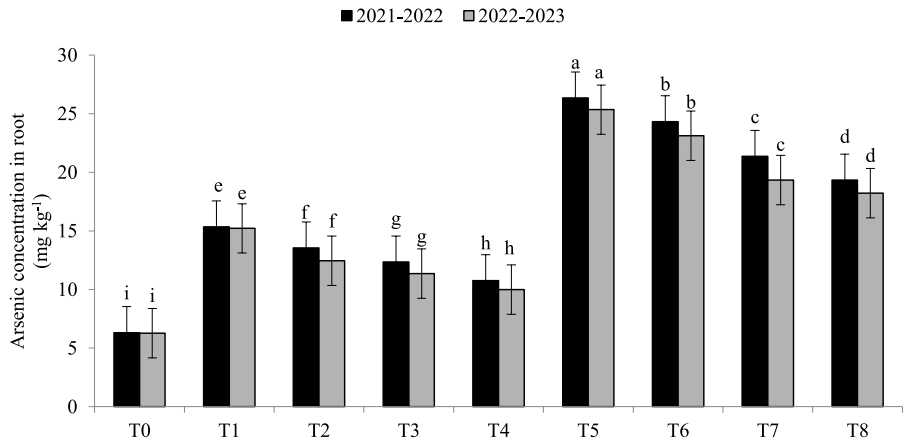


FIGURE 2
Synergistic effect of seaweed extract (SwE) and *Azospirillum brasilense* (B) on arsenic concentration in the root (mg kg^{-1}) of wheat at flag leaf stage under arsenic (As) soil toxicity.

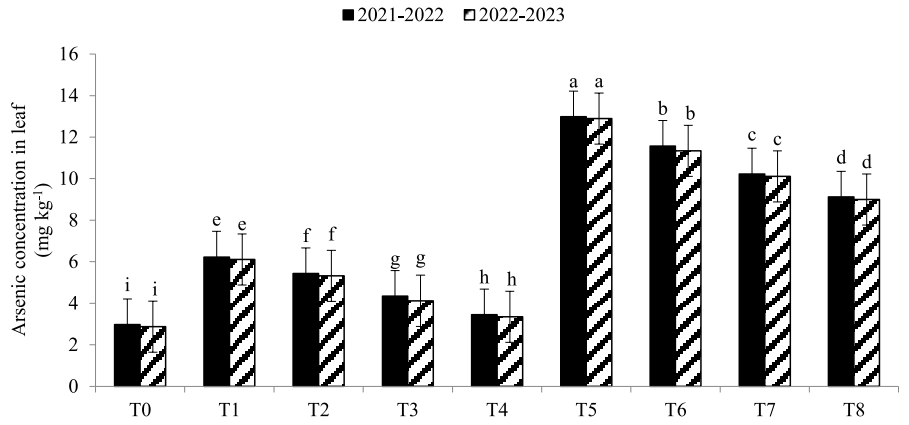


FIGURE 3
Synergistic effect of seaweed extract (SwE) and *Azospirillum brasilense* (B) on arsenic concentration in leaf (mg kg^{-1}) of wheat at flag leaf stage under arsenic (As) soil toxicity.

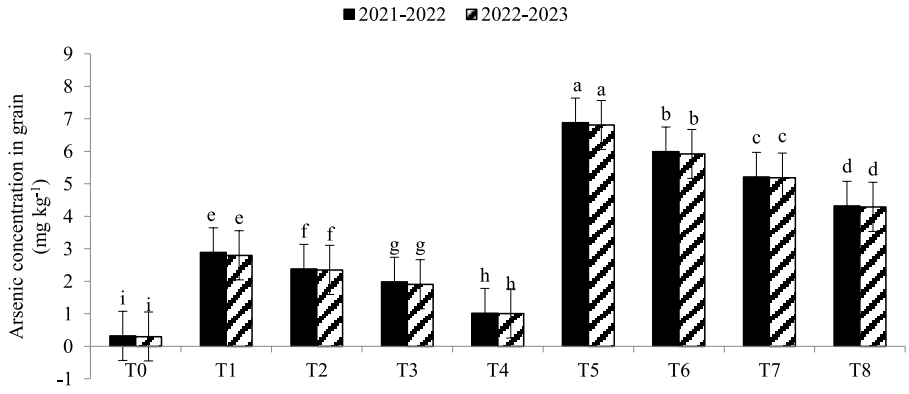


FIGURE 4
Synergistic effect of seaweed extract (SwE) and *Azospirillum brasilense* (B) on arsenic concentration in grains (mg kg^{-1}) of wheat at flag leaf stage under arsenic (As) soil toxicity.

TABLE 6 Synergistic effect of seaweed extract and *Azospirillum brasilense* on available soil nitrogen (N), phosphorus (P), and potassium (K) on wheat-grown soil under arsenic soil toxicity.

Treatments	Available N (mg kg^{-1})		Available P (mg kg^{-1})		Available K (mg kg^{-1})	
	2021–2022	2022–2023	2021–2022	2022–2023	2021–2022	2022–2023
T_0 = control	33.45 a (±1.046)	33.32 a (±1.057)	16.34 a (±0.128)	16.25 a (±0.109)	61.34 a (±0.623)	58.23 a (±0.512)
T_1 = As 50 μM	29.34 e (±1.032)	28.23 e (±1.052)	12.33 e (±0.134)	11.21 e (±0.108)	54.23 e (±0.670)	53.12 e (±0.532)
T_2 = As 50 μM + seaweed extract	30.12 d (±1.034)	29.23 d (±1.058)	13.23 d (±0.124)	12.23 d (±0.111)	55.13 d (±0.613)	54.23 d (±0.609)
T_3 = As 50 μM + *A. brasilense*	31.24 c (±1.053)	30.33 c (±1.053)	14.54 c (±0.128)	13.43 c (±0.112)	56.32 c (±0.622)	55.34 c (±0.576)
T_4 = As 50 μM + seaweed extract + *A. brasilense*	32.33 b (±1.046)	31.21 b (±1.046)	15.21 b (±0.126)	14.65 b (±0.106)	57.33 b (±0.632)	56.43 b (±0.512)
T_5 = As 70 μM	24.56 i (±1.035)	23.65 i (±1.048)	09.34 i (±0.128)	09.21 i (±0.109)	49.32 i (±0.666)	50.23 i (±0.513)
T_6 = As 70 μM + seaweed extract	25.43 h (±1.048)	24.12 h (±1.049)	09.43 h (±0.131)	09.45 h (±0.108)	50.33 h (±0.612)	51.21 h (±0.532)
T_7 = As 70 μM + *A. brasilense*	26.33 g (±1.049)	25.34 g (±1.047)	10.11 g (±0.134)	10.13 g (±0.108)	51.34 g (±0.631)	50.12 g (±0.532)
T_8 = As 70 μM + seaweed extract + *A. brasilense*	27.54 f (±1.046)	26.23 f (±1.043)	11.34 f (±0.126)	10.98 f (±0.102)	52.33 f (±0.634)	51.67 f (±0.543)

Different letters indicate significant differences between treatments.

TABLE 7 Synergistic effect of seaweed extract and *Azospirillum brasilense* on soil organic carbon (SOC), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON) of wheat-grown soil under arsenic soil toxicity.

Treatments	SOC (g kg^{-1})		DOC (μg g^{-1})		DON (μg g^{-1})	
	2021–2022	2022–2023	2021–2022	2022–2023	2021–2022	2022–2023
T_0 = control	1.64 a (±0.026)	1.62 a (±0.031)	152.32 a (±1.023)	150.34 a (±1.032)	23.87 a (±0.624)	21.23 a (±0.623)
T_1 = As 50 μM	1.20 e (±0.028)	1.19 e (±0.032)	141.23 e (±1.012)	139.23 e (±1.035)	17.32 e (±0.612)	15.34 e (±0.655)
T_2 = As 50 μM + seaweed extract	1.29 d (±0.028)	1.28 d (±0.035)	143.45 d (±1.014)	140.21 d (±1.032)	18.34 d (±0.634)	16.34 d (±0.623)
T_3 = As 50 μM + *A. brasilense*	1.36 c (±0.029)	1.33 c (±0.036)	145.33 c (±1.025)	142.34 c (±1.035)	19.54 c (±0.623)	16.99 c (±0.633)
T_4 = As 50 μM + seaweed extract + *A. brasilense*	1.45 b (±0.032)	1.42 b (±0.034)	147.54 b (±1.026)	144.56 b (±1.037)	20.34 b (±0.632)	17.33 b (±0.643)
T_5 = As 70 μM	0.72 i (±0.034)	0.71 i (±0.032)	123.54 i (±1.029)	121.23 i (±1.031)	13.45 i (±0.632)	12.34 i (±0.632)
T_6 = As 70 μM + seaweed extract	0.81 h (±0.032)	0.79 h (±0.036)	125.23 h (±1.028)	123.45 h (±1.032)	14.34 h (±0.634)	13.45 h (±0.612)
T_7 = As 70 μM + *A. brasilense*	0.89 g (±0.043)	0.87 g (±0.038)	127.33 g (±1.023)	124.56 g (±1.034)	15.34 g (±0.632)	14.56 g (±0.623)
T_8 = As 70 μM + seaweed extract + *A. brasilense*	0.97 f (±0.036)	0.95 f (±0.038)	129.45 f (±1.026)	126.34 f (±1.036)	16.34 f (±0.638)	15.66 f (±0.621)

Different letters indicate significant differences between treatments.

seaweed can reduce the damaging effects of arsenic poisoning on wheat growth. Auxins, cytokinins, and gibberellins are just a few of the phytohormones found in seaweed extract that have been shown to have growth-promoting properties (Blunden et al., 1968). Seaweed extract may counteract the growth-inhibiting effects of arsenic by promoting cell elongation and general plant growth when given to wheat plants exposed to arsenic-contaminated soil (Castlehouse et al., 2003). Increased plant height could be the end outcome, resulting in wheat plants that are stronger and healthier overall. *A. brasilense* can improve wheat development by fixing atmospheric nitrogen and making more nutrients available for plants (Zaheer et al., 2019a). The presence of *A. brasilense* in arsenic-toxic soils enhances the availability of more nutrients, whereas nutrient uptake may be hindered due to As toxicity (Zaheer et al., 2022). *A. brasilense* can increase spike length and spikelet development by assuring an adequate supply of crucial nutrients and enhancing growth hormone production (Zaheer et al., 2019b). Wheat plants under arsenic stress may benefit from the synergistic application of seaweed extract and *A. brasilense*. This dual treatment, as opposed to individual treatments, may result in a more notable improvement in plant height, spike length, and spikelets per spike by improving both nutritional availability and growth-promoting chemicals (Zaheer et al., 2019b, 2022).

The production of flowers and grains may be encouraged by seaweed extract, which is a rich source of plant growth regulators, potentially boosting the number of grains per spike (Carvalho et al., 2014). This is particularly crucial in soils with arsenic contamination, where plants' development ability may be compromised (Castlehouse et al., 2003). *A. brasilense* can supplement the benefits of seaweed extract by ensuring that the plant has access to the critical nutrients required for grain growth through its capacity to improve nutrient uptake and stimulate root development (Carvalho et al., 2014; Zaheer et al., 2019a). Applying seaweed extract and *A. brasilense* together increased the number of grains per spike. Micronutrients and trace components found in seaweed extract help plants better digest nutrients, which

increases grain weight (Margal et al., 2023). The ability of *A. brasilense* to fix nitrogen and solubilize nutrients can also improve grain weight (Zaheer et al., 2019a). These treatments can address nutritional deficits, resulting in heavier grains in arsenic-toxic soils. Grain yield can be significantly increased using seaweed extract and *A. brasilense*. *A. brasilense* enables effective nutrient utilization and stress tolerance (Zaheer et al., 2019b), while seaweed extract encourages plant growth and development (Begum et al., 2018). Because of this, wheat plants are more likely to have more well-developed spikes with more grains, increasing the amount of grain produced per plant.

The crop growth rate (CGR) measures how quickly a crop builds its biomass over time. Numerous growth-promoting substances, including phytohormones like auxins, cytokinins, and gibberellins, are present in seaweed extract (Blunden et al., 1968). These substances can promote cell lengthening and division, increasing the biomass produced by wheat plants. A crucial nutrient for plant growth can be made more readily available by *A. brasilense*. By encouraging cell growth and nutrient supply in As-contaminated soil, using seaweed extract and *A. brasilense* together can considerably increase CGR (Castlehouse et al., 2003; Zaheer et al., 2019a). Relative Growth Rate (RGR) is an indicator for evaluating how quickly a plant's biomass increases compared to its starting biomass. Wheat plants can better withstand the negative effects of arsenic toxicity because of the antioxidants and other beneficial substances found in seaweed extract (Deolu-Ajayi et al., 2021). *A. brasilense* can boost nutrient uptake and root growth (Zaheer et al., 2019a), which helps to improve plant vigor and, as a result, increase RGR (Zhang et al., 2023a). This indicates that treated wheat plants are utilizing their resources more effectively while developing quickly. The effectiveness of a plant in turning ingested carbon dioxide into biomass is indicated by its net assimilation rate (NAR). Seaweed extract can enhance NAR by enhancing photosynthetic efficiency, expanding the supply of carbon skeletons, and providing more energy for growth (El-Din, 2015). *A. brasilense* can support NAR by enhancing nutrient uptake, particularly nitrogen, which is crucial for photosynthesis and biomass production (Zaheer et al., 2019a). Combining these treatments in arsenic-contaminated soils can result in a more effective use of resources and a higher NAR. Stomatal conductance (SC) is a key factor governing the exchange of gases, mainly carbon dioxide and water vapor. Frequently decreased stomatal conductance was observed under arsenic toxicity due to the water loss (Bali and Sidhu, 2021). Seaweed extract can enhance SC by possessing certain chemicals that increase plants' tolerance to abiotic stresses. These substances can support or improve stomatal conductance, enabling the plant to continue photosynthesizing effectively even under challenging circumstances (Deolu-Ajayi et al., 2021; Zhao et al., 2023). *A. brasilense* can increase SC by ensuring the plant has enough water necessary for stomatal opening through its influence on root development and nutrient uptake (Zaheer et al., 2022). Plants often find it difficult to maintain relative water content (RWC) in arsenic-contaminated soils due to the damaging impact of arsenic on water intake and plant transport mechanisms. Osmoprotectants and antioxidants found in seaweed extract can help plants retain RWC by reducing the cellular damage brought on by arsenic (Saad-Allah and Nessim, 2016). *A. brasilense* can also help RWC by improving the plant's capacity for water absorption and retention and fostering root growth and nutrient uptake (Zaheer et al., 2019a). Higher As toxicity can lead to a higher reduction in crop growth (Zaheer et al., 2022), and the combined application of seaweed extract and *A. brasilense* is very effective in controlling the negative effect of As toxicity on wheat growth (Deolu-Ajayi et al., 2021; Zaheer et al., 2022). An important sign of a plant's photosynthetic activity and general health is its chlorophyll concentration. As arsenic exposure interferes with photosynthetic activities, chlorophyll concentration frequently decreases (Marcelle et al., 1983). Rich in micronutrients and growth-promoting substances, seaweed extract can boost chlorophyll synthesis and shield chloroplasts from arsenic-induced oxidative damage (Sati et al., 2021). Additionally, *A. brasilense* might indirectly improve CC by increasing the availability of nutrients, especially nitrogen, which is necessary for the synthesis of chlorophyll (Wang et al., 2020; Peng et al., 2021). To maintain or improve CC in wheat plants growing in arsenic-contaminated soil, seaweed extract and *A. brasilense* are used in combination and are very effective.

Seaweed extract and *A. brasilense* can greatly impact how much electrolyte leakage (EL) is in wheat plants cultivated in As-contaminated soil. Electrolyte leakage is a gauge of how well-maintained a plant cell membrane is (Bajji et al., 2002). Cell membrane damages when exposed to As toxicity due to stress conditions that can cause the leakage of electrolytes in plant cells (Hryvusevich et al., 2022). Antioxidants found in seaweed extracts are among the many bioactive substances that can help shield cell membranes from oxidative harm caused by arsenic toxicity. These substances have the capacity to lessen the degree of membrane permeability and the ensuing electrolyte leakage (Deuticke et al., 1987). Osmoprotectants are frequently present in seaweed extracts and might improve a plant's capacity to preserve membrane integrity under adverse conditions (Deolu-Ajayi et al., 2021). *A. brasilense* can decrease electrolyte loss by improving plant health by producing more growth hormones, especially cytokinin, which is helpful in cell division (Zaheer et al., 2022). *A. brasilense* improves nutrient uptake and root growth, both of which help the plant respond to arsenic stress more skillfully (Zaheer et al., 2019b). Wheat plants exposed to arsenic poisoning may experience less electrolyte leakage due to the presence of *A. brasilense* in the soil.

Arsenic concentrations in various areas of wheat plants growing in soil contaminated with arsenic toxicity can be greatly influenced by seaweed extract and *A. brasilense*. Arsenic ions in the soil can be bound by seaweed extract substances, making them less available for plant roots to absorb. Seaweed extracts have the ability to promote root growth, which reduces arsenic uptake by allowing the roots to penetrate less contaminated soil areas (Khan et al., 2009). *A. brasilense* lowers the concentration of arsenic in the roots by increasing the nutrient uptake and dilution of As concentration in the soil (Zaheer et al., 2022). Seaweed extract can reduce the stress caused by arsenic in plants through its antioxidants and stress-response components, resulting in healthier leaves with less arsenic buildup (Castlehouse et al., 2003; Khan et al., 2009). *A. brasilense* helps lower leaf arsenic concentration as it makes a nutrient barrier to uptake less As from the soil, which can assist in reducing arsenic levels (Gomes et al., 2012). Food safety requires lowering the content of arsenic in grains. Seaweed extract promotes grain development and guards against stress brought on by arsenic, resulting in healthier grains that accumulate less arsenic (Castlehouse et al., 2003; Khan et al., 2009). The ability of *A.*

brasilense to withstand stress and absorb nutrients may also reduce the amount of arsenic in grains (Zaheer et al., 2019b, 2022). The mechanism by which seaweed extract and *A. brasilense* mitigate arsenic toxicity involves multiple pathways. Seaweed extract is rich in bioactive compounds, such as polysaccharides and antioxidants (Castlehouse et al., 2003; Khan et al., 2009), which enhance plant tolerance to stress by scavenging reactive oxygen species and improving nutrient uptake. *A. brasilense* enhances root growth and nutrient assimilation, facilitating better arsenic detoxification and reducing uptake (Zaheer et al., 2022). Both treatments improve plant resilience and growth under arsenic stress, as observed in our results.

The solubility and mineralization of nutrients in the soil are improved by the chemicals found in seaweed extract, which increases the availability of nitrogen, phosphorus, and potassium (Zaheer et al., 2022; Zhang et al., 2023b). Applying seaweed extract can offer a conveniently available source of these crucial nutrients to assist wheat plant growth and development in arsenic-contaminated soils (Castlehouse et al., 2003; Cheng et al., 2024). *A. brasilense* contributes to increased nutrient availability in the soil through its function in root growth and nutrient uptake. By fixing atmospheric nitrogen and making it available to plants, this advantageous bacterium can help improve nitrogen availability (Zaheer et al., 2022). When seaweed extract and *A. brasilense* are used together, wheat plants receive access to the critical NPK nutrients.

Organic material found in seaweed extract can raise soil levels of organic carbon (SOC) and dissolved organic carbon (DOC). This organic material acts as a substrate for microbial activity once absorbed into the soil, encouraging the growth of advantageous soil microbes (Witzgall et al., 2021; Qiu et al., 2023). These microbes contribute to the buildup of SOC and DOC by decomposing organic materials. *A. brasilense* can encourage the breakdown of organic compounds, boosting SOC and DOC levels even further (Cook et al., 2022). Applying these treatments can aid in restoring and maintaining SOC and DOC, which are essential for soil structure, water retention, and nutrient cycling, in arsenic-contaminated soils where microbial activity may be impeded (Pal, 2020; Wu et al., 2024). The decomposition of organic matter and microbial activity in the soil is directly related to the availability of dissolved organic nitrogen (DON). By encouraging microbial development and improving nutrient cycling, seaweed extract, and *A. brasilense* can boost the release of DON into the soil solution. To provide a conveniently accessible nitrogen supply for plant absorption, DON must exist.

5 Conclusion

Arsenic toxicity significantly impairs wheat crop growth, exacerbating adverse effects as soil arsenic levels rise. This study highlights the beneficial impact of *Azospirillum brasilense* and seaweed extract in alleviating arsenic toxicity and promoting healthier, more resilient wheat plants. Both *A. brasilense* and seaweed extract individually enhance plant health by improving nutrient uptake, stimulating growth rates, and increasing antioxidant enzyme production, which helps plants cope with stress. The combined application of *A. brasilense* and seaweed extract proves particularly effective in mitigating arsenic toxicity. This synergy results in a more pronounced reduction in arsenic uptake by wheat plants, leading to better overall plant health and productivity.

The combination also enhances soil quality by increasing organic matter content and nutrient availability, fostering a more fertile and sustainable growing environment. The integrated use of *A. brasilense* and seaweed extract offers a robust, organic solution to managing arsenic toxicity in wheat crops. This approach not only supports sustainable agriculture by reducing reliance on chemical treatments but also provides a resilient strategy for maintaining wheat yield and quality in arsenic-contaminated soils. The findings of this study suggest that adopting such practices can significantly benefit farmers and agricultural communities, ensuring the long-term viability and productivity of wheat farming in regions affected by arsenic contamination.

Data availability statement

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

Ethics statement

All local, national, or international guidelines and legislation were adhered to for using plants in this study.

Author contributions

MZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft. NA: Validation, Writing – original draft. AH: Conceptualization, Validation, Writing – review & editing. NB: Validation, Writing – review & editing. SR: Data curation, Validation, Writing – review & editing. MR: Investigation, Validation, Writing – review & editing. AA: Formal analysis, Validation, Writing – original draft. MM: Resources, Validation, Writing – review & editing. MA: Investigation, Resources, Software, Validation, Writing – original draft.

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Conflict of interest

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

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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Nitrogen uptake, grain yield, and oil concentration of dwarf castor beans under nitrogen rates and inoculation of rhizobacteria in grasses–legumes rotation

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Introduction: Plant growth-promoting bacteria (PGPB) have been primarily studied for atmospheric nitrogen (N) fixation but they also have the capacity to improve nutrition and yield of crop plants.

Methods: Therefore, the objective of this research was to investigate the effects of inoculation with PGPB in association with different N rates on N uptake, grain yield, and oil concentration of dwarf castor beans in succession to legumes and grasses in Ilha Solteira, Brazil. The treatments consisted of N rates (0 to 180 kg ha^{-1} of N) and inoculation with three plant growth-promoting bacteria (*Azospirillum brasiliense, Bacillus subtilis*, and *Pseudomonas fluorescens*, applied by leaf) and a control with no-inoculation.

Results: The grain and oil yields of castor beans were increased by 20 and 40% at a rate of 103 kg ha^{-1} of N in succession to grasses as compared to without N application. In addition, the grain yield of castor bean after legumes was increased by 28, 64, and 40% with estimated rates of 97, 113, and 92 kg ha^{-1} of N in combination with inoculations of *A. brasilense*, *B. subtilis*, and *P. fluorescens* as compared to without N application, respectively. Shoot, grain, and total N uptake were improved with foliar inoculation of *A. brasilense*, *B. subtilis*, and *P. fluorescens* at the N rates of 45, 90, and 135 kg ha^{-1}, respectively.

Discussion and conclusions: Topdressing of N at the rate of 103 kg ha^{-1} and foliar inoculation in succession to grasses and 180 kg ha^{-1} of N without the effect of foliar inoculation in succession to legumes are recommended for higher grain and oil yield of castor beans. Foliar inoculations with *A. brasilense*, *B. subtilis*, and *P. fluorescens* increased grain yield under reduced use of N fertilizer by 44,

37, and 49% in dwarf castor cultivation in succession to legumes, potentially contributing to sustainable agriculture.

KEYWORDS

***Ricinus communis* L., nitrogen fertilization, *Azospirillum brasilense*, *Bacillus subtilis*, *Pseudomonas fluorescens*, oil yield**

1 Introduction

Castor beans (*Ricinus communis* L.) is a member of the Euphorbiaceae family and is widely grown in many tropical countries. Castor bean is an important non-edible oilseed plant that contains approximately 40–60% oil and 80–90% ricinoleic acid, which is most commonly used for medicinal and industrial purposes (Mckeon, 2016; Das et al., 2023). It has a unique composition of fatty acids that can be used as one of the competitive feedstocks to produce biodiesel and high-value biopolymers (Ramanjaneyulu et al., 2017). Castor grows at an amazingly fast rate due to its easy adaptability to unfavorable conditions that appeal to its expansion to tropical conditions, being the major cultivated regions in India, China, and Brazil (Omotehinse and Omidih, 2021). Some of the agroclimatic zones of Brazil's central and Cerrado regions are considered ideal conditions with the production of approximately 9.3 thousand metric tons of castor from an area of 5 thousand hectares (CONAB, 2024). The relative social and economic importance of castor in various industries demands the expansion of its cultivation under integrated and sustainable management of nitrogen (N) fertilizers and plant growth-promoting bacteria (PGPB).

Nitrogen is often one of the most limiting factors in the growth and development of crop plants (Galindo et al., 2024). Its deficiency affects the formation of photosynthesis and secondary metabolites, limiting light absorption and consequently plant growth (Urban et al., 2021). However, excessive or inadequate application of N fertilizers is contributing to the volatilization of the environment or leaching, which may pollute the atmosphere and water resources (Galindo et al., 2020). The inflation in socioeconomic costs of N fertilization has increased interest in the development of technologies such as inoculation with PGPB to reduce mineral fertilizer application (Lozada et al., 2018). The use of PGPB of the genera, *Azospirillum, Bacillus,* and *Pseudomonas* allows for crops with high-quality production, aiming at the progress of more sustainable technologies for agriculture under less impactful management (Mortinho et al., 2022). These inoculants exploit soil water and nutrients to increase root architecture and regulate different physiological processes that can contribute to higher use efficiency of nutrients under reduced fertilizer application (Moretti et al., 2020; Jalal et al., 2023a,b).

Castor beans can change the physical and chemical characterizations of soil such as decompression, soil structuring, and recycling nutrients from subsoil to surface soil, and consequently enhancing resource availability for the subsequent crop (Ferreira et al., 2006). The predecessor crop after legume is allowed with higher soil N availability due to higher biological nitrogen fixation (BNF) and straw decomposition (Silva et al., 2020; Te et al., 2022). In contrast, the predecessor crops after grasses/cereals may require greater N supply due to greater affinity for N, low BNF rate, and a higher C/N ratio in straw (Du et al., 2019; Galindo et al., 2020).

In the view of the abovementioned comprehensive literature, few studies have addressed the environmental and economic sustainability of castor cultivation, biomass residue utilization (Parascanu et al., 2019), and biodiesel production (Liang et al., 2013), but lack the impact of integrated use of N rates and foliar inoculation with PGPB on castor cultivation, nutrition, and yield in succussion to grasses/cereals and legumes. In this context, determining N rates that provide higher grain yield and oil yield of dwarf castor in a successor crop to grasses and legumes. In addition, the use of plant growth-promoting bacteria can manage the topdressing of N in dwarf castor beans. The objective of this research was to investigate the reduced rates of N in association with inoculation of *A. brasilense, B. subtilis,* and *P. fluorescens* for improving productive efficiency and nutrient uptake in succession to grasses and legumes in tropical conditions.

2 Materials and methods

2.1 Location and characterization of the experimental area

The research was carried out at the Research Station of the São Paulo State University (UNESP), Ilha Solteira, located in Selvíria–Mato Grosso do Sul–Brazil. The approximate geographical coordinates of the site were 51° 22' W, 20° 22' S, and an altitude of 335 m. This specific region is called "Brazilian Cerrado," which consists of gramineous woody savanna.

The soil of the experimental site is classified as red dystrophic (Santos et al., 2018). The current experiment was conducted in a no-tillage system with successive leguminous crops (soybean/common beans, before castor bean was soybean) and grasses crops (corn/wheat, before castor bean was corn).

Abbreviations: NR-G, number of racemes in succession to grasses; NR-L, number of racemes in succession to legumes; NG-G, number of grains in succession to grasses; NG-L, number of grains in succession to legumes; 100W-G, 100 grains weight in succession to grasses; 100W-L, 100 grains weight in succession to legumes; SDM-G, shoot dry matter in succession to grasses; SDM-L, shoot dry matter in succession to legumes; GY-G, grain yield in succession to grasses; GY-L, grain yield in succession to legumes; %O-G, oil seeds concentration in succession to grasses; %O-L, oil seeds concentration in succession to legumes; OY-G, oil yield in succession to grasses; OY-L, oil yield in succession to legumes; SNC-G, shoot nitrogen concentration in succession to grasses; SNC-L, shoot nitrogen concentration in succession to legumes; GNC-G, grain nitrogen concentration in succession to grasses; GNC-L, grain nitrogen concentration in succession to legumes; SNU-G, shoot nitrogen uptake in succession to grasses; SNU-L, shoot nitrogen uptake in succession to legumes; GNU-G, grain nitrogen uptake in succession to grasses; GNU-L, grain nitrogen uptake in succession to legumes; TNU-G, total nitrogen uptake in succession to grasses; TNU-L, total nitrogen uptake in succession to legumes.

Soil chemical attributes in the 0.0–0.20 m layer were determined before the initiation of the castor beans experiment (van Raij et al., 2001). The following results were obtained: (a) in the area of succession to grasses: 31.4 mg dm^{-3} of P (resin); 4 mg dm^{-3} of S-SO_4; 22 g dm^{-3} of OM; 5.7 of pH ($CaCl_2$); K, Ca, Mg, and H + Al = 2.2, 22.0, 14.0, and 28.0 $mmol_c$ dm^{-3}, respectively; Cu, Fe, Mn, and Zn (DTPA) = 3.1, 27.0, 41.2, and 2.3 mg dm^{-3}, respectively; 0.28 mg dm^{-3} of B (hot water); CEC = 66.2 $mmol_c$ dm^{-3} and 58% of base saturation; (b) in succession to legumes: 37.0 mg dm^{-3} of P (resin); 3 mg dm^{-3} of S-SO_4; 25 g dm^{-3} of OM; 5.7 of pH ($CaCl_2$); K, Ca, Mg, and H + Al = 4.4, 37.0, 24.0, and 31.0 $mmol_c$ dm^{-3}, respectively; Cu, Fe, Mn, and Zn (DTPA) = 5.5, 24.0, 84.7, and 2.5 mg dm^{-3}, respectively; 0.32 mg dm^{-3} of B (hot water); CEC = 96.4 $mmol_c$ dm^{-3} and 68% of base saturation.

The climate region is Aw as per the Köppen classification, which has been characterized as humid tropical with a rainy season in summer and a dry season in winter. The mean annual rainfall is 1,370 mm, the mean annual temperature is 23.5°C, and the relative humidity is between 70 and 80%. Mean rainfall and maximum and minimum temperatures during the cultivation of dwarf castor beans were monitored, as shown in Figure 1.

2.2 Experimental design and treatments

The experiment was designed in randomized blocks, arranged in a 5×4 factorial scheme with four replications. The treatments consisted of five N rates (0, 45, 90, 135, and 180 kg ha^{-1} of N, applied from urea as a source) and inoculation with three plant growth-promoting bacteria (*Azospirillum brasiliense, Bacillus subtilis,* and *Pseudomonas fluorescens*, applied by leaf) and a control with no-inoculation. The experimental field has a history with successive cultivation of legumes in the last 7 harvests (soybean/common bean—2017/2018, soybean/common bean—2018/2019, soybean/common bean—2019/2020, and soybean—2020/2021) and with successive cultivation of grasses in the last 7 harvests (corn/wheat—2017/2018, corn/wheat—2018/2019, corn/wheat—2019/2020, and corn—2020/2021). This allows us to determine and optimize the best rate of N in association with foliar inoculation of PGPB in dwarf castor beans.

2.3 Installation and management of the experiment

The castor bean seeds were mechanically sown in April 2021 with rows space of 0.90 m. The plots consisted of eight rows of 5.0 m length with plants spaced 0.30 m apart. The useful area consisted of four central lines, disregarding 0.50 m at both ends of each line. In addition, the sowing seeds were chemically treated with Standk Top® (fungicide and insecticide) at the rate of 2 g kg^{-1} of seeds an hour before the plantation.

The castor beans hybrid "MIA" from Kaiima®, São Paulo, Brazil with an average productivity of 1,400 kg ha^{-1} in a non-irrigated area and 3,000 kg ha^{-1} in an irrigated area was used in the experiment. It has a life cycle of 120–150 days, height of 100–140 cm, adapted to mechanized harvesting, seed oil content is approximately 45% ± 2%, and has a nematocidal effect, controlling nematodes of the *Meloidogyne incognita*, *M. javanica,* and *Pratylenchus brachyurus* species.

Weed management was carried out with pre-emergence herbicide Dual Gold® at a rate of 1.5 L ha^{-1} and post-emergence herbicide Gladium® at a rate of 20 g ha^{-1}, as recommended for castor bean or soybean crops. The recommended mineral fertilization was carried out in the sowing furrows at the rate of 250 kg ha^{-1} of NPK (8–28-16), providing 20 kg ha^{-1} of N, 70 kg ha^{-1} of P_2O_5, and 40 kg ha^{-1} of K_2O. The topdressing of N fertilizer was performed as per treatments, using urea (46% N) as a fertilizer source.

The supply of water was carried out by a fixed conventional sprinkler irrigation system with an average sprinkler precipitation of 3.3 mm h^{-1} and an average irrigation depth of 13 mm according to the necessity of the crop.

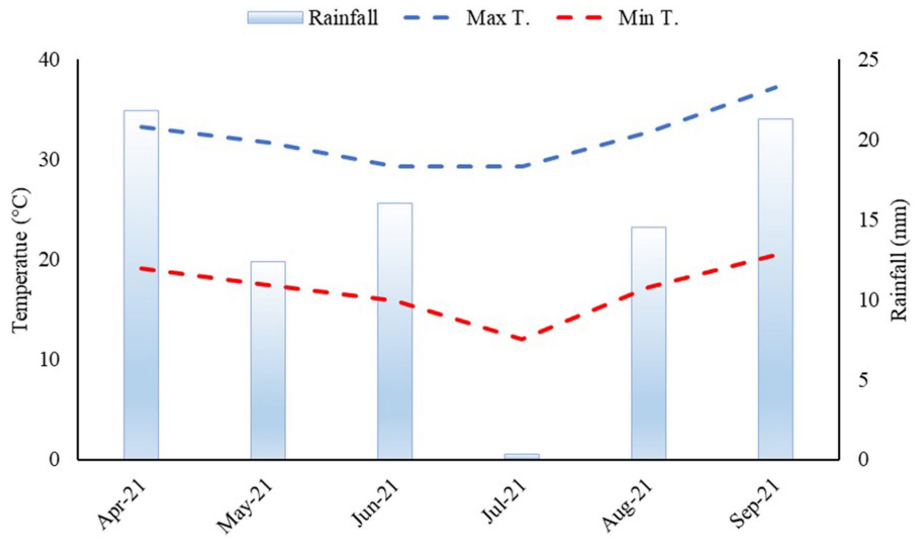


FIGURE 1
Rainfall, maximum, and minimum temperatures were acquired from the weather station of the Extension Research Farm of the Faculty of Engineering—UNESP during a dwarf castor cultivation period from April to September 2021.

2.4 Plant growth-promoting bacterial strains and their application

The PGPB treatments were applied as a foliar spray at the fourth-leaf vegetative growth stage (V4) of the dwarf castor beans. The solution was diluted with tap water and sprayed in the morning with the aid of a backpack sprayer at a flow rate of 300 L ha^{-1}. The droplet size was carefully regulated to prevent rolling-off of the leaf surface. We did not find an optimized foliar dose–response of PGPB, hence, doses for the foliar spray of the inoculants were selected following the manufacturer recommendations[1] and previous experiments with PGPB via seeds inoculation (Rosa et al., 2022; Jalal et al., 2023b).

The foliar application with *Azospirillum brasilense* strains, Ab-V5 and Ab-V6, was carried out at a rate of 100 mL of liquid inoculant ha^{-1} (which is equivalent to 100 mL of inoculant per 37,000 castor bean seeds, when applied to seeds) making a concentration of 2×10^8 forming unit of colony (CFU) mL^{-1}. Inoculation with *Bacillus subtilis* strain CCTB04 via leaf was performed at a rate of 100 mL of liquid inoculant ha^{-1} (equivalent to 100 mL of inoculant per 37,000 castor bean seeds, when applied by seeds) to get a concentration of 1×10^8 CFU mL^{-1}. Inoculation with *Pseudomonas fluorescens* strain CCTB03 via leaf was performed at a rate of 100 mL of liquid inoculant ha^{-1} (equivalent to 100 mL of inoculant per 37,000 castor bean seeds, when applied by seeds) to get a concentration of with 1×10^8 CFU mL^{-1}. All these inoculant strains are commercially registered with the Ministry of Livestock and Agriculture (MAPA), Brazil, and available under the trade names of AzoTotal® (MAPA PR Registration: 93923–10,074-1; *A. brasilense*), Vult® (MAPA PR Registration: 001593–8.000004; *B. subtilis*) and Audax® (MAPA PR registration: 16920; *P. fluorescens*).

2.5 Data collection and sample processing

The number of primary, secondary, and total racemes per plant was quantified at the end of the castor beans cycle. The number of grains was quantified by counting grains in primary and secondary racemes. A hundred-grain weight was estimated from the mean weight of one hundred grains by collecting and weighing 100 grains from each plot. The hundred grains weight was collected in both seasons, succession to cultivation of grasses (100W-G) and succession to legumes (100W-L). The grain yield was quantified by harvesting two central lines, threshed, and processed to measure the grain weight of each treatment with a precise scale.

The oil content of castor bean seeds was determined through the oil capture method by cold pressing. A 100 g of shelled castor beans were weighed and placed in a filter press adapted with a 10-ton mechanical press. The concentration of oil was weighed with a semi-analytical balance (g). The oil content in triplicate is expressed in percentage (%) of the product, adopting the simple arithmetic mean as the result. Oil yield was calculated via the formula.

$$\text{Oil yield}\left(\text{kg ha}^{-1}\right) = \text{Oil production}\left(\text{kg ha}^{-1}\right) \times \text{Oil content}\left(\%\right)$$

In addition, plant material was dried, weighed, and ground in a Wiley mill to determine N concentrations (Malavolta et al., 1997) in the shoot and grain of dwarf castor beans. The shoot N uptake and grain N uptake of the plants were calculated from the fraction of shoot dry matter and grain yield through the following equations:

$$\text{Shoot N uptake}\left(\text{kg ha}^{-1}\right) = \text{shoot dry matter}\left(\text{kg ha}^{-1}\right) \times \text{N concentration}\left(\text{g ha}^{-1}\right)$$

$$\text{Grain N uptake}\left(\text{kg ha}^{-1}\right) = \text{Grain yield}\left(\text{kg ha}^{-1}\right) \times \text{N concentration}\left(\text{g ha}^{-1}\right)$$

2.6 Statistical analysis

The results were analyzed using the F test in the analysis of variance. When the F test indicated significant differences, the means were compared using Tukey's test with a significance level of $p < 0.05$. This comparison was performed for the means of inoculations with PGPB while adjusting regression equations for the effect of nitrogen rates. All the statistical analyses were performed using the Sisvar statistical program (Ferreira, 2019).

3 Results

3.1 Effect of N rates and inoculations on the productive components

The effect of inoculations with PGPB and N rates and their interaction were not significant ($p > 0.05$) on the number of racemes (NR) and number of grains (NG) per plant of dwarf castor bean in succession to grasses and legumes (Table 1). The number of grains of castor beans in succession to grasses (NG-G) was adjusted to a linear increase in N rates (Table 1). The increasing N fertilizer in topdressing at a rate of 180 kg ha^{-1} was observed with higher calculated NG-G (109.6 grains) per plant of castor beans (Figure 2).

In addition, the effect of inoculation with PGPB and N rates, and their interaction was not significant ($p > 0.05$) on 100 grains weight in succession to grasses (100W-G). The 100 grains weight in succession to legumes (100W-L) was significant ($p < 0.01$) in the treatments with inoculations PGPB while not significant with N rates (Table 1).

Shoot dry mass of dwarf castor bean plants in succession to grasses (SDM-G) was significantly ($p < 0.01$) influenced by N rates and their interaction with inoculations of PGPB while shooting dry mass in succession to legumes (SDM-L) was significantly ($p < 0.01$) increased by interaction and treatments effects (Table 2). The SDM-G was increased with increasing N application in topdressing in the treatments without foliar inoculation. Inoculations with *A. brasilense, B. subtilis,* and *P. fluorescens* at estimated increasing N rates of 103, 99, and 63 kg ha^{-1} were observed with greater SDM-G as compared to N fertilization at a rate of 0 kg ha^{-1} (Figure 3A). The treatments with inoculation of *P. fluorescens* at the rates of 0 and 45 kg ha^{-1} of N

1 https://topbiobrasil.com.br/produtos/micro-bio/

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TABLE 1 Effect of nitrogen rates and inoculations of PGPB on the castor beans number of racemes (NR-G), number of grains (NG-G), and weight of 100 grains (100 W-G) in succession to grasses and number of racemes (NR-L), number of grains (NG-L), and weight of 100 grains (100 W-L) in succession to legumes.

Treatments	NR-G	NG-G	100 W-G	NG-L	NR-L	100 W-L
	N° plant^{-1}		g	N° plant^{-1}		g
Foliar inoculation						
Non-inoculation	5.83 a	97.70 a	38.36 a	82.41 a	5.40 a	36.50 ab
A. brasilense	6.08 a	103.27 a	36.61 a	93.17 a	5.13 a	35.75 b
B. subtilis	6.00 a	103.48 a	37.22 a	84.19 a	5.27 a	37.56 a
P. fluorescens	5.77 a	102.67 a	36.50 a	90.79 a	5.27 a	37.59 a
Nitrogen (N) rates (kgha^{-1})						
0	5.87	95.44	36.45	81.22	5.17	36.33
45	6.15	94.29	37.30	89.59	5.21	36.79
90	5.94	103.04	37.39	83.78	5.44	36.37
135	6.10	106.50	36.80	97.37	5.50	37.24
180	5.54	109.63	37.93	86.24	5.02	37.51
Mean	5.92	101.78	37.17	87.64	5.26	36.85
Standard error (±)	0.21	4.93	1.11	4.48	0.18	1.15
Source of variance						
Block	0.03*	0.07ns	0.05*	0.62ns	0.00**	0.08ns
Inoculation (I)	0.86ns	0.75ns	0.06ns	0.16ns	0.89ns	0.01**
N rates (R)	0.71ns	0.11ns	0.46ns	0.09ns	0.69ns	0.35ns
I*R	0.32ns	0.07ns	0.19ns	0.09ns	0.21ns	0.06ns
Linear	0.50ns	0.01**	0.19ns	0.20ns	0.99ns	0.07ns
Quadratic	0.30ns	0.86ns	0.95ns	0.23ns	0.23ns	0.62ns
CV (%)	22.30	18.92	6.38	19.75	20.28	5.32

ns, not significant, * and ** significant at $p < 0.05$ and $p < 0.01$ probability using the F test. Distinct letters in the column differ from each other using Tukey's test. PGPB, plant growth-promoting bacteria; CV, coefficient of variance.

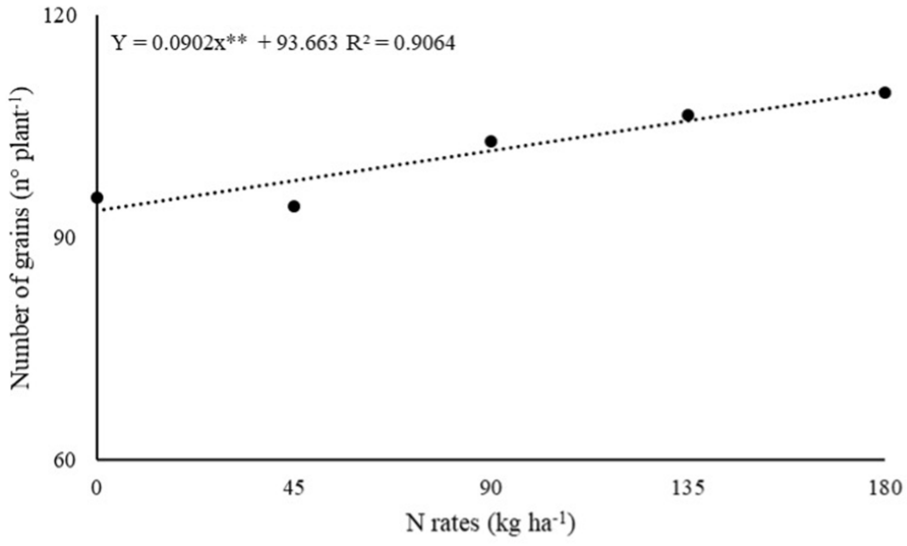


FIGURE 2
Effect of N rates under number of grains of dwarf castor grown in succession to grasses in a no-tillage system.

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TABLE 2 Effect of nitrogen rates and inoculations of PGPB on the castor beans shoot dry matter grown with grasses (SDM-G) and grain yield in succession to grasses (GY-G), shoot dry matter (SDM-L), and grain yield in succession to legume (GY-L).

Treatments	SDM-G	GY-G	SDM-L	GY-L
	cm	kg ha^{-1}	cm	kg ha^{-1}
Foliar inoculation				
Non-inoculation	10,586 a	3,339 d	10,122 b	3,743 b
A. brasilense	10,717 a	4,849 b	10,971 a	4,413 a
B. subtilis	10,870 a	5,397 a	11,421 a	4,556 a
P. fluorescens	10,567 a	4,214 c	9,483 c	4,590 a
Nitrogen (N) rates (kgha^{-1})				
0	8,812	3,999	8,749	3,566
45	10,974	4,440	9,583	4,142
90	12,070	4,886	11,072	4,792
135	11,424	4,563	12,275	4,842
180	10,147	4,361	10,819	4,285
Mean	10,685	4,450	10,499	4,325
Standard error (±)	258.7	122.1	234.4	117.4
Source of variance				
Block	0.24ns	0.19ns	0.28ns	0.22ns
Inoculation (I)	0.58ns	0.00**	0.00**	0.00**
N rates (R)	0.00**	0.00**	0.00**	0.00**
I*R	0.00**	0.23 ns	0.00**	0.00**
Linear	0.00**	0.02*	0.00**	0.00**
Quadratic	0.00**	0.00**	0.00**	0.00**
CV (%)	7.27	9.92	8.06	5.21

ns, not significant, * and ** significant at $p < 0.05$ and $p < 0.01$ probability using the *F* test. Distinct letters in the column differ from each other using Tukey's test. PGPB, plant growth-promoting bacteria; CV, coefficient of variance.

increased SDM-G by 23 and 28% as compared to no-inoculation, while inoculation with *A. brasilense* in combination with 135 kg ha^{-1} of N increased SDM-G by 43% as compared to the treatments with inoculation of *P. fluorescens* (Figure 3A). The treatment with no foliar spray with PGPB was observed with greater SDM-G at the rate of 180 kg ha^{-1} of N as compared to the other treatments (Figure 3A).

Shoot dry mass of dwarf castor beans in succession to legumes (SDM-L) was increased with inoculation with *A. brasilense* at increasing rates of N (Figure 3B). The treatments with no-inoculation, inoculation with *B. subtilis* and *P. fluorescens* increased SDM-L by 42, 65, and 18% at the estimated topdressing rates of 113, 85, and 160 kg ha^{-1} of N in relation to 0 kg ha^{-1} of N, respectively (Figure 3B). Inoculation with *B. subtilis* at a rate of 135 kg ha^{-1} of N was observed with 26% greater SDM-L as compared to without foliar spray of PGPB. Inoculation with *A. brasilense* and *B. subtilis* at a rate of 180 kg ha^{-1} of N increased SDM-L by 24 and 22% as compared to other inoculation and non-inoculation, respectively (Figure 3B).

The interaction for the grain yield of castor beans in succession to grasses (GY-G) was not significant whereas inoculation with PGPB and N rates and their interaction were observed significant ($p < 0.01$) for castor bean grain yield in succession to legumes (GY-L; Table 2). The GY-G was increased by 20% at the estimated topdressing rate of 103 kg ha^{-1} of N in relation to 0 kg ha^{-1} of N (Figure 3C). There was a linear increase in dwarf castor GY-L with increasing topdressing rates of N as compared to non-inoculated treatment (Figure 3D). Inoculations with *A. brasilense, B. subtilis,* and *P. fluorescens* increased GY-L by 28, 64, and 40% at the estimated rates of 97, 113, and 92 kg ha^{-1} of N in relation to 0 kg ha^{-1} of N, respectively (Figure 3D).

Inoculations with *A. brasilense, B. subtilis,* and *P. fluorescens* increased GY-L of castor beans by 37, 21, and 33%, while the same inoculations at the rate of 90 kg ha^{-1} of N increased GY-L by 33, 25, and 40% in relation to no-inoculation at 0 kg of N, respectively. Inoculation with *B. subtilis* increased GY-L by 41% at a rate of 135 kg ha^{-1} of N, whereas, inoculation with *P. fluorescens* at a rate of 45 kg ha^{-1} of N increased GY-L by 50% as compared to without foliar inoculation with PGPB (Figure 3D).

3.2 Effect of N rates and inoculations on the oil content and oil yield

The oil concentrations in castor bean grains in succession to grasses (%O-G) and legumes (%O-L) were significantly ($p < 0.05$) affected by N rates and inoculation with PGPB via leaf, respectively (Table 3). The %O-G was set to linear increase at the rate of 36 kg ha^{-1} of N and further increase in N rates led to the reduction of %O-G (Figure 4A).

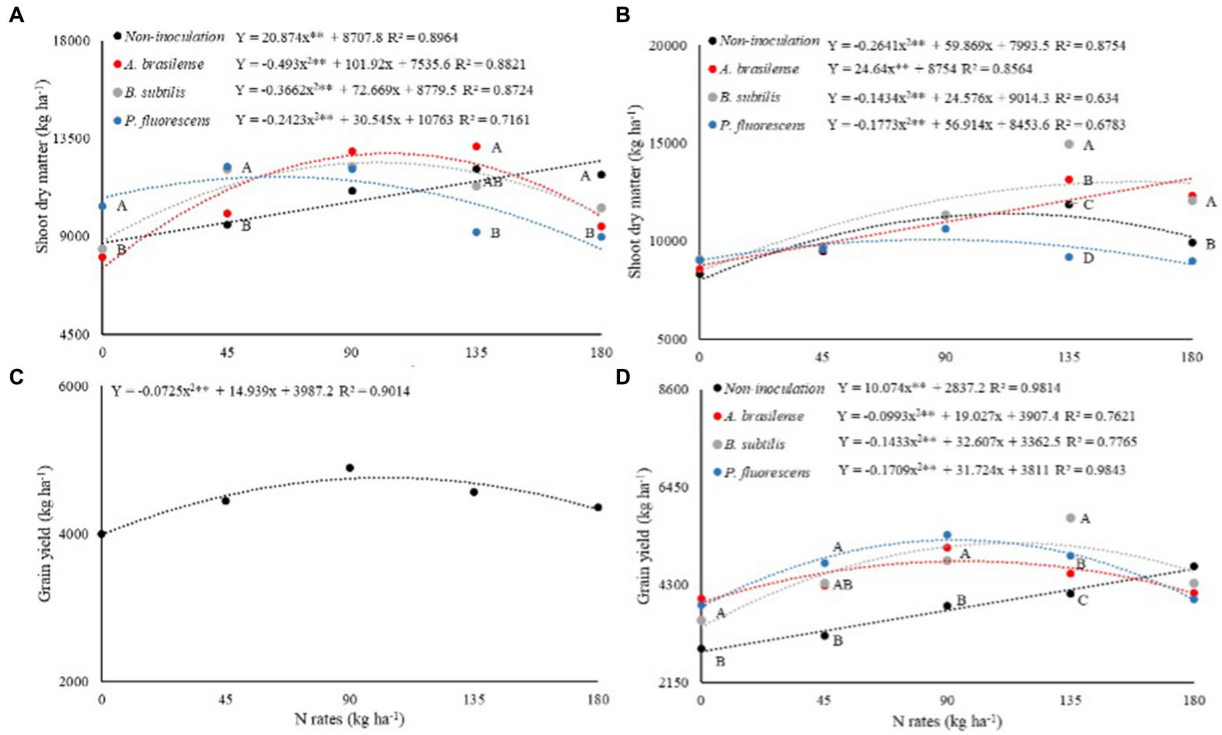


FIGURE 3
Effect of N rates and inoculations under shoot dry matter of dwarf castor plants grown in succession to grasses **(A)** and grown in succession to legumes **(B)**, grain yield of dwarf castor plants grown in succession to legumes **(D)**. Effect of N rates on grain yield of dwarf castor **(C)** cultivated in succession to cultivation with grasses in a no-tillage system.

Castor oil yield in succession to grasses (OY-G) and legumes (OY-L) were significantly ($p < 0.01$) affected by N rates and inoculation with PGPB, while their interaction between two factors was significant ($p < 0.01$) for castor oil yields in succession to legumes (OY-L; Table 3). Higher OY-G was observed at the estimated rate of 103 kg ha^{-1} of N (Figure 4B). Inoculations with *A. brasilense, B. subtilis,* and *P. fluorescens* at the rate of 0 and 45 kg ha^{-1} of N increased OY-L by 27, 18, and 27%, and 28, 34, and 41% in relation to without foliar inoculation of PGPB, respectively. Inoculation with *P. fluorescens* at a rate of 90 kg ha^{-1} of N enhanced OY-L by 37% while inoculation with *B. subtilis* at a rate of 135 kg ha^{-1} of N improved OY-L by 44% as compared to no-inoculation (Figure 4C). Inoculations with *A. brasilense, B. subtilis,* and *P. fluorescens* increased OY-L by 23, 72, and 55% at the calculated topdressing rates of 92, 64, and 93 kg ha^{-1} of N in relation to 0 kg ha^{-1} of N, respectively (Figure 4C).

3.3 Effect of N rates and inoculations on the N concentration and N uptake

There was a significant effect ($p < 0.05$) of the interaction between N rates and PGPB inoculations on shoot N concentration of dwarf castor in succession to grasses (SNC-G; Table 4) while shooting N concentration in succession to legumes (SNC-L) set to linear regression (Figure 5A). The highest SNC-G of dwarf castor was observed with no-inoculation treatment and inoculation with *B. subtilis* at the N rate of 135 kg ha^{-1}in relation to inoculation with *P. fluorescens*. The SNC-G of castor bean was increased to a maximum calculated N concentration of 62.36 g kg^{-1} up to a topdressing N rate of 40 kg ha^{-1} at inoculation with *B. subtilis* (Figure 5A). Higher SNC-L of dwarf castor was observed with increasing N supply in topdressing (Figure 5B).

The verified significant effect ($p < 0.01$) of N rates on grain nitrogen concentration in succession to grasses (GNC-G) and grain nitrogen concentration in succession to legumes (GNC-L) was significantly ($p > 0.01$) influenced by inoculation and interaction of N rates and inoculations (Table 4). The GNC-G of dwarf castor was increased with increasing N supply in topdressing (Figure 5C). Foliar inoculation with *A. brasilense, B. subtilis,* and without inoculation treatments at rates of 0 and 90 kg ha^{-1} of N were observed with higher GNC-L of dwarf castor bean than inoculation with *P. fluorescens*. Inoculation with *A. brasilense* at the topdressing N rate of 45 kg ha^{-1} was observed with greater castor bean GNC-L than other inoculations. The greater castor GNC-L was observed with increasing N supply in topdressing and foliar inoculation with *B. subtilis*. Inoculation with *A. brasilense* at an estimated topdressing N rate of 116 kg ha^{-1} was observed with maximum calculated GNC-L (58.22 g kg^{-1}) in dwarf castor (Figure 5D).

There was a significant effect ($p < 0.01$) of N rates and interaction between N rates and inoculations on shoot N uptake dwarf castor plants in succession to grasses (SNU-G). There was a significant effect of N rates, inoculations with PGPB, and their interaction on shoot N uptake in succession to legumes (SNU-L) and grain N uptake in succession to grasses (GNU-G) and legumes (GNU-L) (Table 5).

TABLE 3 Effect of nitrogen rates and inoculations of PGPB on the castor beans seeds oil concentration (%O-G) and oil yield (OY-G) in succession to grasses and seeds oil concentration (%O-L) and oil yield (OY-L) in succession to legumes.

Treatments	%O-G	OY-G	%O-L	OY-L
	%	kg ha^{-1}	%	kg ha^{-1}
Foliar inoculation				
Non-inoculation	40.16 a	1,340 c	40.32 a	1,507 c
A. brasilense	40.64 a	1969 a	37.16 b	1,638 b
B. subtilis	39.48 a	2,127 a	39.06 ab	1783 a
P. fluorescens	40.26 a	1,693 b	38.90 ab	1788 a
Nitrogen (N) rates (kgha^{-1})				
0	40.57	1,621	39.20	1,404
45	41.47	1842	39.03	1,605
90	38.70	1887	38.86	1833
135	38.97	1779	38.69	1906
180	40.96	1780	38.52	1,468
Mean	40.13	1782	38.86	1,679
Standard error (±)	2.15	93.26	1.89	80.14
Source of variance				
Block	0.95ns	0.32ns	0.10ns	0.09ns
Inoculation (I)	0.64ns	0.00**	0.02*	0.00**
N rates (R)	0.03*	0.01**	0.81 ns	0.00**
I*R	0.94ns	0.41ns	0.59ns	0.00**
Linear	0.45ns	0.13ns	0.49ns	0.00**
Quadratic	0.05*	0.00**	0.73 ns	0.00**
CV (%)	7.17	11.91	7.86	10.32

ns, not significant, * and ** significant at $p < 0.05$ and $p < 0.01$ probability using the F test. Distinct letters in the column differ from each other using Tukey's test. PGPB, plant growth-promoting bacteria; SOV, source of variance; CV, coefficient of variance.

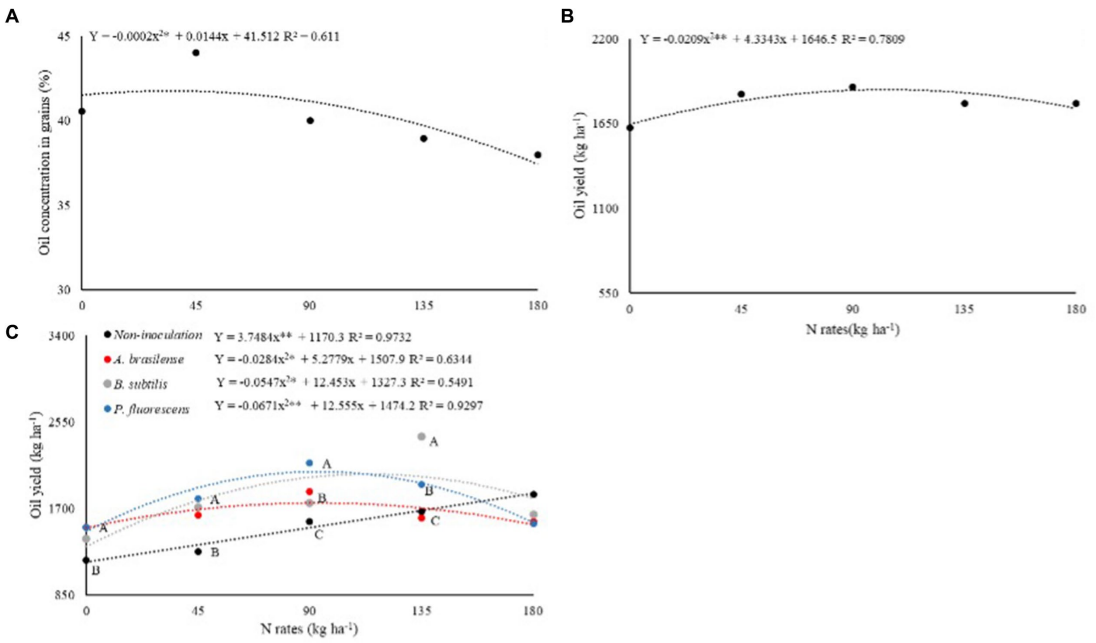


FIGURE 4
Effect of N rates under oil concentration in dwarf castor seeds **(A)** and on oil yield of dwarf castor **(B)** grown in succession to cultivation with grasses. Effect of N rates and inoculations on oil concentration in dwarf castor seeds grown in succession to legumes **(C)** in a no-tillage system.

The SNU-G was increased with increasing N rates in topdressing in the no-inoculated treatments (Figure 6A). Inoculations with *A. brasilense, B. subtilis,* and *P. fluorescens* at the calculated rates of 99, 106, and 75 kg ha^{-1} of N improved SNU-G by 69, 44, and 30%, respectively. Inoculation with *B. subtilis* and *P. fluorescens* were with 26 and 25% higher SNU-G than no foliar inoculation. Inoculation with *A. brasilense* at a rate of 45 kg ha^{-1} of N and without foliar inoculation at a rate of 180 kg ha^{-1} of N were observed with higher SNU-G in relation to other inoculations. Inoculation with *A. brasilense* increased SNU-G by 47% in relation to inoculation with *P. fluorescens* at a rate of 135 kg ha^{-1} of N (Figure 6A).

Shoot N uptake in succession to legumes (SNU-L) was increased with increasing rates of N and inoculation with *A. brasilense* (Figure 6B). Treatments with no-inoculation and foliar inoculation with *B. subtilis* and *P. fluorescens* at the estimated topdressing rates of 118, 159, and 95 kg ha^{-1} of N increased SNU-L by 45, 67, and 32%, respectively (Figure 6B). Inoculation with *B. subtilis* at the rate of 135 kg ha^{-1} of N was observed with 24% SNU-L of dwarf castor as compared to non-inoculated. Inoculations with *A. brasilense* and *B. subtilis* at the rate of 180 kg ha^{-1} of N increased SNU-L by 21 and 18% as compared to no-inoculation, respectively (Figure 6B).

Grain N uptake in succession to grasses (GNU-G) was increased by 21 and 39% with inoculations of *A. brasilense* and *B. subtilis* at calculated rates of 95 and 126 kg ha^{-1} of N, respectively. There was a linear increase in GNU-G with increasing rates of N and inoculation with *P. fluorescens*. Inoculation with *B. subtilis* and *A. brasilense* at a rate of 45 kg ha^{-1} of N enhanced GNU-G by 39 and 60% as compared to no-inoculation. In addition, inoculation with *B. subtilis* at rates of 90, 135, and 180 kg ha^{-1} of N was observed with 60, 83, and 73% higher GNU-G than no-inoculation, respectively (Figure 6C).

There was an increase in the grain N uptake in succession to legumes (GNU-L) of dwarf castor as the N rates increased under the non-inoculated treatment. Inoculations of *A. brasilense, B. subtilis,* and *P. fluorescens* at the estimated rates of 101, 112, and 98 kg ha^{-1} of N increased GNU-L by 27, 37, and 35% in relation to 0 kg ha^{-1} of N, respectively (Figure 6D). Inoculation with *P. fluorescens* at a rate of 90 kg ha^{-1} of N increased GNU-L by 22% while inoculation with *B. subtilis* at a rate of 135 kg ha^{-1} of N increased GNU-L by 47% over non-inoculated treatments (Figure 6D).

There was an increase in the total N uptake in succession to grasses (TNU-G) with an increase in topdressing N rates in the non-inoculated treatments. Foliar inoculations with *A. brasilense, B. subtilis,* and *P. fluorescens*. The foliar inoculation of *A. brasilense, B. subtilis,* and *P. fluorescens* at the rate of 90 kg ha^{-1} of N provided higher TNU-G in relation to non-inoculated treatments. Inoculations with *A. brasilense* and *B. subtilis* at the rate of 135 kg ha^{-1} of N were observed with higher TNU-G than non-inoculated treatments (Figure 6E).

Foliar inoculation with *A. brasilense* and *B. subtilis* at the rates of 135 and 180 kg ha^{-1} of N was observed with higher total N uptake in succession to legumes (TNU-L) than non-inoculated (Figure 6F). The highest calculated TNU-L (896.01 kg ha^{-1}) was verified at the estimated topdressing rate of 133 kg ha^{-1} of N under the non-inoculated treatments. Foliar inoculations with *A. brasilense, B. subtilis,* and *P. fluorescens* at estimated topdressing rates of 163, 140, and 96 kg ha^{-1} of N were observed with higher TNU-L (970.1, 1026.6, and 868.8 kg ha^{-1} of N), respectively (Figure 6F).

4 Discussion

Inoculation with *P. fluorescens, A. brasilense,* and *B. subtilis* is considered one of the diverse strategies of agricultural cultivation systems with a positive effect on shoot dry mass, grain yield, and other productive components (Andrade et al., 2019; Chu et al., 2020; Rosa et al., 2020; Akhtyamova et al., 2021; Jalal et al., 2021; Fonseca et al., 2022; Galindo et al., 2022; Jalal et al., 2023b), and a foliar inoculation with *A brasilense* in corn (Pereira et al., 2020). In addition, the use of these bacteria in association with N rates has been studied in several studies with the intention of reducing N consumption in the agriculture crop production system (Prasad and Babu, 2017; Galindo et al., 2020). However, there exists a research gap on the foliar inoculations with these bacteria and their ability to promote the growth of castor beans in Brazilian Cerrado.

The current results indicated that inoculation with *A. brasilense* at the rates of 90 and 135 kg ha^{-1} of N and inoculation with *P. fluorescens* at the rates of 0 and 45 kg ha^{-1} were observed with greater shoot dry matter of dwarf castor in succession to grasses. However, the highest shoot dry matter of dwarf castor beans in succession to legumes was noted with inoculations of *B. subtilis* and *A. brasilense* at the estimated rates of 135 and 180 kg ha^{-1} of N (Figures 3A,B). As previously reported, *A. brasilense, P. fluorescens,* and *B. subtilis* have the ability to produce different plant hormones in the root rhizosphere of crop plants that may promote the growth of roots and shoots (Mekonnen and Kibret, 2021).

The increased production of plant hormones by the inoculated plants could be linked to the higher distribution of photo-assimilates by the plant, especially in the period of greatest plant demand (flowering and fruiting), which may positively affect grain yield (Mortinho et al., 2022; Jalal et al., 2023a). This effect was observed in GY-G of castor bean with foliar inoculation of *B. subtilis* while inoculation with *A. brasilense*, *B. subtilis,* and *P. fluorescens* at the rates of 45 and 90 kg ha^{-1} of N were observed with higher GY-L of dwarf castor. The highest GY-L of dwarf castor was observed with inoculation of *B. subtilis* at the rate of 135 kg ha^{-1} of N (Figure 3). In addition, the highest OY-L of castor beans observed was with inoculation of *P. fluorescens* at a rate of 90 kg ha^{-1} of N and inoculation with *B. subtilis* at a rate of 135 kg ha^{-1} of N as compared to non-inoculated treatments (Figure 4).

The beneficial effects of inoculation with PGPB can be impaired by the high supply of N, which was the case in the present study that the highest rates of N impair the performance of PGPB in castor bean cultivation, by reducing grain yield and oil yields (Figure 4). The high supply of N fertilizer can increase the competition of bacteria and roots for the large amount of N present in the soil, which may affect the desired mechanisms of biological fixation of N_2 by bacteria (Galindo et al., 2021).

An adequate supply of N can increase nutrient uptake by plants, as reported that PGPB can improve plant's N acquisition through biological N fixation (BNF) (Fukami et al., 2018; Galindo et al., 2021) and greater root biomass through physiological changes in plants (Eckshtain-Levi et al., 2020). This could influence the ability of plant roots to penetrate the soil for greater water and nutrient absorption (Li et al., 2016). The present result verified greater N uptake with higher N supply in topdressing. The highest SNU-G was observed with inoculations of *B. subtilis* and *P. fluorescens* at a rate of 45 kg ha^{-1}

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TABLE 4 Effect of nitrogen rates and inoculations of PGPB on the castor beans shoot nitrogen concentration (SNC-G) and grain nitrogen concentration in succession to grasses (GNC-G), shoot nitrogen concentration (SNC-L), and grain nitrogen concentration in succession to legumes (GNC-L).

Treatments	SNC-G	GNC-G	SNC-L	GNC-L
	g kg^{-1}			
Foliar inoculation				
Non-inoculation	59.92 a	48.39 a	60.23 a	53.24 b
A. brasilense	59.42 a	50.39 a	58.30 a	56.53 a
B. subtilis	60.85 a	50.75 a	59.21 a	55.59 a
P. fluorescens	61.11 a	48.21 a	58.51 a	51.24 c
Nitrogen (N) rates (kgha^{-1})				
0	60.98	45.90	57.67	53.35
45	61.68	47.84	59.16	53.73
90	59.09	49.65	59.09	54.72
135	59.10	51.21	59.52	55.31
180	60.67	52.58	59.88	53.84
Mean	60.32	49.44	59.06	54.19
Standard error (±)	3.08	2.41	2.78	2.91
Source of variance				
Block	0.24ns	0.27ns	0.24ns	0.99ns
Inoculation (I)	0.29ns	0.07ns	0.06ns	0.00**
N rates (R)	0.09ns	0.00**	0.11ns	0.28ns
I*R	0.04*	0.26ns	0.22ns	0.00**
Linear	0.22ns	0.00**	0.01*	0.00**
Quadratic	0.15ns	0.69ns	0.44ns	0.00**
CV (%)	5.20	7.58	4.06	5.20

ns, not significant, * and ** significant at $p < 0.05$ and $p < 0.01$ probability using the F test. Distinct letters in the column differ from each other using Tukey's test. PGPB, plant growth-promoting bacteria; CV, coefficient of variance.

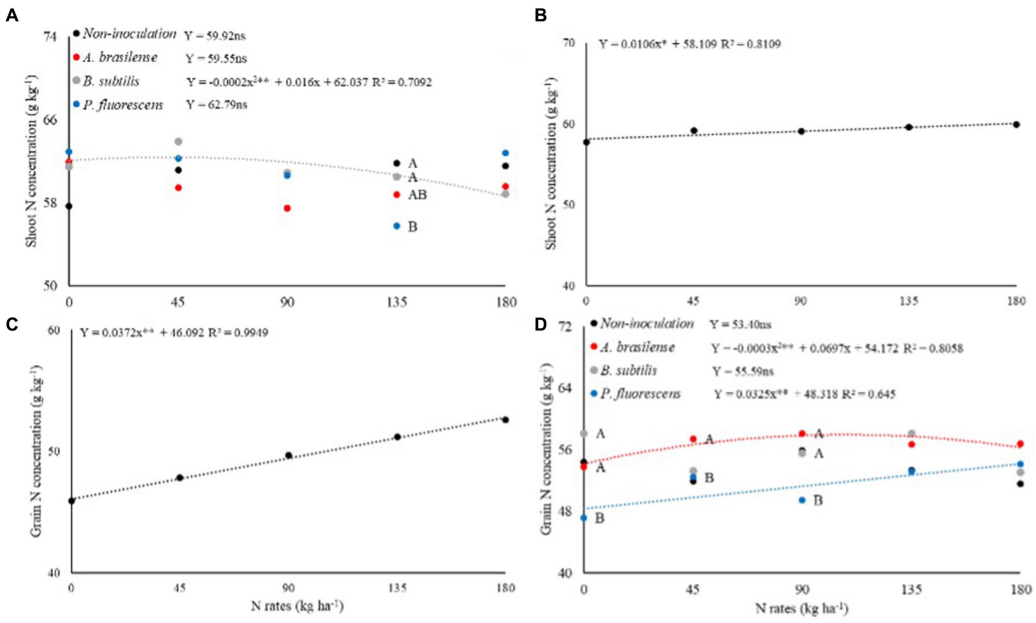


FIGURE 5
Effect of N rates and inoculations PGPB under shoot N concentration of dwarf castor plants grown in succession to grasses **(A)** and grain N concentration of dwarf castor grown in succession to legumes **(D)**. Effect of N rates under shoot N concentration of dwarf castor plants grown in succession to legumes **(B)** and grain N concentration of dwarf castor grown in succession to grasses **(C)** in a no-tillage system.

TABLE 5 Effect of nitrogen rates and inoculations of PGPB on the castor bean shoot nitrogen uptake (SNU-G), grain nitrogen uptake (GNU-G) and total nitrogen uptake (TNU-G) in succession to grasses and shoot nitrogen uptake (SNU-L), grain nitrogen uptake (GNU-L), and total nitrogen uptake (TNU-L).

Treatments	SNU-G	GNU-G	TNU-G	GNU-L	SNU-L	TNU-L
	kg ha^{-1}					
Foliar inoculation						
Non-inoculation	638.3 a	167.2 c	805.48 b	194.9 b	610.5 b	805.38 b
A. brasilense	625.2 a	248.7 a	873.82 a	220.2 a	640.9 ab	861.16 a
B. subtilis	643.4 a	273.4 a	916.82 a	228.6 a	676.7 a	905.40 a
P. fluorescens	618.4 a	208.5 b	826.87 ab	233.1 a	556.1 c	789.15 b
Nitrogen (N) rates (kgha^{-1})						
0	505.8	195.2	701.05	176.9	504.9	681.79
45	649.2	216.5	865.64	207.3	566.9	774.29
90	712.6	248.5	961.08	240.9	654.1	894.99
135	681.1	232.8	913.86	253.6	732.4	986.04
180	607.9	229.2	837.12	217.3	646.9	864.27
Mean	631.3	224.4	855.75	219.21	621.1	840.28
Standard error (±)	26.24	14.18	38.89	19.56	24.44	36.22
Source of variance						
Block	0.47ns	0.11ns	0.48ns	0.85ns	0.59ns	0.60ns
Inoculation (I)	0.37ns	0.00**	0.00**	0.00**	0.00**	0.00**
N rates (R)	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
I*R	0.00**	0.04*	0.00**	0.00**	0.00**	0.00**
Linear	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
Quadratic	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
CV (%)	7.88	11.24	6.42	7.94	9.47	7.25

ns, not significant, * and ** significant at $p < 0.05$ and $p < 0.01$ probability using the F test. Distinct letters in the column differ from each other using Tukey's test. PGPB, plant growth-promoting bacteria; CV, coefficient of variance.

of N and inoculation with *A. brasilense* at a rate of 135 kg ha^{-1} of N as compared to non-inoculated treatments (Figures 6A,B). Inoculation with *A. brasilense* and *B. subtilis* provided greater GNU-G at all rates of N; however, inoculation with *B. subtilis* and *P. fluorescens* was observed with greater GNU-L at rates of 45 and 135 kg ha^{-1} of N (Figures 6C,D). Although BNF is a determining factor for increasing N use efficiency and N uptake by plants, these bacteria are still functionally contributing to some other mechanisms (production of gibberellins, auxins, and cytokinins) that could increase plant growth and productivity (Fukami et al., 2018; Poveda and González-Andrés, 2021). Previous studies reported that inoculation of *A. brasilense* and *B. subtilis* has increased N use efficiency and recovery of applied N in cereal crops that contribute to sustainable grain production under reduced N fertilization (Galindo et al., 2022; Gaspareto et al., 2023).

The isolated inoculation of *A. brasilense* and *B. subtilis* proved to be effective in increasing the recovery of applied N, N use efficiency, shoot N uptake, grain N uptake, productive components, and the grain yield of wheat (Gaspareto et al., 2023). Curiously, it is possible to highlight the highest TNU-G of 51, 42, and 32% at rates of 104, 104, and 85 kg ha^{-1} of N under foliar inoculations of *A. brasilense, B. subtilis,* and *P. fluorescens,* respectively. There was an increase of 50, 68, and 33% in TNU-L under the inoculations of *A. brasilense, B. subtilis,* and *P. fluorescens* at rates of 163, 140, and 96 kg ha^{-1} of N in dwarf castor bean (Figures 6E,F). Castor bean plants are tolerant to water scarcity, which allows them to be cultivated in both arid regions and regions with adequate water availability. However, under the appropriate conditions of cultivation system and water supply, these plants exhibit enhanced development (Zoz et al., 2021). In addition, castor bean plants are responsive to nitrogen fertilization, with greater N acquisition by plants and being translocated to plant tissues (Cavalcante et al., 2020). Inoculations with *B. subtilis, P. fluorescens,* and *A. brasilense* increased the efficiency of the applied N, managing to reduce N fertilization under proper management practices (Fukami et al., 2018; Galindo et al., 2020; Lee et al., 2020; Blake et al., 2021).

5 Conclusion

Topdressing nitrogen fertilization recommended in the crop after succession with grasses is 103 kg ha^{-1} because it provides higher oil yield and grain yield. The recommended topdressing fertilization with nitrogen in the cultivation in succession to legumes without the effect of foliar inoculation is 180 kg ha^{-1} of N. The use of foliar inoculations with *A. brasilense, B. subtilis,* and *P. fluorescens* provided a reduction of 44, 37, and 49% of nitrogen fertilization for grain yield and 49, 40, and 48% of nitrogen fertilization for oil yield in castor bean cultivation in succession to legumes. Foliar inoculation with *B. subtilis* and *A. brasilense*

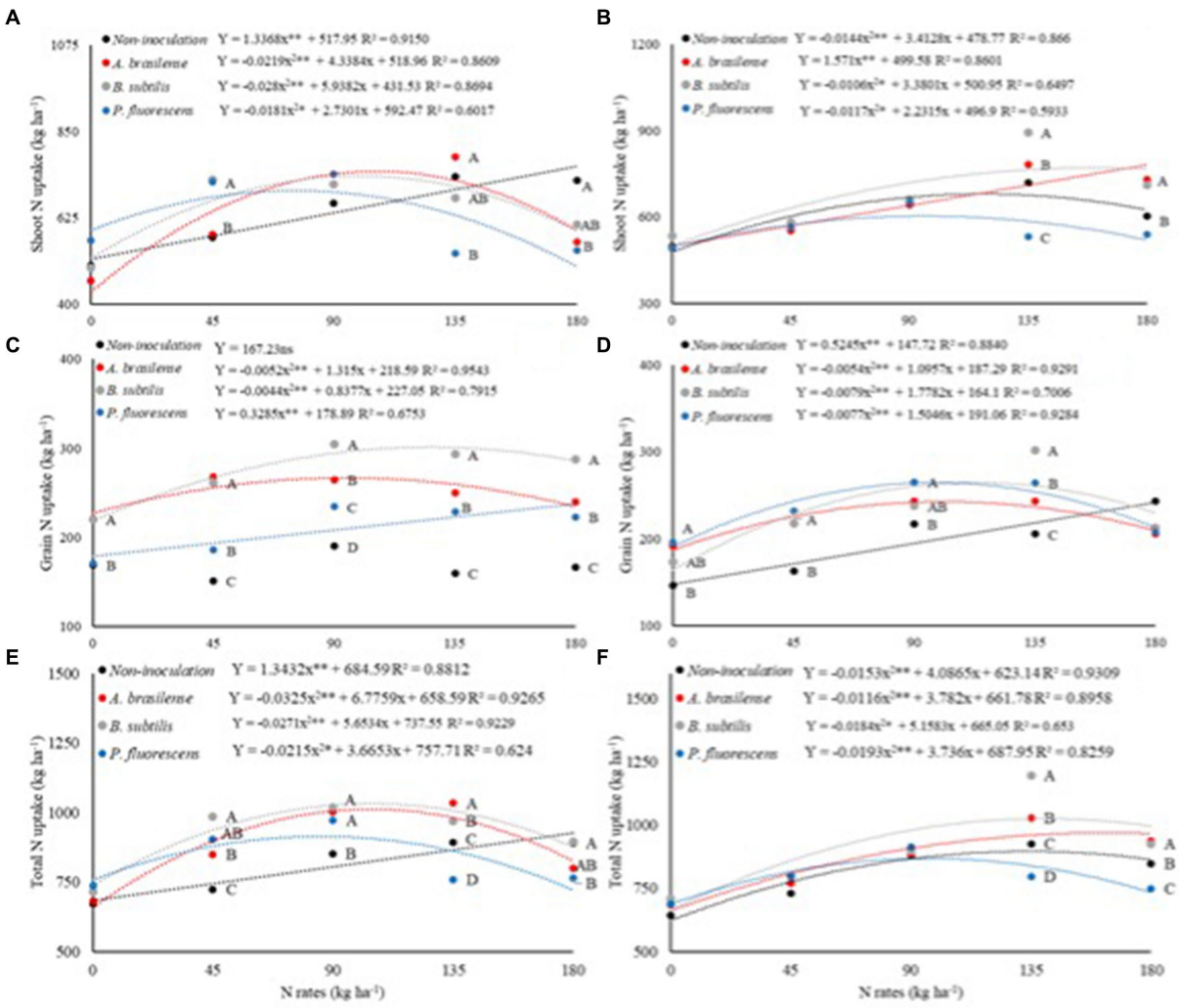


FIGURE 6
Effect of N rates and inoculations PGPB under shoot N uptake in dwarf castor plants grown in succession to grasses **(A)** and grown in succession to legumes **(B)**, grain N uptake of dwarf castor grown in succession to grasses **(C)** and grown in succession to legumes **(D)**, total N uptake of dwarf castor grown in succession to grasses **(E)**, and grown in succession to legumes **(F)** in a no-tillage system.

provided the highest grain yield and castor oil yield in cultivation in succession to grasses.

Foliar inoculation with *A. brasilense, B. subtilis,* and *P. fluorescens* provided higher shoot N uptake, grain N uptake, and total N uptake at topdressing rates of 45, 90, and 135 kg ha^{-1} of N in both systems of succession of plants (legumes and grasses).

Despite different aspects of the use of PGPB in a new way in the field condition, our study was limited with a significant progression from *in vitro* conditions to field applications and constraints within the rhizosphere like soil microbiota structure and enzymes. Research with PGPB should focus on understanding the genetic mechanisms regulating growth-promoting processes, genetic modification of plants, chemical genomics strategies, rhizospheric engineering, and colonization with large subpopulations of rhizomicrobiomes may help overcome these constraints. This approach could be essential for evaluating critical microbial molecular components that regulate plant development and facilitate effective PGPB application in the field.

Data availability statement

All the data generated and analyzed during this study are included in this article.

Ethics statement

Experimental research and field studies on plants (cultivated), including the collection of plant material, comply with relevant institutional, national, and international guidelines and legislation.

Author contributions

IG: Conceptualization, Formal analysis, Investigation, Methodology, Software, Writing – original draft. CO: Conceptualization, Investigation, Methodology, Software, Visualization, Writing – review & editing. AJ:

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Microbial strategies for lead remediation in agricultural soils and wastewater: mechanisms, applications, and future directions

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High lead (Pb) levels in agricultural soil and wastewater threaten ecosystems and organism health. Microbial remediation is a cost-effective, efficient, and eco-friendly alternative to traditional physical or chemical methods for Pb remediation. Previous research indicates that micro-organisms employ various strategies to combat Pb pollution, including biosorption, bioprecipitation, biomineralization, and bioaccumulation. This study delves into recent advancements in Pb-remediation techniques utilizing bacteria, fungi, and microalgae, elucidating their detoxification pathways and the factors that influence Pb removal through specific case studies. It investigates how bacteria immobilize Pb by generating nanoparticles that convert dissolved lead (Pb-II) into less harmful forms to mitigate its adverse impacts. Furthermore, the current review explores the molecular-level mechanisms and genetic engineering techniques through which microbes develop resistance to Pb. We outline the challenges and potential avenues for research in microbial remediation of Pb-polluted habitats, exploring the interplay between Pb and micro-organisms and their potential in Pb removal.

KEYWORDS

lead pollution, environmental toxicity, detoxification mechanisms, microbial and molecular remediation, environmental restoration

1 Introduction

Higher concentrations of heavy metals, the predominant contaminants in the environment, pose a significant hazard to soil and water due to their heightened toxicity levels (Ahamad et al., 2024; Razzaq et al., 2024; Zhao et al., 2024). Lead (Pb) has attracted considerable research attention worldwide due to its high toxicity, persistence, and accessibility (Futsaeter and Wilson, 2013; Raza Altaf et al., 2021; Shan et al., 2023). Hou et al. (2020) emphasized a substantial 232%

rise in worldwide Pb production in the last five decades, reaching 11.3 Mt. annually due to industrial growth. The Pb contamination globally originates from natural and human-induced sources. Volcanic eruptions release natural Pb sources through dust emissions. Still, human activities, including mining, waste disposal, chemical plants, and fertilizer usage, have recently been the main causes of Pb pollution (Kushwaha et al., 2018). Different sources of Pb pollution in ecosystems are shown in Figure 1.

The progressive movement of elevated amounts of Pb contamination from the atmosphere, ground, and water sources into the food web and ultimately into body parts of humans presents heightened dangers. Previous research has shown that Pb poisoning can result in anemia, developmental issues, neurological disorders, and deaths in different animals (Wani et al., 2015) Similarly, Mitra et al. (2021) discovered that children in almost four million families globally are subjected to increased Pb levels, affecting their development and well-being. Pb contamination is a crucial issue in the worldwide environmental preservation system. Rahman and Singh (2020) classified methods for addressing Pb pollution into three main categories: physical, chemical, and biological treatments. Conventional physicochemical methods to remove Pb ions, such as chemical precipitation, ion exchange, membrane processing, and adsorption, encounter challenges due to high costs and inadequate Pb ion elimination (Dhankhar and Hooda, 2011).

Microbial remediation is a simple, cost-effective, and efficient procedure compared to other alternatives (Adil, 2021; Raza Altaf et al., 2021; Wang et al., 2024). It entails adjusting environmental factors to stimulate the proliferation of micro-organisms and remove impurities. Critical techniques for eliminating Pb include biomineralization, bioprecipitation, biosorption, bioaccumulation, and efflux mechanisms, which convert soluble Pb ions into insoluble states (Figures 2–4). Studies have demonstrated that bacteria such as *Azotobacter chroococcum*, *Paenibacillus jamilae,* and fungus like *Aspergillus niger* are efficient in eliminating Pb from the environment (Xu et al., 2021; Shan et al., 2023). Progress in microbial technology has resulted in the creation of vigorous bacteria and the identification of associated genes, which may improve the effectiveness of removal procedures (Morillo Pérez et al., 2008).

This review suggests information about micro-organisms and their use in environmental cleanup, explicitly focusing on different types of bacteria, fungi, and microalgae recognized for their effectiveness in removing Pb. The study investigates the impact of environmental elements on the effectiveness of remediation, assesses procedures, and appraises the appropriateness of microbial-based techniques for Pb-contaminated locations. The article also addresses the obstacles and possibilities for extensive adoption.

2 Micro-organisms assisted remediation of lead-II

2.1 Bacteria assisted remediation

Bacteria can thrive in diverse environmental conditions, making them the most prevalent microbes on earth (Shan et al., 2023). Their varied origins, rapid growth, robust durability, and notable efficiency contribute to their extensive use in eliminating HMs. As shown in Table 1, recent global scientific studies have focused on finding native bacterial species that can eliminate the hazardous impacts of Pb. These include *Paenibacillus jamilae, Azotobacter chroococcum,* and *Sporosarcina pasteurii*. Bacterial assembly and adsorption are the primary techniques for eliminating Pb-II from the environment. Figure 2 shows the different bioremediation methods, such as bioaccumulation, bioaccumulation, biomineralization, bioleaching, biotransformation, and biosorption, performed by the microbial system to remove or transform toxic Pb from contaminated sites.

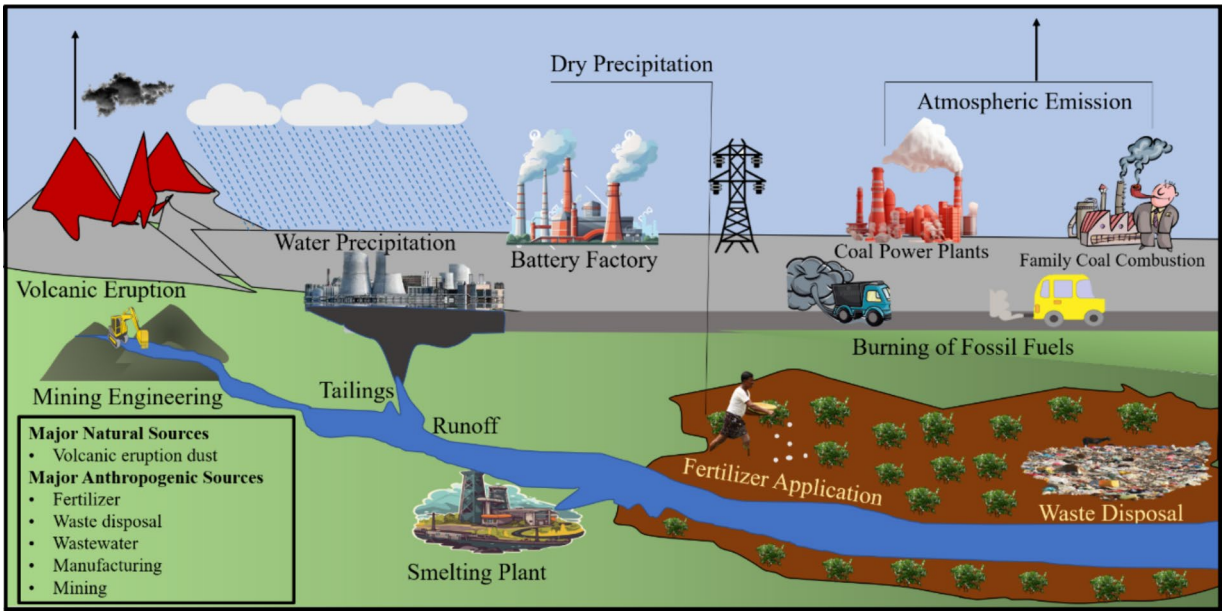


FIGURE 1
Presents the different sources of Pb pollution in the ecosystem [Modified from Figures in Hou et al. (2020) and Shan et al. (2023)].

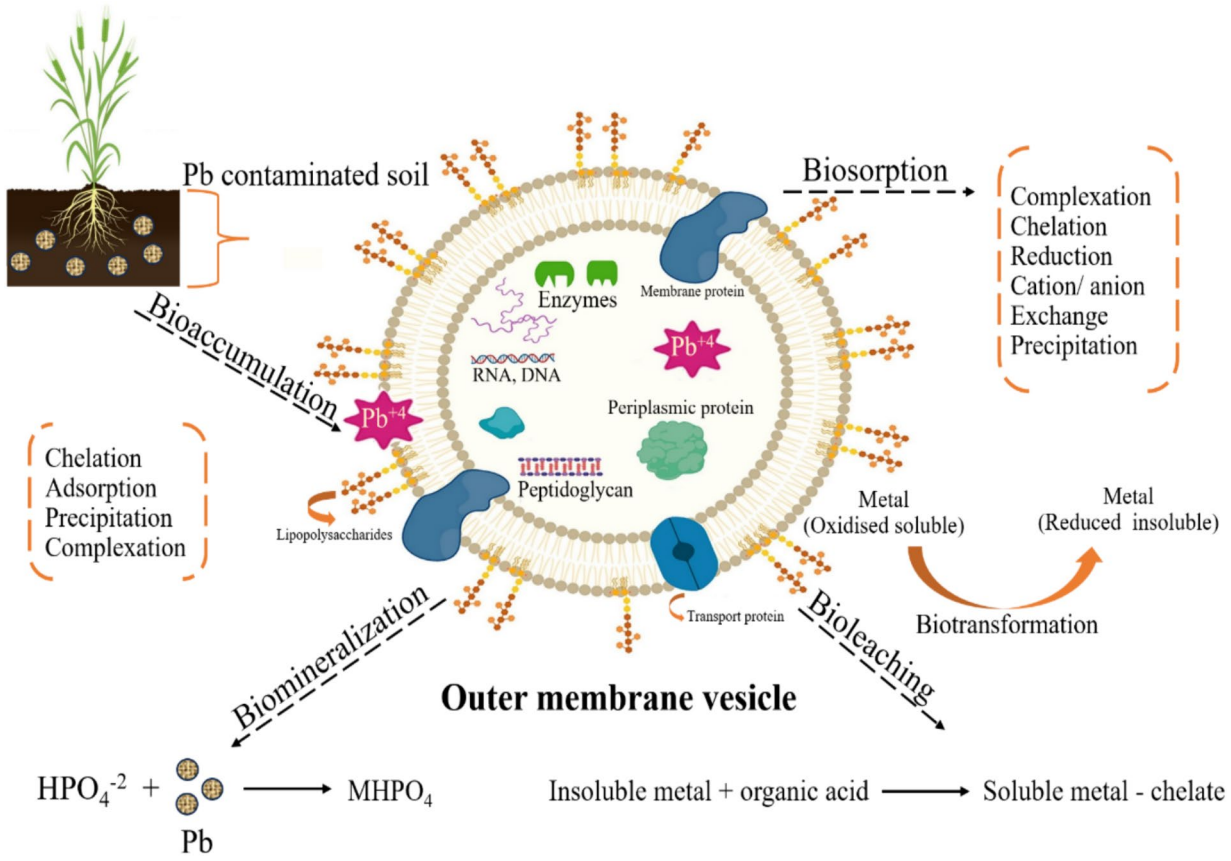


FIGURE 2
Different bioremediation methods, such as bioaccumulation, bioaccumulation, biomineralization, bioleaching, biotransformation, and biosorption, performed by the microbial system to remove or transform toxic Pb from contaminated sites [Modified from Figure in Joshi et al. (2023)].

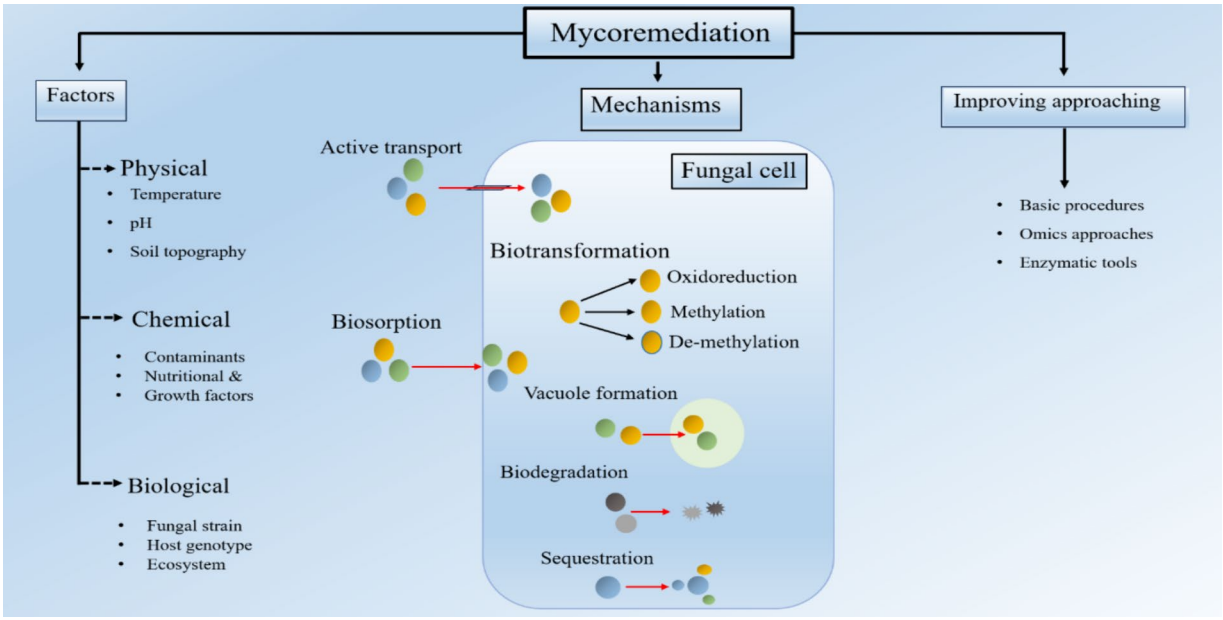


FIGURE 3
Fungi-assisted remediation of HM involves several mechanisms, such as biosorption, biodegradation, biotransformation, and sequestration [Modified from Figure in Kumar et al. (2021)].

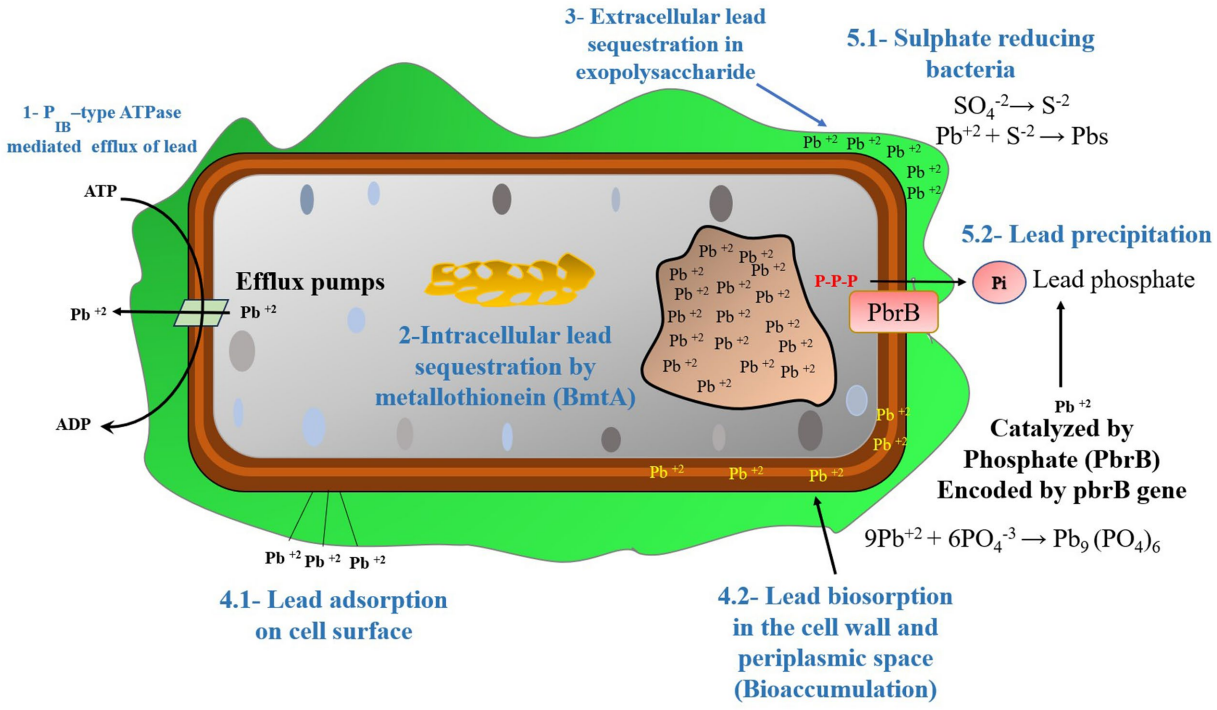


FIGURE 4
The Pb resistant mechanisms operational in bacteria, (1) PIB-type ATPase mediated efflux of Pb, (2) Pb sequestration by metallothionein (BmtA), (3) Pb sequestration in exopolysaccharide, (4.1) Cell surface adsorption of Pb, (4.2) Biosorption of Pb in the cell wall and periplasmic space (bioaccumulation), (5.1) Pb precipitation by sulfate-reducing bacteria, (5.2) Pb precipitation catalyzed by Phosphatase enzyme (PbrB) [Modified from Figure in Ashkan (2023)].

Moreover, both living and dead bacterial biomass have significant capacities to absorb Pb-II, as Li et al. (2017) highlighted the exceptional biosorption abilities of *Pseudomonas* sp. -I3, a psychrotrophic bacterium tolerant to Pb-II. Similarly, the bacteria exhibited substantial Pb biosorption rates of 49.48 mg g^{-1} with living biomass and 42.37 mg g^{-1} with dead biomass. Bacterial cell walls include essential functional groups such as phosphate, carboxyl, sulfate, and amino groups that are important for Pb-II adsorption. Different types of bacteria have metal-binding spots on their cell walls and peptidoglycan such as the binding sites of Pb on the cell wall of Gram-positive bacteria and Gram-negative bacteria differ. In the cell wall of Gram-positive bacteria, the carboxyl group of peptidoglycans is the primary binding site for Pb, whereas in Gram-negative bacteria, the phosphate group plays a significant role. In addition, *Pseudomonas aeruginosa* PU21 has several negatively charged groups on its surface, which enhances its efficacy in extracting Cd, Pb, and Co from wastewater that is polluted with these metals (Shan et al., 2023).

Micro-organisms secrete extracellular polymeric substances, also known as EPS in a laboratory, mainly composed of nucleic acids, proteins, lipids, polysaccharides, and humic chemicals (Shan et al., 2023). EPS is crucial for heavy metal adsorption and the production of biofilms (Czaczyk and Myszka, 2007). Bacteria synthesize EPS to withstand environmental stress, exhibiting a high capacity to sequester metals inside the EPS framework. Moreover, the EPS has many ionizable functional groups that can bind metals better (Flemming and Wingender, 2010). Spectroscopic research has demonstrated that Pb-II has a higher affinity for phosphoryl groups in EPS produced by bacteria such as *Shewanella oneidensis* strain MR-1 (Ha et al., 2010). Molecular size and the presence of proteins, polysaccharides, and lipids influence the adsorption capacity of EPS (Comte et al., 2006). Chemicals like amino acids, sulfate esters, and high-nitrogen pyruvates help metals and ligands bind together (Loaëc et al., 1997).

2.2 Fungi assisted remediation

Fungi possess exceptional resistance to high amounts of heavy metals compared to bacteria and are very efficient at reducing these toxic substances in the environment. Fungi have more functional groups that can bind Pb-II and sequester it more effectively because their cell walls can make up to 30% of their dry mass (Dhankhar and Hooda, 2011). Similarly, Dhankhar and Hooda (2011) have demonstrated the performance benefits of an extensive fungi culture and short-cycle multiplication. In addition to this, many fungal biosorbents have non-pathogenic characteristics, making them ideal for engineering purposes. Shan et al. (2022) found that some fungi, like *Cunninghamella echinulate, Penicillium polonicum,* and *Aspergillus tubingensis*, absorb Pb-II well. Moreover, *Aspergillus niger* is a commonly used biosorbent for removing Pb-II.

Xu et al. (2021) exhibited that living and modified (high-temperature, freeze-dried, alkali treatment) *Aspergillus niger* has a great capacity to remove Pb(II) present in aqueous solution, and the rates of Pb(II) removal were 96.21, 8.76, 25.02, 15.05% under initial 828 mg L^{-1} Pb(II), respectively.

Using a response surface approach to improve the Pb-II adsorption by *Aspergillus niger* biomass intended changing the pH of the solution, the amount of Pb in it, and the dose of biomass, which

TABLE 1 Microbes-mediated removal of Pb with different environmental factors.

Micro-organism	Species	Temp (°C)	Optimal pH	Initial Pb concentration (mg L^{-1})	Pb removal rate	Removal mechanisms	References
Bacteria	*Pseudomonas stutzeri*	30	7	103.5	> 99.0%	Biosorption; bioaccumulation; bioprecipitation (calcium carbonate crystals containing Pb)	Wang et al. (2015)
	Enterobacter cloacae KJ-46	30	7	7.2	68.1%	Biosorption; bioprecipitation ($PbCO_3$)	Kang et al. (2015)
	Serratia marcescens	28	7	100	97.57%	Biosorption; bioprecipitation ($Pb_5(PO4)_3Cl$)	Zhu et al. (2019)
	Alishewanella sp. WH16-1	37	6	100	84.13%	Biosorption; bioprecipitation (PbS)	Zhou et al. (2016)
	Microbacterium oxydans CM3	30	7.59	400	58.0%	Biosorption; bio accumulation; Bioprecipitation) ($Pb_3(PO_4)_2$	Dabir et al. (2019)
	Pseudomonas sp.	20	7	50	> 90.0%	Biosorption	Gallardo-Rodríguez et al. (2019)
	Bacillus subtilis X3	37	4	300	192.05 mg g^{-1}	Biosorption, biomineralization ($Pb_5(PO_4)_3OH$, $Pb_{10}(PO_4)_6(OH)_2$ and $Pb_5(PO_4)_3Cl$)	Qiao et al. (2019)
	Bacillus cereus	30	5	100	75.6%	Biosorption; complexing	Murthy et al. (2011)
Fungi	*Penicillium polonicum*	30	5	828.8	90.3%	Biosorption; bioprecipitation (PbC_2O_4, $Pb_5(PO_4)_3Cl$)	Xu et al. (2020)
	Aspergillus tubingensis	30	5	828.8	> 90.0%	Biosorption; bio accumulation; Bioprecipitation (PbS, $PbCO_3$, $Pb_2O_{3.333}$)	Shan et al. (2022)
	Aspergillus niger	30	5	828.8	97.0%	Biosorption; Bioprecipitation (PbC_2O_4)	Ding et al. (2019), Ding et al. (2019)
	Trichoderma asperellum	30	7	250	18.71%	Biosorption	Sun et al. (2018)
	Rhizopus oryzae	30	7	450	33.76%	Biosorption	Sun et al. (2018)
	Mucor irregularis	30	7	100	17.37%	Biosorption	Sun et al. (2018)
Microalgae	*Phormidium* sp.	25	5	10	2.305 mg g^{-1}	Biosorption; Bio-accumulation	
	Oscillatorialaetevirens	25	5	60	20.36 mg g^{-1}	Biosorption	
	Pseudochlorococcu typicum	20	7	10	4.49 mg g^{-1}	Biosorption; Bio-accumulation	Shanab et al. (2012)
	Spirulina (Arthrospira platensis)	25	7	100	188 mg g^{-1}	Bioaccumulation	Arunakumara et al. (2008)
	Synechocystis sp.	25	7	8	155.63 mg g^{-1}	Bioaccumulation	Arunakumara et al. (2007)
	Chlorella vulgaris CCAP211/11B (Non-living)	28	7	600	39 mg g^{-1}	Biosorption	Kumar et al. (2015)
	Scenedesmus acutusI FRPD1020 (Non-living)	28	7	600	90 mg g^{-1}	Biosorption	Kumar et al. (2015)
	Arthrospira (Spirulina) *platensis* (Non-living)	25	5–5.5	About 621	102.56 mg g^{-1}	Biosorption	Ferreira et al. (2011)
	Aulosira fertilis-sima (Non-living)	25	5	200	31.12 mg g^{-1}	Biosorption	Singh et al. (2007)
	Calothrix parietina TISTR 8093	28	7	600	45 mg g^{-1}	Biosorption	Kumar et al. (2015)
	Chlamydomonas reinhardtii (Caalginate immobilized)	25	6	100	230.7 mg g^{-1}	Biosorption	Bayramoğlu et al. (2006)

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led to a maximum adsorption capacity of 13.3 mg g^{-1} under pre-optimized circumstances (Amini et al., 2008).

Recent research has investigated using microbial composite methods for Pb cleanup. These methods involve mixing charcoal, sodium alginate, carbon fiber, and minerals with micro-organisms (Akar et al., 2013; Wang et al., 2022). Researchers have used fungal species like *Mucor plumbeus* and *Aspergillus niger* to create microbial composites that effectively remove Pb. Narayanan et al. (2021) demonstrated that the combination of *Aspergillus niger* and water hyacinth-made biochar can effectively adsorb and reduce pollutants. Ding et al. (2019) found that synthetic anatase may enhance the ability of *Aspergillus niger* to remove Pb(II) from aquatic environments. Although the final adsorption capacity did not show any noticeable fluctuation, the speed at which *Aspergillus niger* adsorbed Pb(II) increased by 204%. (Figure 3). The results highlight the possibility of using microbial composite techniques to enhance the removal of Pb pollutants from the atmosphere by leveraging the combined effects of micro-organisms and different materials.

2.3 Microalgae assisted remediation

Researchers worldwide are interested in microalgae because of their exceptional biological features, such as higher photosynthetic efficacy and strong growth in harsh atmospheres with high HMs levels, limited nutrients, and extreme temperatures (Shan et al., 2022). Because of their high tolerance and large number of surface binding sites, researchers increasingly use microalgae for the remediation of HMsin polluted areas. Priatni et al. (2018) stated that removing Pb through microalgae involves two steps. Initially, the external environment quickly absorbs Pb-II, gradually diffusing over the cell membrane and accumulating within the cell. The microalgae's cell wall comprises laminaran, monomeric alcohols, deprotonated sulfate, and different functional groups, such as hydroxyl, amino, and carboxyl, which are the essential components for the Pb adsorption (Pradhan et al., 2019).

Several studies have shown that microalgae species like *Chlamydomonas reinhardtii*, *Aphanothece* sp., *Isochrysis galbana*, and *Chlorella sorokiniana* are effective in removing Pb-II from polluted environments (Table 1) (Hu et al., 2018; Keryanti and Mulyono, 2021; Li et al., 2021; Tan et al., 2022; Ye et al., 2022). Moreover, Zeraatkar et al. (2016) showed that variations in the cell wall composition and amount of various microalgae affect their ability to absorb Pb such as at the initial Pb-II level of 10 mg L^{-1}, a removal efficiency of 92.2% was recorded.

3 Microbial Pb-II remediation mechanisms

Micro-organisms use the following remediation mechanisms to reduce the harmful effects of Pb (Figure 4).

3.1 Biosorption

Biosorption is vital for immobilizing Pb outside the cell to prevent its entry, which is achieved by many procedures, such as ion exchange, electrostatic interactions, and the cell wall binding of Pb-II (Chia et al., 2020; Shan et al., 2023). Pb-II biosorption often happens sequentially. The Pb-II biosorption rate first rises due to the abundance of accessible cell surface binding sites. However, the adsorption rate significantly decreases toward the end of the process as the binding sites fill up (Shan et al., 2023). Repulsive interactions between ions with similar charges may hinder the Pb-II adsorption on the cell surface (Sevak et al., 2021).

Researchers found *Pseudomonas aeruginosa,* a Pb-resistant strain, also known as 4EA, in polluted soil at an automobile battery disposal location in India. When researchers grew the cells in a solution comprising 166 mg L^{-1} of Pb-II, they noted a notable buildup of Pb on the cells' surface (Naik and Dubey, 2011). The primary constituents of the cell wall consist of polysaccharides, chitin, and cellulose derivatives. These components include several functional groups that have the ability to effectively adsorb Pb(II). Several research have shown that carboxyl, hydroxyl, sulfhydryl, amine, and phosphonate groups have a role in the adsorption of Pb(II). These functional groups have the ability to form complexes with Pb(II) based on their ion exchange potential (Ha et al., 2010; Jin et al., 2016). The biosorption method for Pb(II) is not uniform but rather differs depending on the specific micro-organisms involved.

Mota et al. (2016) investigated the process by which *Cyanothece* sp. CCY 0110 absorbs Pb(II) ions; the results obtained from the research showed that the stretching vibrations of the hydroxyl and carboxyl groups on the cytoderm of the cell made it better at absorbing Pb-II onto its surface. Furthermore, several adsorption tests have conclusively shown that both living and non-living microbes can adsorb Pb(II). This is because high temperatures during the inactivation process remove some functional groups from dead biomass, making living biomass better at adsorbing Pb-II (Rahman et al., 2019). However, under some circumstances, dead biological material can adsorb a greater amount of metal ions than live organic matter due to pH and temperature control (Srinath et al., 2002). -The surfaces of microbes can adsorb Pb-II through covalent bonding, or non-covalent interactions. An adsorption isotherm, which demonstrates experimental behavior and allows for adsorption process prediction, assesses the biosorbent's ability to adsorb Pb-II. Following the Freundlich model, the dissolved lead (Pb-II) sticks to different surfaces by multilayer adsorption.

Moreover, Guan et al. (2005) discovered that *Sphaerotilus natans* can take in all Pb-II at levels below 20 mg L^{-1}. They also found that the process of absorption follows the Freundlich isotherm model. When there is minimal contact force between Pb-II molecules and microbial surfaces, the adsorption process adheres to the Langmuir model. Bacillus strain MRS-2 (ATCC 55674) tended toward the Langmuir isotherm model while adsorbing Pb-II, indicating a monolayer adsorption process (Hoyle-Gardner et al., 2021).

Fungus like *Saccharomyces cerevisiae* has shown the ability to capture and retain 65–79 percent of Pb and Cd from soil that is polluted (Lee et al., 2001) The biosorption process involves the utilization of fungal cell walls, which consist of chitin, proteins, glucans, lipids, pigments, and polysaccharides. These cell walls possess functional groups such as hydroxyl, carboxyl, amino, sulphate, or phosphate, and the process is facilitated by interactions such as adsorption, ion exchange, and complexation (Ojuederie and Babalola, 2017). Wood-decaying species, such as white-and brown-rot fungi, as well as mushrooms and other fungi, are used in mycoremediation due

to their capacity to absorb heavy metals in their fruiting bodies (Jeyakumar et al., 2023).

The biosorption was first noticed in several microalgae during the early 1970s, when radioactive substances and heavy metals released from a nuclear reactor were accumulated in microalgae (Abdelfattah et al., 2023). The cell wall of microalgae is directly accountable for biosorption, and its chemical composition plays a crucial part in the process. Furthermore, microalgal surfaces possess holes, and the presence of surface charge facilitates biosorption. The cell wall of microalgae contains many chemical groups, including carboxyl, hydroxyl, and sulfate. These groups serve as binding sites and also act as ion exchangers, facilitating the complexation of metal ions and the adsorption of organic compounds from contaminated water (Soto-Ramírez et al., 2021).

Moreover, the cell surface's active binding sites have the capability to create complexes with certain contaminants found in water. This process triggers flocculation and leads to a decrease in the overall amount of dissolved and suspended solids (Al-Tohamy et al., 2022). The process of HMs ions biosorption by microalgae occurs via a two-step method. The process comprises two stages. The first stage is metabolism-independent and involves the quick and reversible binding of adsorbate onto active sites on the surface of microalgae. The second step is slower and involves positive intracellular diffusion, predominantly driven by the metabolic activity of microalgae (Abdelfattah et al., 2023).

3.2 Bioaccumulation

Bioaccumulation refers to the accumulation of elements or compounds by organisms from their environment as they grow, storing these substances in their bodies. Transporters and passive diffusion facilitate the transfer of Pb ions into microbial cells, which move from zones of high absorption to zones of low absorption. The dissolved lead (Pb-II) can penetrate the cell core and build up even after adhering to the cell surface (Arifiyanto et al., 2017). Pb ions can form associations with cytoplasmic molecules upon entering the cell or diffuse into vacuoles. Active microbes accumulate more Pb in their cells than inactive biomass due to proteins with vital biological roles. *Bacillus coagulans* R11 cells that were active could eliminate a lot of Pb up-to 17.53 mg g^{-1} of Pb-II in the best conditions (Xing et al., 2021).

According to Xing et al. (2018), bacteria in active growth accumulated more Pb within their cells than dormant spores, suggesting an active transport system for Pb-II absorption into the cell. Further, Liu and Yen (2016) studied the sulfate-reducing bacteria, specifically *Shewanella oneidensis,* in which the cells absorbed Pb-II by passive diffusion. The growth stage of micro-organisms influences the rate of Pb bioaccumulation, peaking during the logarithmic growth phase and then declining over time (Sizentsov et al., 2019). Biological components such as cysteine, cytosolic polyphosphates, sulfide, and glutathione can combine with Pb to protect against Pb-II exposure. Huang et al. (2016) suggested three conserved cysteines might interact with Pb-II in a trigonal-pyramidal coordination configuration. Similarly, Gadd and White (1993) discovered some proteins exhibiting distinct reactions to Pb ions, while Li et al. (2022) elucidated how cytoplasmic proteins, including thioredoxin (TXN) and formaldehyde-activated enzyme (GFA), assist in the interaction of Pb-II with glutathione within cells, offering protection against toxicity. Cells contain a significant amount of metallothioneins (MTs), characterized by their high sulfhydryl amount and low molecular weight. Metallothioneins can accumulate heavy metal ions in living organisms, assisting in their growth and chemical processes (Blindauer et al., 2002; Liu et al., 2003).

Pseudomonas aeruginosa strain WI-1 may accumulate Pb-II internally using the bmtA gene, which produces MTs, with a maximum capacity of 26.5 mg g^{-1} (Naik et al., 2012a). Similarly, *Salmonella choleraesuis* strain 4A was shown to have genomic DNA including MTs (SmtA), associated with Pb resistance, and capable of accumulating up to 19 mg g^{-1} (Naik et al., 2012b). Arifiyanto et al. (2017) observed the presence of microtubules in *Bacillus* sp. following exposure to Pb. These MTs helped make Pb complexes inside the cytoplasm.

Previous studies by Xu et al. (2014) and Zhang et al. (2016) confirm that certain fungi have the ability to gather significant quantities of heavy metals by binding them with glutathione (GSH) within their cells without causing any damage to the cell structure. However, the majority of research found in the literature about the removal of Pb(II) primarily concentrate on the choice of fungal strains, the optimization of environmental conditions, and the measurement of removal effectiveness. Only a small number of studies have examined the processes involved in the elimination of Pb(II). In addition, a smaller number of articles examined the processes of Pb(II) elimination specifically from the perspective of minerals containing Pb. *Penicillium polonicum*, a filamentous fungus, was obtained from the effluent of a lead-zinc mine located in Dexing City, Jiangxi Province, China (Xu et al., 2020). The fungus was confirmed to have the ability to tolerate Pb(II) concentrations of up to 12 mmol L^{-1} (2486.4 mg L^{-1}) 13 and had a high efficiency in removing Pb (Yang et al., 2012).

Understanding the bioaccumulation mechanism is essential for effectively addressing Pb pollution in affected areas. Researching micro-organisms that can collect Pb can potentially improve Pb cleanup methods. Enhancing our understanding of Pbaccumulation and identifying micro-organisms with exceptional abilities will help us develop precise and efficient cleaning strategies. This field of study shows promise for mitigating the detrimental effects of Pbpoisoning on the environment and human health. A list of microbes reported to increase plant growth and productivity under Pb stress compared to control is shown in Table 2.

Microalgae have the ability to gather various contaminants together with nutrients and microelements that are already present (Mustafa et al., 2021). Microalgae has the ability to adapt to their surroundings, enabling them to withstand low quantities of contaminants. In addition, microalgae have a high level of tolerance to various contaminants originating from residential, agricultural, and industrial sources, hence enhancing their potential to remediate these pollutants (Wu et al., 2012; Mojiri et al., 2020). In order to enhance the effectiveness of microalgae in bioremediation, it is essential to adjust the physicochemical conditions. This is because the pace and capacity of microalgae in the bioaccumulation process are dependent on these factors. Furthermore, the process of selecting microalgae species that can withstand high levels of pollutants is a successful approach to increase the ability and speed of bioaccumulation (Abdelfattah et al., 2023).

TABLE 2 A list of microbes reported to increase plant growth and productivity under Pb stress compared to control.

Microbial inoculants	Plant species tested	Effect on plants	References
Pseudomonas putida KNP9	*Vigna radiata*	Increase in root growth-20% Increase in shoot growth-19%	Tripathi et al. (2005)
Burkholderia sp. J62	*Zea mays*	Overall increase in plant growth and productivity-75%	Jiang et al. (2008)
	Solanum lycopersicum	Overall increase in plant growth and productivity-30–54%	
Pseudomonas fluorescens	*Brassica napus*	Overall increase in plant growth and productivity-21%	Sheng et al. (2008)
Microbacterium sp. G16		Overall increase in plant growth and productivity-35%	
Bacillus sp. SC2b	*Sedum plumbizincicola*	Overall increase in plant growth and productivity-42-46%	Ma et al. (2015)
Funneliformis mosseae	*Robinia pseudoacacia*	Increase in root growth-28%, Increase in shoot growth-43%	Yang et al. (2015)
Rhizophagus intearadices		Increase in root growth-38% Increase in shoot growth- 75%	
Phialocephala fortinii	*Clethra barbinervis*	Overall increase in plant growth and productivity- 376%	Yamaji et al. (2016)
Rhizodermea veluwensis		Overall increase in plant growth and productivity- 157%	
Rhizoscyphus sp.		Overall increase in plant growth and productivity-213%	
Bradyrhizobium japonicum	*Lactuca sativa*	Increase in root growth- 42%, Increase in shoot growth-28%	Seneviratne et al. (2016)
Bacillus sp.QX8 and QX13	*Solanum nigrum*	Overall increase in plant growth and productivity- 1.36 fold	He et al. (2020)
Bacillus spizizenii DSM (SN36)	*Spinaacia oleracea* L	Overall increase in plant growth and productivity- 170%	Desoky et al. (2020)
Paenibacillus jamilae		Overall increase in plant growth and productivity- 179%	
Pseudomonas aeruginosa		Overall increase in plant growth and productivity- 205%	
Pseudomonas spp.	*Anethum gravelens* L	Overall increase in plant growth and productivity- 117%	Rahbari et al. (2021)

3.3 Bioprecipitation and biomineralization

Biosorption and bioaccumulation frequently occur alongside precipitation and mineral formation, which may postpone toxicity's inception. Bioprecipitation and biomineralization are vital in decreasing Pb-II availability and aiding in environmental reuse (Figure 3). Micro-organisms can immobilize Pb-II by causing it to precipitate, a process known as intracellular or extracellular biomineralization (Figure 4).

3.3.1 Micro-organisms engaging in extracellular biomineralization

Micro-organisms frequently excrete oxalic acid as an external metabolite. The solubility of oxalic acid reduces dramatically when it forms a chelating complex with Pb-II metal cations. Xu et al. (2020) showed that Pb-II notably boosts the release of oxalic acid by *Penicillium polonicum*. The response is due to the fungal stress reaction, which involves creating lead oxalate minerals outside the cell to reduce Pb toxicity. Ding et al. (2019) found that living cells of *Aspergillus niger* may trap Pb-II as lead oxalate on their cell wall.

According to Park et al. (2011), microorganism-induced phosphate precipitation is a cost-effective, viable, and ecologically friendly approach to address Pb pollution. Phosphate-solubilizing bacteria (PSB) are essential for breaking down phosphate using enzymes such as phytase or phosphatase, which helps increase phosphorus availability in soil. Scientists worldwide are interested in immobilizing Pb-II by using available phosphates to react with Pb, transforming it into less soluble forms such as lead phosphates (Wang et al., 2020). Although immobilizing Pb-II with phosphates may lead to a decrease in plant-accessible phosphorus, it remains a feasible method in agricultural areas when handled with caution. Farmers may reduce Pbhazards and preserve soil fertility and crop production by using accurate application techniques, utilizing alternate phosphorus sources, adopting effective crop management practices, and implementing constant monitoring. The effectiveness of the phosphorus amendment in lead-contaminated soil is contingent upon the soil type, as well as the characteristics and magnitude of the contamination. Thorough analysis should be conducted on the kind and rate of the P source, as well as the application management, for soil amendment (Miretzky and Fernandez-Cirelli, 2008).

The microbes release enzymes such as phosphatases to break down β-glycerol phosphate, releasing PO_4^{3-}ions that react with Pb-II to form a precipitate (Naik and Dubey, 2013). General anions such as fluoride (F^-), chloride (Cl^-), and bromide (Br^-) can assist in PbII mineralization when PSB is present. Qiao et al. (2019) proposed that *Bacillus subtilis* X3 may convert Pb-II into $Pb_5(PO_4)_3OH$ and $Pb_5(PO_4)_3Cl$ by mineralization, such as Su et al. (2020) found $Pb_5(PO_4)_3OH$ on the *Rhodobacter sphaeroides* SC01 cell membrane. This is primarily because of the complex interaction between the phosphate group and Pb ions. Researchers have already found that microbial metabolites and organic chemicals can speed up the breakdown of pyromorphite, which lets the Pb out (Debela et al., 2010; Topolska et al., 2013). Thus, using PSB for Pb cleanup still presents a notable issue.

Moreover, micro-organisms containing urease enzymes may efficiently trap Pb-II together with PSB. They drive the process by catalyzing urea hydrolysis, increasing pH levels to between 8.0 and 9.1, and generating CO_3^{2-} ions, which encourage the creation of calcium and lead carbonates (Shan et al., 2021). Stocks-Fischer et al. (1999) found that when conditions are alkaline, calcium ions in the calcium carbonate lattice may eventually be replaced by Pb ions. This can cause composite calcium-lead carbonate precipitates to form. Achal et al. (2012) were the first to introduce microbial carbonate precipitation procedures for treating Pb-contaminated soils. They showed that *Kocuria flava* efficiently traps Pb by generating lead oxide and carbonate. Furthermore, lead ions (Pb-II) can transform into calcite crystals when they touch a cell wall. The carbonate precipitation induced by Microbes for remediating Pb pollution is mostly experimental or restricted in scale

due to restrictions linked to nutrition supply, calcium supplementation, urea availability, and microbial mobility.

3.3.2 Micro-organisms engaging in intracellular biomineralization

Micro-organisms can precipitate Pb ions by generating extracellular metabolites. Additionally, the movement of Pb ions into cells might result in their immobilization via biological mechanisms. Xu et al. (2020) showed that *Penicillium polonicum* could transfer Pb from the external environment into the cytoplasm and then transform it into Pb(0) with the help of reductase enzymes. Sani et al. (2010) highlighted the importance of goethite and lead chloride treatment in producing compact lead/iron sulfide precipitates in the cytoplasm and periplasm of *Desulfovibrio desulfuricans* G20. Levinson et al. (1996) found that high concentrations of $Pb(NO_3)_2$ can lead to the movement of Pb-II into the cytoplasm of *Staphylococcus aureus*, leading to the creation of lead phosphate $[Pb_3(PO_4)_2]$ deposits.

Providentia alcalifaciens may immobilize the Pb strain 2EA and *Vibrio harveyi*, creating $Pb_9(PO4)_6$ precipitates inside the cells. Li et al. (2022) conducted a proteomics study that showed how enzymes triggered by thioredoxin and glutathione in the presence of formaldehyde may help create Pb-glutathione composite precipitates within cells. Shan et al. (2022) used Selected Area Electron Diffraction (SAED) patterns and Field Emission High-Resolution Transmission Electron Microscopy (FE-TEM) to study the formation of unique lead oxides ($Pb_2O_{3.333}$) in *Aspergillus tubingensis* cells grown in a solution with 828 mg L^{-1} of Pb-II. Their research exhibited that Pb-II was oxidized, leading to the creation of lead oxide precipitates inside *Aspergillus tubingensis* cells. The exact process of how Pb forms within cells is not well known because of the intricate interaction between minerals and bacteria.

Sayer et al. (1999) exhibited that an *Aspergillus niger* produced lead oxalate and lead oxalate dihydrate through microbial phosphate-solubilizing mechanisms during the transformation of pyromorphite $[Pb_5(PO_4)_3Cl]$. This discovery marked the first recorded instance of the biogenesis of this mineral. Subsequent studies have revealed that numerous bacteria and fungi, when exposed to different environmental conditions, are capable of immobilizing Pb ions. They achieve this by converting inorganic phosphate sources, such as apatite minerals, or organic phosphate sources, such as phenolphthalein diphosphate, glycerophosphate, acephate, glycerol 2-phosphate, and phytic acid, into phosphate. This conversion process occurs in the presence of either phosphatase or phytase enzymes.

3.4 Efflux mechanisms

For optimal growth of micro-organisms, it is crucial to manage the concentration of harmful heavy metals within cells using efflux mechanisms (Nies, 1999). Various micro-organisms, especially those in polluted settings, exhibit heavy metal outflow (Yin et al., 2019). Multiple groups of membrane transporters facilitate exocytosis at the plasma membrane. The transporters can be classified into different groups, including the ATP-binding cassette (ABC), multidrug endosomal transporter (MET), and resistance-nodulation-cell division (RND) group. Resistance genes carried on plasmids primarily serve to control the activity of metal ion transporters.

Nevertheless, the particular micro-organism and heavy metal ions can impact the nature of this interaction. Specific transporter proteins hinder the excessive buildup of Pb-II in cellular structures. P-type ATPases, classified as transmembrane transport proteins, enable the transportation of tiny organic molecules and ions across cellular membranes (Coombs and Barkay, 2004).

According to Hynninen et al. (2009), the *Cupriavidus metallidurans* CH34 can tolerate Pb-II by removing it from the cytoplasm via P-type ATPase (Figure 5).

The zntA gene in *Escherichia coli* makes Pb-II-translocating ATPases, similar to the cadA gene in the pI258 plasmid from *Staphylococcus aureus*. Both genes are implicated in Pb-II translocation, as reported by Rensing et al. (1999). Furthermore, P-type ATPases transport HMs from the cytoplasm to the periplasm. An ion-proton exchanger called the CBA efflux pump moves metal ions out of the periplasm and into the extracellular space. Among the P-type ATPases, PIB-type ATPases are significant because they eliminate Pb-II, maintain homeostasis, and prevent Pb-II poisoning (Rensing et al., 1999). Similarly, Coombs and Barkay (2004) revealed that PIB-type ATPases facilitate Pb-II transport via the cell wall. Most sequenced archaea, bacteria, and eukaryote genomes have around one hundred genes that encode PIB-type ATPases. Moreover, Argüello et al. (2003) studied how heavy metals affect PIB-type ATPases in the thermophile *Archaeoglobus fulgidus*, focusing on the CopA enzyme. Researchers discovered that certain heavy metals could potentially trigger the CopA enzyme. The operon pbrUTRABCD in *Ralstonia metallidurans* was sequenced by Taghavi et al. (2009) to control Pb-II and lessen toxicity and hazardousness.

4 Factors affecting Pb-II elimination by micro-organisms

4.1 Effects of pH

The pH of the environment significantly influences the availability of Pb to micro-organisms, affecting microbial biomass and enzyme activity. Scientists who study how pH affects oxidation–reduction enzymes like phosphatase and urease have pointed out how important they are for microbes to keep Pb ions from moving. pH has a considerable impact on the precipitation of Pb (Shan et al., 2022). The cell wall's functional groups strongly bind to H3O+ ions at low pH, generating repulsive interactions restricting the Pb-II adsorb and precipitating on the cell wall. The best pH range for removing Pb-II from microalgae-immobilized biomass is between 5 and 6. As the pH rises, $Pb(OH)_2$ precipitates form. Decreased pH levels reduce the effectiveness of removing Pb-II because Pb ions and hydrogen ions compete for adsorption sites (Akhtar et al., 2004).

Moreover, *Bacillus subtilis* FZUL-33 has been shown to co-precipitate Pb-II when the pH is above 5.5, and the mineral's shape is strongly influenced by pH (Lin et al., 2016). Li et al. (2013) found that *Sporosarcina pasteurii* strains speed up the biomineralization process of Pb. This process turns Pb into solid lead carbonate crystals when the pH level is between 8 and 9. pH affects the crystallization of Pb ions and also influences the production of humic and fulvic acid by bacteria. Pérez-Esteban et al. (2019) suggested a direct relationship between pH levels and the amounts of humic and fulvic acids in the environment. Humic chemicals can decrease the movement of Pb ions in the

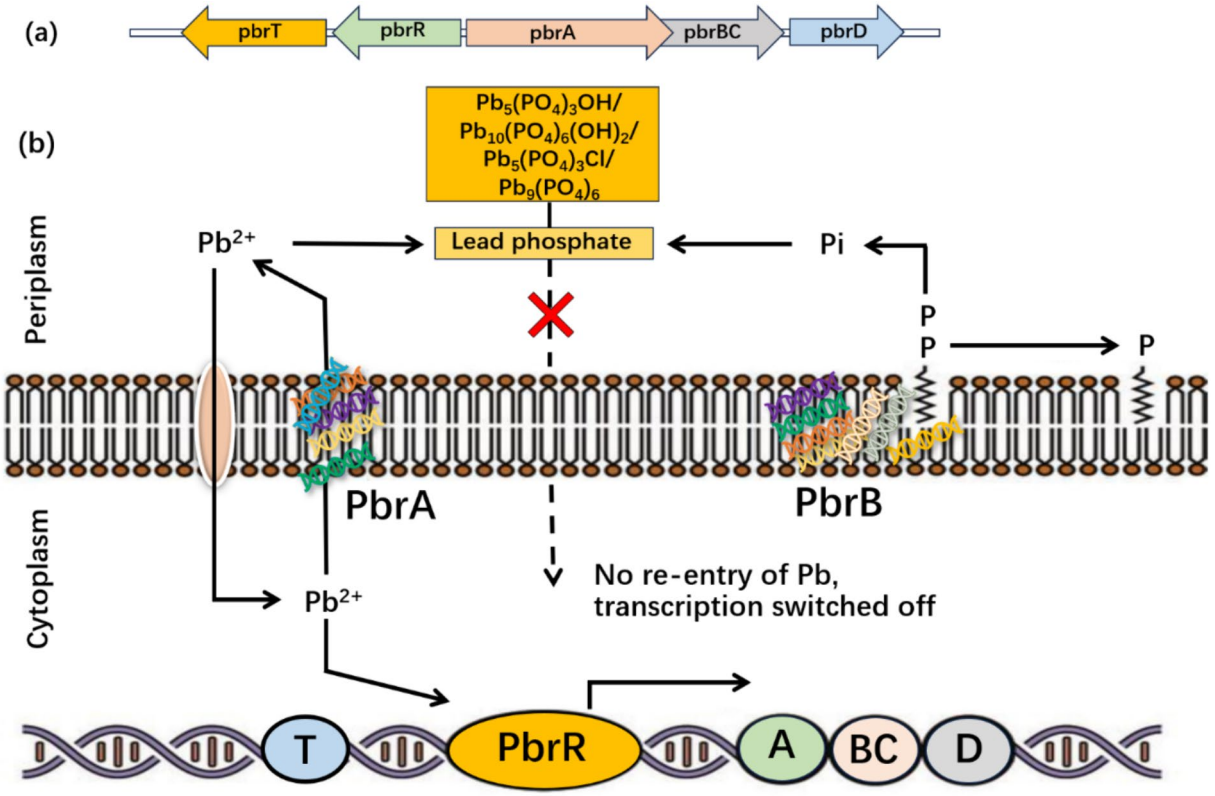


FIGURE 5
Bacterial response to Pb toxicity at the molecular level. **(A)** Pb-resistant genes pbrTRABCD in *Cupriavidus metallidurans* CH34 and **(B)** stimulation of Pb-resistant genes and generation of lead phosphate in bacteria [Modified from Figure in Hynninen (2010)].

environment by forming complexes (Shan et al., 2022). To find the best pH to eliminate Pb-II, it's essential to do a complete analysis of different micro-organisms, considering the amount of secretion, the level of ionization, the adsorption sites, and the surface charge of the adsorbent. The pH of the environment significantly influences the availability of Pb to micro-organisms, affecting microbial biomass and enzyme activity. Scientists who study how pH affects oxidation–reduction enzymes like phosphatase and urease have pointed out how important they are for microbes to keep Pb ions from moving. pH has a considerable impact on the precipitation of Pb (Shan et al., 2022).

4.2 Effects of temperature

The efficiency of removing Pb-II differs across various microbial species and is influenced by temperature changes. Temperature impacts microbial activity, including secretion content, biomass, and enzyme activities related to Pb-II binding. Temperature significantly influences the rate of growth of phosphate-mineralizing bacteria (PMB) (Qian and Zhan, 2016). The biomass of PMB reached its highest point at 30°C, leading to a substantial production of alkalinity material and phosphatase throughout the growth process. Zucconi et al. (2003) discovered that treating Paecilomyces lilacinus with high temperatures made living cells adsorb more Pb-II than dead cells. This suggests that high temperatures may stop enzymes that bind Pb from working. Micro-organisms showed an enhanced capacity to adsorb Pb-II within a certain temperature range as temperature increased (Bandowe et al., 2014).

Furthermore, extracellular polymeric substances (EPS) significantly impact Pb-II adsorption, and their formation is linked to temperature. EPS production by different bacteria takes place between −2°C and 42°C, with the amount of EPS generated being influenced by the ideal temperature for microbial development. Most microbes usually release more EPS at 25°C and 30°C (Takeda et al., 1991; Nichols et al., 2005; Zheng et al., 2008; Buthelezi et al., 2010). Choosing the best temperature for EPS production and microbial growth is crucial, considering the appropriate microbial species and environmental conditions. The efficiency of removing Pb-II differs across various microbial species and is influenced by temperature changes. Temperature impacts microbial activity, including secretion content, biomass, and enzyme activities related to Pb-II binding.

4.3 Biostimulation and bioaugmentation

Micro-organisms that can handle Pb are often found in places where Pb is present. These micro-organisms are very resistant to oxidation and have ways to get rid of Pb (Li et al., 2020). These native organisms are essential in the biogeochemical process of heavy metal remediation. Researchers have proven that biostimulation techniques,

such as providing more nutrients, electron donors, or acceptors, enhance the resilience of micro-organisms in Pb polluted areas, leading to higher immobilization or transformation of Pb pollutants (Hou et al., 2020). Similarly, Huang et al. (2006) grew *Phanerochaete chrysosporium* in Pb-contaminated fields, which led to the breakdown of straw and the creation of humus, which may bind and trap Pb-II ions.

Moreover, biofertilizers stimulate the growth and development of native micro-organisms and increase the synthesis of organic matter that interacts with soil to create significant clusters of organic minerals and materials (Wang et al., 2019). Bioaugmentation, also known as *in-situ* bioremediation, introduces lab-grown bacteria capable of handling Pb in polluted areas (Yu et al., 2016). Current bioaugmentation approaches mainly include utilizing laboratory-cultivated microbial strains to create biofertilizers that enhance plant development. However, these laboratory-cultivated micro-organisms typically face challenges when competing with natural species.

Additional study is necessary to enhance the practicality and effectiveness of lab-grown microbes in immobilizing Pb in polluted areas. Due to the complex biological structures of contaminated areas and their adjacent surroundings, achieving the desired results with only one remediation method might be difficult. Integrating several approaches customized to the individual needs of real-time remediation is essential for maximizing the effectiveness of Pb-contamination cleanup.

5 Genetic-engineering techniques

Bioremediation research has turned to genetic engineering as a viable method because it enhances microbes' resistance to metal stress, boosts the production of metal-binding proteins, and expands the storage capacity of metal. Genetic engineering methods usually entail inserting individual genes or operons and altering current gene sequences to create new strains with distinct metal-binding properties.

Four primary techniques are being considered in developing genetically engineered microbes for bioremediation (Jeyakumar et al., 2023). These techniques include: (1) using bio-affinity bioreporter sensors to sense chemicals, analyze end points, and reduce toxicity; (2) creating, monitoring, and controlling bioprocesses; (3) enhancing affinity and enzyme specificity; and (4) constructing and regulating routes. The choice of cellular factories is a crucial element that must be taken into account. Within the realm of fungus, bacteria, and algae strains, the bacterial system has been shown to possess significant promise due to its inherent characteristics, including a faster growth rate, confinement, and ease of genetic modification. However, cyanobacteria and microalgae contribute to sustainability and economic feasibility due to their photosynthetic activities and sophisticated metabolic pathways, similar to those found in the plant kingdom. The main function of biosorption and bioaccumulation is to remediate heavy metals. Efforts are being made to develop techniques that increase the adsorption of heavy metals on cell surfaces and boost the capacity to accumulate these metals by introducing porters (Diep et al., 2018). For instance, certain bacteria absorb and increase the expression of metal ion import systems such as channels, main active transporters, and secondary carriers to enhance the absorption of particular heavy metals.

Additionally, there is a strong focus on significantly reducing or treating heavy metals after their buildup in living organisms. Enzymes and proteins that are specially intended to decrease heavy metal complexes are added to various species to improve their ability to remediate these contaminants. Metal importers, which rely on enhanced diffusion processes mediated by channel proteins, were incorporated to enhance the absorption of arsenic and mercury ions (Diep et al., 2018). Researchers have shown increasing interest in cell surface engineering, specifically in expressing metal-specific peptides in the extracellular phase to improve adsorption and remediation processes. A wide variety of cell surface peptide display systems are found in many microbial species and are well documented (Wang et al., 2021). *Escherichia coli* is a system in which various techniques of cell surface engineering are experimented with and confirmed. The metal-binding protein EC20, siderophore-binding protein, and CueR were individually examined for their ability to bind Pb, iron, and copper, respectively (Wang et al., 2021). The CadR gene from the wild-type *Pseudomonas putida* strain was modified in *Saccharomyces cerevisiae* and exhibited about a six-fold increase in the binding effectiveness for cadmium compared to the wild type strain (García-Hernández et al., 2017).

Additionally, Wei et al. (2014) changed *Escherichia coli* to make a strain that selectively takes Pb from solutions containing other heavy metals. They added the promoter section for PbrR and Pb-specific binding proteins. Similarly, Almaguer-Cantú et al. (2011) showed that incorporating a gene that expresses metallothionein from mice into *Escherichia coli* greatly improved the absorption of Pb-II.

Furthermore, Jafarian and Ghaffari (2017) found that adding a gene that codes for metallothionein (CgMT) to *Escherichia coli* BL21 (DE3) made it better at absorbing Pb ions, which helped get rid of Pb from polluted areas. Transgenic micro-organisms have benefits in the remediation of heavy metals, such as enhanced efficiency, resilience, and ecological preservation. However, their practical use is mainly restricted to laboratory trials. Before realizing widespread practical deployment, we need to conduct additional research to address safety, regulatory permission, and field application issues.

6 Research gaps and challenges

6.1 Mechanistic understanding at the molecular level

Although several methods of Pb resistance and remediation by microbes have been identified, there is still a dearth of full knowledge of the molecular processes involved. A comprehensive understanding of gene regulation, protein interactions, and metabolic alterations in response to Pb stress is crucial for improving and maximizing microbial remediation techniques. Furthermore, it is essential to conduct comprehensive genomic, transcriptomic, and proteomic research to discover and describe the genes and proteins that play a role in Pb detoxification. These studies may assist in developing micro-organisms that have improved capacities to remove Pb from the environment.

6.2 Optimization of microbial strain

The progress in creating genetically engineered or hybrid strains with enhanced capacities to remove Pb is still in the early stages.

Further research is required to improve the efficiency and stability of these strains under various environmental situations. Further investigation is required to explore the possible advantages of using microbial consortia, consisting of numerous microbial species working together instead of a single strain, for Pb remediation. Gaining insight into the interactions among microbial consortia can enhance the efficacy of remediation efforts.

6.3 Interaction with native microbiota

The understanding of the interaction between imported Pb-remediating micro-organisms and native microbiota in contaminated environments is limited. Future research should investigate possible ecological disturbances and strategies to minimize adverse effects on the indigenous microbial populations. It is necessary to conduct long-term studies on the adaptability and development of microbial communities continuously exposed to Pb to comprehend microbial remediation's long-term sustainability and dependability.

6.4 Environmental and operational factors

Further systematic research is required to determine the influence of several environmental parameters, such as pH, temperature, the presence of other heavy metals, and organic matter, on the effectiveness of microbial Pb remediation. Validating field and laboratory results is essential. Transferring successful experiments conducted in the laboratory to practical solutions that can be implemented on a broader scale in the field presents considerable difficulties. The research should prioritize the development of scalable procedures, including the design of bioreactors, conducting field experiments, and assessing the economic viability of large-scale operations.

6.5 Comparative studies with other remediation methods

There is a need for systematic comparative studies to evaluate the effectiveness of microbial remediation compared to standard physical and chemical techniques. Those studies should prioritize the evaluation of cost-effectiveness, environmental impact, and long-term sustainability to establish microbial remediation as a feasible option.---.

7 Conclusion and future perceptions

This review highlights the substantial potential of microbial remediation as a successful approach for reducing Pb contamination in agricultural soils and wastewater. Using bacteria, fungi, and microalgae makes it feasible to convert the toxic Pb into less harmful forms by employing processes such as biosorption, bioprecipitation, biomineralization, and bioaccumulation. The findings highlight the versatility and efficiency of microbial systems in immobilizing Pb, notably through the generation of nanoparticles that convert dissolved lead (Pb-II) into stable, less toxic states. Notably, *Pseudomonas* sp., *Bacillus* sp., and *Aspergillus niger* are among the commonly studied biosorbents for Pb-II removal. Exploring genetic engineering techniques and molecular-level mechanisms has provided a deeper understanding of how microbes develop resistance to Pb, revealing intricate detoxification pathways and resistance genes. Despite the promising advancements, challenges remain in the practical implementation of microbial remediation. Choosing the best temperature for EPS production and microbial growth is crucial, considering the appropriate microbial species and environmental conditions. The efficiency of removing Pb-II differs across various microbial species and is influenced by temperature changes. Temperature impacts microbial activity, including secretion content, biomass, and enzyme activities related to Pb-II binding. Addressing these challenges requires further research to develop robust, adaptable microbial consortia capable of thriving in diverse, contaminated environments. In conclusion, microbial remediation is promising for sustainable Pb detoxification in polluted environments. Continued interdisciplinary research and innovation are essential to overcome current limitations and fully realize the potential of microbial systems in restoring Pb-contaminated ecosystems.

Author contributions

MA: Conceptualization, Formal analysis, Methodology, Project administration, Writing – original draft, Writing – review & editing. IG: Formal analysis, Software, Writing – review & editing. FL: Resources, Writing – review & editing. TL: Resources, Writing – review & editing. YC: Resources, Writing – review & editing. HL: Funding acquisition, Writing – review & editing. MIA: Writing – original draft, Investigation. SL: Data curation, Methodology, Writing – review & editing. WF: Resources, Writing – review & editing.

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Conflict of interest

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

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Significance of zinc-solubilizing plant growth-promoting rhizobacterial strains in nutrient acquisition, enhancement of growth, yield, and oil content of canola (*Brassica napus* L.)

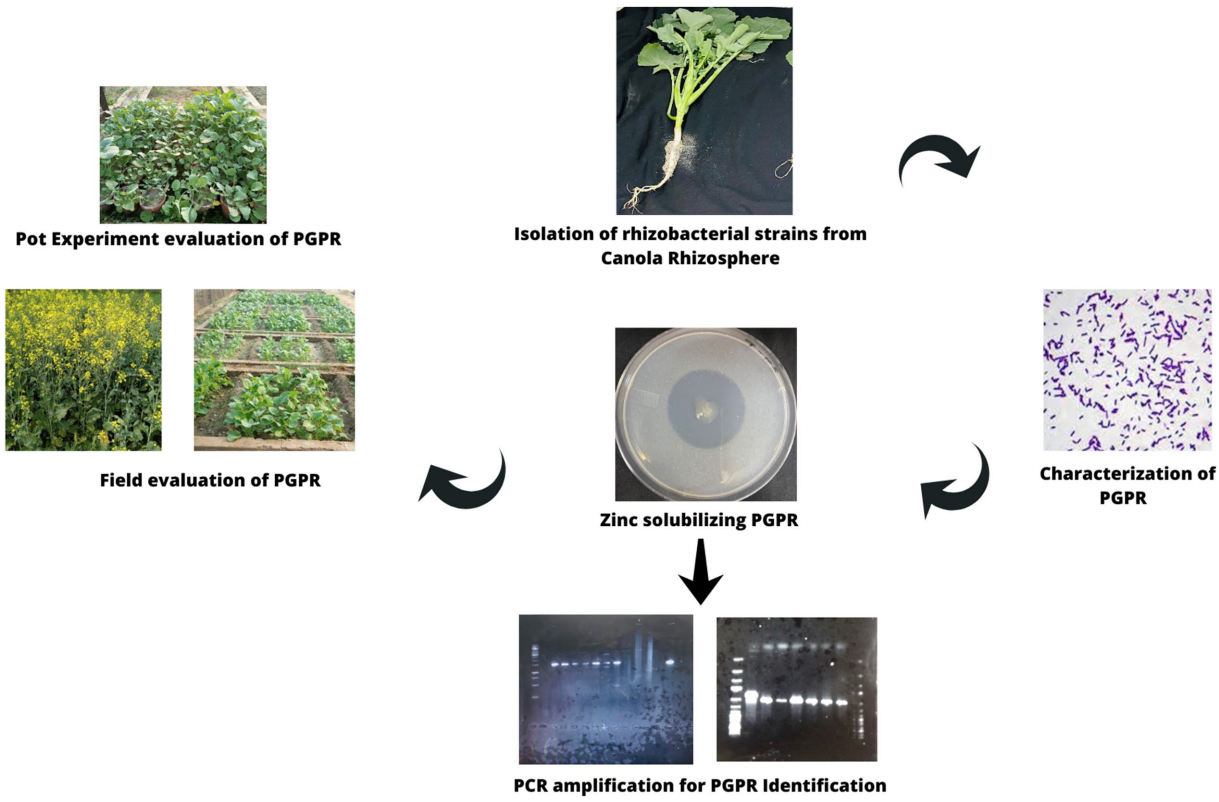
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The present study was conducted with the aim to isolate, characterize, and identify the promising zinc-solubilizing rhizobacteria found naturally in the rhizosphere of canola (*Brassica napus* L.) plants. The study investigated the roles of these strains in nutrient acquisition and assimilation of extracellular molecules such as hormones and secondary metabolites. Ten isolated promising zinc-solubilizing strains (CLS1, CLS2, CLS3, CLS6, CLS8, CLS9, CLS11, CLS12, CLS13, and CLS15) were selected and characterized biochemically. Almost all the tested strains were Gram-positive, could fix nitrogen, and were positive for indole acetic acid, HCN, exopolysaccharides, and siderophore production. These effective zinc-solubilizing strains were identified through 16S rRNA gene sequencing. Based on the amount of solubilized zinc and halo zone diameter, four potent strains (CLS1, CLS2, CLS3, and CLS9) were selected for pot and field evaluation. Among all the identified bacterial genera isolated from the rhizosphere of the same host plant at different sampling sites, *Priestia aryabhattai* was found most abundant and found at all three sampling sites. The strains *Priestia megaterium*, *Staphylococcus succinus*, and *Bacillus cereus* were found at two different sites. *Bacillus subtilis* was found at only one site. These strains have a number of plant growth-stimulating characteristics as well as the ability to colonize plant roots successfully. The results indicated that inoculation of all these four zinc-solubilizing tested strains enhanced the plant growth, oil contents, and yield attributes of canola as compared to non-inoculated control with fertilizer levels. *Staphylococcus succinus* (CLS1) was first reported as a zinc solubilizer and associated with canola. *Priestia aryabhattai* (CLS2) and *Priestia megaterium* (CLS9) were found to be the best strains, with the most pronounced beneficial effect on canola growth and yield traits in both pot and field conditions. The site-specific dominance of these strains observed in this study may contribute toward decision-making for the development of specific inocula for canola. Therefore, identification of these strains could help in providing adequate amount of soluble zinc along with enhanced plant growth, yield, and oil content of canola.

KEYWORDS

zinc-solubilizing rhizobacteria, canola, oil content, IAA synthesis, root colonization, biofilm production and sustainable agriculture



GRAPHICAL ABSTRACT
Isolation and identification of zinc-solubilizing rhizobacterial strains and their evaluation for pot and field trials.

1 Introduction

Canola (*Brassica napus* L.) is an economic agricultural and major oilseed crop in Pakistan and South Asia with a rich source of nutritive qualities (Noreen et al., 2016). Since canola oil has a high percentage of unsaturated fatty acids, therefore it is a nutritious food source for both humans and animals (Nega and Woldes, 2018; Ahmad et al., 2020). Pakistan produced 0.46 million tons of edible oil domestically, which was not enough and fulfilled only 23% of the country's requirement. Due to its increasing demand and production gap, Pakistan is facing a severe scarcity of edible oil. Soil productivity decreases as a result of rapid industrialization and vigorous cultivation practices. Due to losses caused by fixation, denitrification, leaching, precipitation, erosion, runoff, volatilization, and other processes, the majority of applied nutrients are not accessible to the plants (Gourley et al., 2012; Ghavami et al., 2016). Moreover, the improper utilization of synthetic fertilizers in agriculture is an important factor leading to changes in soil pH and ultimately has some adverse environmental impacts (Suthar, 2009; Reda and Hailu, 2017; Świątczak et al., 2023). The scientific community is seeking alternative methods that could substantially play a role in promoting and sustaining the production of oil seed crops. One promising method for reducing the adverse environmental impacts caused by improper use of chemical fertilizers is seed inoculation with plant growth-promoting rhizobacteria (PGPR). Rhizosphere-associated microorganisms may efficiently improve agricultural production quality and yield and significantly reduce the use of pesticides and chemical fertilizers (Premachandra et al., 2020). Zinc is one of the key micronutrients, and it is utilized by tissues in very small amounts (30–100 mg kg^{-1} dry matter of plant) for optimal plant development and reproduction, whereas Zn contents above 300 mg/kg are toxic for plants. It normally exists in Asian soil at a concentration of 69–89 mg kg^{-1} (Kiekens, 1995). Soil deficient in Zn contents has 10–30 mg kg^{-1} of zinc (Alloway, 2008). For plants, accessible forms of zinc occur in free ions (Zn^{2+} and $ZnOH^{+}$) and organically complexed Zn solution in soil. Zinc precipitates as sulfides and forms complexes with organic matter in organic soil due to its achalcophile components (Vodyanitskii, 2010).

Zinc deficiency is a well-known problem that affects both plants and humans (Yadav et al., 2023). Its shortage in plants retards nitrogen metabolism and photosynthesis, causes a reduction in fruit development and flowering, limits the synthesis of phytohormones and carbohydrates, and delays crop maturity, leading to decrease in nutritional quality of grains and crop yield (Singh et al., 2005). Additionally, zinc activates many enzymes and plays a key role as cofactor in the enzyme tryptophan synthetase, which is involved in the production of IAA (Hafeez et al., 2013). Plants can absorb zinc in the form of the divalent cation Zn^{2+} and chelate form, although most of the zinc in the soil is found in insoluble forms $Zn(OH)_2$, ZnO, and $ZnCO_3$ (Abaid-Ullah et al., 2015). The cultivation of zinc-solubilizing bacteria is a significant substitute for chemical fertilizers, which stabilize soil characteristics over time by gradually releasing the nutrients found in the soil and preserving the soil microflora (Dinesh et al., 2018). Through bioaugmentation of microbial isolates with a

capability to solubilize insoluble Zn compounds, the unavailable Zn compounds can be transformed back into accessible form (Saravanan et al., 2007; Yadav et al., 2022). Instead of inadequate Zn availability in soil, low Zn solubility is the primary cause of the widespread Zn deficit in crops (Iqbal et al., 2010; Barbagelata and Mallarino, 2013; Gontia-Mishra et al., 2016). A number of PGPR have been reported for their ability to solubilize the insoluble form of zinc in soil (Hussain et al., 2015; Rahman et al., 2024). Inoculating crops with Zn-solubilizing isolates may not only help us to resolve the issue of malnutrition through enhancing the nutritional contents in grains, but they may also serve as essential equivalents for zinc fertilizers. Zinc PGPR regulates the soil characteristics for a longer period of time by gradually releasing the nutrients found in soil with inaccessible Zn concentrations (Sindhu et al., 2019). Hence, exploitation of zinc-solubilizing bacteria for zinc fortification in food grains like canola as well as for alleviating the zinc deficiency in canola plants could be a promising agronomical approach. The plant's growth is facilitated by rhizobacteria directly by fixing atmospheric nitrogen, simulating the synthesis of plant hormones, and alleviating nutrient uptake through zinc solubilization (Akbar et al., 2019; Kumar et al., 2019). They also facilitate the plant growth indirectly by preventing the deleterious effects of phytopathogenic organisms through antibiotics, hydrolytic enzymes (amylases, cellulases, proteases, and dehydrogenases), volatile compounds (hydrogen cyanide and ammonia), and siderophores (Kumar and Dubey, 2012; Backer et al., 2018; Gouda et al., 2018). Indole acetic acid has a direct effect on plant cell division, differentiation, and extension (Shoebitz et al., 2009). It promotes root development, xylem formation, and seed germination. Moreover, it regulates photosynthesis, formation of different metabolites, and pigment production. However, the accumulation of IAA that has been released by soil bacteria could influence the naturally occurring IAA reservoir of plants (Afzal et al., 2015). In agro-biotechnology industry, *Bacillus* is an adaptable genus and one of the most commercially exploited bacteria. Members of the genus *Bacillus* are considered to have a number of beneficial characteristics that support plants through protection from pathogens, recovery of nutrients by cycling of nutrients, and overall enhancement in growth by phytohormone production (Mumtaz et al., 2020). These strains could be employed as biofertilizers to enhance growth and productivity of agricultural crops, giving a replacement to the application of synthetic nitrogen fertilizers (Defez et al., 2017). These microorganisms promote plant growth and maximize yield of crops (Babalola, 2010; Kumar et al., 2016; Kumar et al., 2017). Microbes have evolved a number of iron acquisition mechanisms for their existence and adaptation to environment, helping the plant overcome this challenge by obtaining iron. One of these mechanisms is the formation of these molecules known as siderophores, small organic compounds synthesized by bacteria in iron-limiting conditions to increase the iron sequestration ability (Dimkpa et al., 2009; Aznar and Dellagi, 2015; Li et al., 2016). Most extensively studied genera *Bacillus* have multiple growth-promoting characteristics, such as the ability to solubilize zinc, fix nitrogen, produce antibiotics, siderophores, and secondary metabolites that inhibit soil-borne plant pathogens (Chen et al., 2007; Mumtaz et al., 2018; Shahid et al., 2022). Extracellular polymeric substances (EPS) are polymers of carbohydrate released by a wide range of zinc-solubilizing rhizobacteria. These plant-associated rhizobacteria are additionally employed to promote systemic tolerance by the synthesis of EPS (Naseem et al., 2022). Surface-associated microbial cells that form biofilms are enveloped in a self-produced EPS that mostly contains polysaccharide, extracellular DNA, lipids, and proteins (Flemming and Wingender, 2010). Biofilm PGPR showed exceptionally high levels of IAA generation, nitrogenase activity, phosphate solubilization, and siderophore formation (Zhang et al., 2015). The prospect of zinc-solubilizing rhizobacteria in agriculture is gradually increasing since it presents an appealing substitute to the excessive use of zinc fertilizers, pesticides, and other supplements. A great deal of research work has been done to isolate and characterize rhizobacteria that solubilize zinc, and numbers of strains are identified for other crops. However, canola has garnered little attention in this context even though it is the world's second largest oilseed crop after soybeans (Martínez-Hidalgo et al., 2021). Previously, limited work has been done on isolation and identification of microbial flora of canola (Valetti et al., 2018). To date, most research works have focused on Gram-negative endophytic bacteria in case of canola (Farina et al., 2012). Scant information has been found in regards to Gram-positive endophytes impacting canola growth (Jimenez-Gomez et al., 2020). Hence, the importance of indigenous bacterial isolates in increasing canola crop productivity prompted us to investigate the ability of locally isolated zinc-solubilizing PGPR, which would perform better due to being adaptable to local climatic conditions. Therefore, it is essential to explore and discover the region-specific plant growth-promoting rhizobacterial strains because they will be more effective, best fitting and unique for promoting crop growth and yield in that region. Keeping in view the significance of Gram-positive rhizobacteria in enhancing growth and yields of canola, the current research was conducted to isolate the indigenous bacteria from the rhizosphere of canola plants that are host-specific and identify the novel strains through 16S RNA gene amplification and sequencing. Moreover, we also evaluated the selected PGPR for plant growth-promoting (PGP) attributes such as zinc solubilization, root colonization, siderophore, biofilm, EPS, and phytohormone (IAA) production. This research is also focused on exploiting these promising zinc-solubilizing strains for improving growth and yield attributes such as seed oil level and fattyacid profile of canola for enhanced canola production under pot and field conditions.

2 Materials and methods

The present study was carried out in the Microbial Physiology and Biocontrol Lab of Plant Microbiomes, Soil and Environmental Biotechnology Division of NIBGE, Faisalabad, Pakistan.

2.1 Bacterial isolation

Canola (*Brassica napus* L.) plants along with rhizosphere were collected from canola fields in the different districts of Punjab Province, Liyyah (30°46′02.0″N 70°55′16.2″E), Dera Ghazi Khan (30°09′26.8″N,70°43′48.3″E) and Faisalabad (31°240 32.2100″N 73° 040 5.8600″E), Pakistan. Rhizosphere soil suspensions were diluted to a concentration of 10^{-1} to get single colonies. Fifty µl of these dilutions were then spread onto Luria-Bertani (LB) agar medium (tryptone 10 g, NaCl 5 g, yeast extract 5 g, nutrient agar 20 g, distilled water 1 L, pH 7) plates and incubated at 28 ± 2°C for 48 h. From the liquid cultures, 0.8 ml of bacterial cultures that had been incubated for 48 h

were combined with 0.2 ml of sterile glycerol and then preserved at −80°C for further investigation.

2.2 Morphological cultural characterization

Bacterial isolates were characterized on the basis of their size, shape, color, margin, pigmentation, and surface. These characteristics were documented following the guidelines provided by Holt et al. (1994) according to Bergey's Manual of Determinative Bacteriology. The standard protocol outlined by Dubey and Maheshwari (2011) was used to perform Gram staining for the unknown bacterial isolates.

2.3 Production of Indole-3-acetic acid by bacterial isolates

Bacterial isolates were evaluated for their capability to synthesize indole-3-acetic acid (IAA) employing a colorimetric detection assay in liquid culture by utilizing the Salkowski reagent as exemplified by Shao et al. (2015). Each strain cultures were incubated in 5 ml of LB broth medium at 28°C for 48 h, after which 20 µl of culture cells were placed in Dworkin and Foster's (1958) salt minimal media enriched with and without L-tryptophan (5 mg L^{-1}) at 28°C for 48 h on an orbital shaker (Thermo Scientific™, MaxQ™, 40,000®). By measuring the absorbance at 535 nm, spectrophotometer (GBC-Avanta AA series) was used to quantify the intensity of the pink-red color. The standard curve was constructed through IAA production (µg ml^{-1}) by bacterial isolates and standard solution. Three replicates of each bacterial strain were employed, and the experiment was repeated.

2.4 Nitrogenase activity

Nitrogen fixation was evaluated to test the efficiency of bacterial strains to convert nitrogen gas from the atmosphere into nitrate that is accessible by plants. Nitrogenase activity was determined by employing nitrogen-free medium (Hafeez and Malik, 2000) with three replicates. Quantitative estimation of nitrogen-fixing capacity (nmol h^{-1} mg^{-1}) of 10 rhizobacterial isolates was tested employing the acetylene reduction assay (ARA), according to the protocol provided by Sajjad Mirza et al. (2001).

2.5 Iron Solubilization assays

The chrome azurol sulfonate (CAS) assay is a valuable method for evaluating prospective biocontrol microorganisms that produce siderophores. The tested isolates were subjected to CAS assay by following the protocol as reported by Schwyn and Neilands (1987). The CAS assay is based on the color change around the microbial colony of CAS iron complex from blue to orange following the association of the bound iron by siderophores. The magnitude of the yellowish-orange haloes around rhizobacterial colonies was suggested to be positive for siderophore production.

2.6 Exopolysaccharide synthesis

Tested isolates were evaluated for exopolysaccharide (EPS) production by Gerhardt (1994) on yeast extract mannitol agar medium (mannitol 10 g, K_2HPO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, NaCl 0.1 g, yeast extract 0.5 g, agar 15 g, distilled water 1 L). For 3 days, plates were incubated at 28 ± 2°C. The production of mucoid was considered as positive for EPS.

2.7 Hydrogen cyanide production

All of the isolates have been analyzed for the formation of HCN employing the Castric (1975) methodology. Bacterial strains were spread on nutritional media plates containing 4.4 g/L glycine. On the lid of the Petri plate, a piece of sterilized filter paper soaked in 1% picric acid and 2% sodium carbonate solution was inserted. These plates were incubated for 4 days at 28–30°C. The transformation of the color from orange to reddish-brown suggested that bacterial strains were producing HCN.

2.8 Evaluating the rhizobacterial isolates for hydrolytic enzyme synthesis

In this study, bacterial strains were analyzed for their ability to produce hydrolytic enzymes such as protease, cellulase, and amylase.

2.8.1 Protease

Bacterial isolates were evaluated for their ability to produce proteolytic enzymes on the skim milk agar (3% v/v) media by following the standard procedures of Chang et al. (2009).

2.8.2 Cellulase

Cellulose degradation ability of bacterial isolates was detected by streaking culture on cellulose Congo red agar medium ($MgSO_4{\cdot}7H_2O$ 0.25 g, K_2HPO_4 0.5 g, cellulose 2 g, Congo red 0.2 g, gelatin 2 g, agar 15 g, distilled water 1 L, pH 6.8–7.2). Discoloration of Congo red indicator in an agar medium by cellulolytic bacteria provides rapid and sensitive screening (Gupta et al., 2012).

2.8.3 Amylase

The hydrolysis of starch by bacterial strains was examined using starch agar medium (starch 20 g, tryptone 10 g, NaCl 5 g, yeast extract 5 g, 20 g nutrient agar, distilled water 1 L, pH 7) and incubated for 48–72 h at 28 ± 2°C. Following incubation, starch agar plates were flooded with autoclaved Lugol's solution [iodine (5%), KI (10%), distilled water (100 ml)], allowed to keep for 1 min, and then drained off. After the reaction of iodine with starch, a blue product is formed. This blue color abruptly vanishes. Following that, colorless area surrounding the bacterial colonies showed starch hydrolysis (Gupta et al., 2003).

2.9 Calcium solubilization assay

The calcium-dissolving bacteria (CDB) differentiating media (NaCl 5 g, $CaCO_3$ 5 g, glucose 5 g, K_2HPO_4 0.4 g, $MgSO_4$ 0.01 g,

peptone 1 g, $(NH_4)_2SO_4$ 0.05 g, yeast extract 1 g, agar 7.5 g, distilled water 1 L, pH 7.0) supplemented with calcium in the form of calcium carbonate (Peper et al., 2022). It was used to identify the bacterial isolates having ability to solubilize insoluble calcium and convert it into plant-available soluble form. The freshly grown bacterial culture was inoculated on the CDB medium plates and incubated for 3–4 days at 28 ± 2°C. Clear zones were observed around the bacterial colony, indicating that the bacterial strains solubilize calcium complexes.

2.10 Zinc solubilization assay

Ten strains were examined in Mineral Salt Medium (MSM) described by Saravanan et al. (2008) to determine the zinc solubilization capability. Insoluble zinc ZnO was added to the medium (NaCl 1 g, $CaCl_2$ 0.1 g, KH_2PO_4 1 g, K_2HPO_4 0.1 g, $MgSO_4$ 0.5 g, ZnO 0.1%, yeast extract 4 g, agar 18 g, distilled water 1 L, pH 7.0) and autoclaved at 121°C for 30 min. Overnight-produced fresh bacterial cultures were spot inoculated in triplicate on the sterilized media containing Petri plates with sterile toothpicks. The spotted plates were incubated in the dark for 1 week at 28 ± 2°C to detect the formation of distinct halo zones surrounding colonies. After 7 days, the diameter of the halo zone around the colonies and the diameter of the colonies were measured. The zinc solubilization efficiency (ZSE) and zinc-solubilizing index (ZSI) were determined using the methods of Khanghahi et al. (2018) and Rafique et al. (2022). Zinc solubilizers having the highest value of ZSE are believed to be efficient.

$$ZSI = \frac{\text{Colony Diameter} + \text{Halo Zone Diameter}}{\text{Colony Diameter}}$$

$$ZSE = \frac{\text{Diameter of halo zone of solubilization}}{\text{Colony Diameter}} \times 100$$

Quantitative zinc solubilization by rhizobacterial isolates was estimated by using a mineral salt medium broth provided with 0.1% insoluble zinc compounds. Overnight grown culture of zinc-solubilizing isolates (1×10^4 CFU ml^{-1}) was inoculated into each flask containing 50 ml of MSM broth and then incubated for 2 weeks at 30°C, then on rotary shaker for 48 h at 180 rpm. After 14 days of incubation, 10 ml of samples were removed and centrifuged for 10 min at 10,000 rpm and filtered using Whatman filter paper of 11 µm (Cytiva, Whatman1001-045 US). Following that, 2 ml of supernatant was transferred to a 100-ml flask, and distilled water was used to dilute the volume up to 100 ml. The culture supernatant was fed directly to atomic absorption spectrophotometer at 230 nm to quantify the level of soluble zinc (Dinesh et al., 2018). Highly zinc-solubilizing strains were picked for further evaluation. A medium with no culture was used as a control. The amount of solubilized zinc was estimated as (mgL^{-1} = ppm) culture by subtracting the soluble Zn of the inoculated sample from the equivalent non-inoculated control. A pH meter was also used to measure the pH of the culture and control. The experiments were conducted in triplicate.

2.11 Evaluation of rhizobacterial strains for potassium solubilization

Initially, selected bacterial strains were subjected to K solubilization screening test based on halo zone production on modified Aleksandrov's agar medium via spot test method following the modified standard methodology of Meena et al. (2015). Spot-inoculating Petri plates were incubated for 7 days at 28 ± 2°C. The halo zone appearance around the bacterial colonies indicated the positive results.

2.12 Phosphate solubilization assays

To detect the qualitative phosphate solubilization potential of isolated bacterial strain, liquid Pikovskaya's agar medium [yeast extract 0.5 g, dextrose 10 g, $Ca(PO_4)_2$ 3 g, $(NH_4)_2SO_4$ 0.5 g, KCl 0.2 g, $MgSO_4$ $7H_2O$ 0.1 g, $MnSO_4$ 0.0001 g, Fe-EDTA 0.0001 g, $CaCo_3$ 0.3 g, agar 18 g, distilled water 1 L, pH 7] was used in triplicate (Pikovskaya, 1948). After sterilization and pouring, bacterial strains were spot inoculated on the Pikovaskaya's agar medium and incubated at 28 ± 2°C for 5–7 days. Halos zone formed around the bacterial colony after the incubation. This halo zone formation indicated the positive results.

2.13 PCR amplification of 16S rRNA gene sequencing and phylogenetic analysis

The selected promising rhizobacterial isolates having growth endorsing characters in canola were identified through amplification, sequencing, and bioinformatics analysis of its 16S rRNA gene sequence. For this purpose, crude DNA of the selected isolates was extracted from the bacterial isolates by CTAB method (Wilson, 1987). DNA concentration was determined through the comparison of the isolated DNA with the DNA intensity marker on agarose gel (0.8%), and the DNA was preserved at −20°C. For amplification, the 16S rRNA gene with PCR reagents was amplified in a thermalcycler (SuperCycler, Model: SC300G R2, Australia) using the 16S primers: fD1-5′-AGA GTT TGA TCC TGG CTC AG-3′ and rD1-5′-AAG GAG GTG ATC CAG CC-3′. The thermocycling conditions involved an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 5 min. Amplified PCR product (1.5 kb) was eluted from the gel and cleaned using commercial QIA Quick Gel Extraction Kit (QIAGEN Sciences, Maryland 20,874, USA). Amplification product (1,500 bp) was sent to Macrogen Laboratories Inc., Seoul, Korea[1] for sequencing. Basic Local Alignment Search Tool (BLAST) was used to identify nucleotide-related sequence similarities which were obtained from the GenBank database "National Centre for Biotechnology Information (NCBI) database https://blast.ncbi.nlm.nih.gov/Blast.cgi." Species were assigned based on the highest sequence identity, highest E-value, and coverage. Phylogenetic relationship between the representative bacterial species and identified strains based on 16S rRNA gene sequences was developed with the

1 http://macrogen.com/eng/

ClustalW program as described by Roohi et al. (2012). A phylogenetic tree was constructed by maximum likelihood method among different isolates using MEGA X version 11.0.13 (Kumar et al., 2018). Confidence in the tree topology was evaluated by bootstrap analysis with 1,000 replicates (Tamura et al., 2013). The isolates identified in the current study are indicated in bold text. The comparison of almost complete gene sequences has been used to establish taxonomic relationships between prokaryotic strains with 98.65% similarity currently recognized as the cutoff for delineating species (Kim and Chun, 2014).

2.14 Biofilm formation

Zinc-solubilizing rhizobacterial strains were evaluated for their ability to form biofilm using the procedure described by Haque et al. (2020). Overnight-grown cultures in LB broth having optical density (OD_{660}) reached 0.7–0.8. Then 1 ml of culture of each strain was centrifuged at 10,000 rpm for 10 min. The pellet was diluted (ca. 106 CFU/ml). Glass test tubes were filled with 5-ml salt-optimized broth plus glycerol (SOBG) medium (tryptone 20 g, KCl 0.186 g, $MgSO_4.7H_2O$ 2.4 g, NaCl 0.5 g, yeast extract 5 g, 40% glycerol 50 ml, H_2O 1,000 ml, pH 7.00) and 50 µl of each bacterial cultures were then kept in an incubator at 28°C. Air Liquid (AL) biofilm-producing rhizobacteria were selected after 72 h. Biofilm of selected strains was removed from glass test tubes and washed with sterile water three times. After that, 1 ml of sterile distilled water and 14 glass beads (3 mm) were added to each glass tube. By vortexing at high speed for 50 s, biofilm was detached. Then optical density was measured by reading the absorbance at 660 nm with spectrophotometer (CamSpec M350 double beam UV–visible). Biomass of biofilms was estimated by employing the methodology presented by Mosharaf et al. (2018). Three replicates for every rhizobacterial isolate were used, and the experiment was repeated.

2.15 Root colonization assay

Roots of canola plants that were growing in sterilized sand were used in this assay. Surface-sterilized canola roots (200 mg) were macerated and ground in 6 ml sterilized distilled water with sanitized mortar and pestle. Subsequently, tubes were shaken at 250 rpm for 20 min at room temperature. After shaking, suspensions were serially diluted 10^{-1} to 10^{-7}. A volume of 20 µl of diluted root suspension was spot inoculated into sterilized LB media Petri plates and incubated at 28 ± 2°C for 48 h. After that, the bacterial population was counted in terms of colony-forming units (CFU). The number of bacteria colonizing the root was calculated as CFU/mg root. Logarithm of CFU/mg of root was taken.

$$\log\text{CFU} = \frac{\text{No. of colonies} \times \text{Total dilution factor}}{\text{mg of root}}$$

2.16 Pathogenicity test

The pathogenicity test was performed employing the methodology described by Chahad et al. (2012). The bacterial isolates were spot inoculated on blood agar plates supplemented with 5% (v/v) sheep blood to determine their hemolytic activity (peptone 10 g, NaCl 5 g, beef extract 3 g, agar 15 g, distilled water 1 L). Sheep blood was added after autoclaving and before pouring it onto the plates. Plates were incubated at 28 ± 2°C for 48 h. The findings were recorded for the appearance of zones with difference in color.

2.17 PGP potential of zinc-solubilizing strains in pot trial

The zinc-solubilizing ability of these selected rhizobacterial strains was checked in a pot experiment. During the canola growing season (October–March), pot trial was carried out in a net house under natural temperature and light conditions using tap water at NIBGE, Faisalabad. The experiment was conducted in sterilized sand with three replicates. Autoclave sand (10 kg) was filled in 18 cm diameter pots. Six treatments were planned for the experiment, including inoculation of four most promising zinc-solubilizing strains, CLS1, CLS2, CLS3, and CLS9, and two non-inoculated control (with or without fertilizer treatment) with three replicates per treatment. A single colony of each strain was added to four sterilized conical flasks holding 250 ml of nutrient broth and incubated for 7 days at 28°C in a rotary shaking incubator. Surface sterilization of canola seeds of two cultivars was performed by first immersing them in ethanol (95%) for 1 min, followed by dipping in 0.2% solution of $HgCl_2$ for 3 min. Then the seeds of canola were washed three times with sterile distilled water. Sterilized filter mud was used as carrier material. For one acre of seedlings, 1 kg of carrier material was used. After sterilizing the filter mud, it was placed in sterile sealed plastic bags. The fresh culture broth (250 ml) of selected rhizobacterial strains (1×10^4 CFU ml^{-1}) was injected into the bag of sterile mud. After that, canola seeds were immersed in these bags and then left for 2 h before sowing in pots. In the net house, pots were arranged in a complete randomized design. Eight coated seeds were sown at equal intervals in every pot and watered regularly. Each treatment was used in triplicate. After 10 days of germination, plants were thinned to a maximum of 5 plants in every pot.

2.18 Field evaluation of zinc-solubilizing strains for their PGP potential

Two field experiments were carried out in Dera Ghazi Khan to investigate the efficiency of zinc-solubilizing strains in increasing canola plant growth and crop production under field conditions. Experiments were planned in a randomized complete block design (RCBD), including three replicates. This trial has same non-inoculated and inoculated treatments and replicates that were evaluated in the pot experiment. Three plants from each treatment were selected at random to determine the impact of the treatments on crop growth. To determine the dry weight of shoots and roots, samples from each treatment and replication were dried in an oven (FELISA, model 242-A®) at 67°C. The number of grains/pod, number of pods/plant, grain yield/plant, and weight of thousand seeds were all collected and statistically examined. All data were acquired using standard methods (Namvar and Khandan, 2014).

2.19 Oil quality analysis

The protein, total seed oil contents, and fatty acid profile were tested by the method illustrated by da Silva Medeiros et al. (2022). Protein and oil content of seeds were determined using oilseeds calibrated near-infrared reflectance (NIR) spectroscopy (Model: Perten DA 7250, USA) at Hi-Technology Laboratory of Oilseeds Research, Ayub Agricultural Research Institute, Faisalabad, Pakistan.

2.20 Statistical analysis of data

For statistical analyses, the data regarding *in vitro* experiment were compared through one-way analysis of variance (ANOVA). The data regarding *in vivo* trials were statistically analyzed, and the Statistix 8.1 computer software was employed to evaluate the significance among treatments. The obtained means of three replications were subjected to the Tukey test at 5% probability (Steel et al., 1997).

3 Results

3.1 Isolation, purification, and screening of PGPR

The present study was conducted to investigate an eco-friendly method of using plant growth-promoting rhizobacteria as a bioinoculant to enhance canola nutrition, growth, and yield. The main objective of this study was to isolate and characterize Zn-solubilizing strains from different cities in Punjab, Pakistan. A total of 84 rhizobacterial strains were isolated from the canola rhizosphere. These strains were purified on separate fresh agar plates and stored at 4°C for further experimentations.

3.2 Morphological and biochemical characterization of selected rhizobacterial strains for PGP attributes

Out of 84, only 10 indigenous potent zinc-solubilizing rhizobacterial isolates were selected and subjected to detailed characterization for their plant growth-enhancing attributes. Morphologically, most of these rhizobacterial isolates were motile, Gram-positive, and rod-shaped (Table 1). To determine the plant growth-stimulating characteristics of the most auspicious rhizobacterial strain, biochemical characterization was carried out, and potent isolates were selected for screening trials. These rhizobacterial strains were evaluated for zinc, phosphate, potassium, and calcium solubilization, HCN, siderophore, exopolysaccharide, protease, and cellulose production, and starch hydrolysis, as depicted in Table 2. The potential of rhizobacterial isolates to solubilize phosphate in the Pikovskaya medium was examined. Seven rhizobacterial isolates that showed phosphate solubilization are listed in Table 2. Among the 10 rhizobacterial isolates, CLS8 and CLS11 were not able to produce siderophore, while the other eight isolates showed positive results in producing siderophores. Four rhizobacterial isolates, CLS8, CLS9, CLS11, and CLS12, could not produce HCN; however, all other tested isolates were well capable of producing HCN (Table 2). Amylase production of isolates was determined by starch hydrolysis test. Production of exopolysaccharides was checked by the formation of precipitation in broth inoculated with the selected isolates. Some of the isolates CLS2, CLS6, CLS8, and CLS11 produced exopolysaccharides and showed positive results, while the rest of the isolates were unable to produce exopolysaccharides (Table 2).

3.3 Zinc solubilization by rhizobacterial isolates from canola

In the current study, 10 isolates (CLS 1, CLS 2, CLS 3, CLS 6, CLS 8, CLS 9, CLS11, CLS12, CLS 13, and CLS15) demonstrated high solubilization of insoluble Zn compound (ZnO) during agar plate assay. Among 10 rhizobacterial isolates, CLS2 from the *Bacillus* genus showed the greatest ability for Zn solubilization. *In vitro* analysis of selected isolates for their ability to solubilize ZnO indicated that zinc solubility ranged from 7.90 to 29.63 mm halo zone diameter (Table 3). Data regarding solubilization efficiency (ZSE) and zinc solubilization index (ZSI) based on halo zone and colony diameter indicated that the most effective isolates were CLS2, CLS6, and CLS13, which were statistically non-significant to each other (Table 3). The isolate CLS12 also showed good Zn solubilization zone and was followed by CLS9. The inoculation of isolates CLS8 and CLS11 produced lowest solubilization zone. Rhizobacterial isolates that displayed an increase

TABLE 1 Morphological and cultural characteristics of rhizobacterial strains isolated from Canola (*Brassica napus* L.) rhizosphere.

Sr. No.	No. of isolates	Colony size	Colony colour	Colony margin	Colony shape	Surface	Motility	Gram reaction	Shape	Pigmentation
1.	CLS1	Small	White	Entire	Round	Smooth, opaque	Nonmotile	Positive	cocci	White
2.	CLS2	Large	Off white	Entire	Round	Smooth, opaque	Motile	Positive	Rod	Non pigmented
3.	CLS3	Medium	milky white	Irregular	Round	Rough, opaque	Nonmotile	Positive	Rod	Fuzzy White
4.	CLS6	Large	Off white	Entire	Round	Smooth, opaque	Motile	Positive	Rod	Non pigmented
5.	CLS8	Large	Grey	Irregular	Round	Rough, opaque	Motile	Positive	Rod	Dull Grey
6.	CLS9	Medium	White	Regular	Irregular	Smooth, opaque	Motile	Positive	Rod	Cream
7.	CLS11	Large	Grey	Irregular	Round	Rough, opaque	Motile	Positive	Rod	Dull Grey
8.	CLS12	Medium	Dull white	Regular	Irregular	Smooth, shiny	Motile	Positive	Rod	Cream
9.	CLS13	Large	Off white	Entire	Round	Smooth, opaque	Motile	Positive	Rod	Non pigmented
10.	CLS15	Small	White	Entire	Round	Smooth, opaque	Nonmotile	Positive	cocci	White

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TABLE 2 Biochemical and biocontrol activities of PGPR isolates from Canola (*Brassica napus* L.)

Isolates	Zinc solubilization	Phosphate solubilization	Potassium solubilization	Calcium solubilization	HCN production	Siderophore production	EPS production	Protease production	Cellulose production	Starch hydrolysis
CLS1	+	–	–	+	+	+	–	+	+	+
CLS2	+	–	–	–	+	+	+	+	+	+
CLS3	+	–	–	–	+	+	–	+	+	+
CLS6	+	–	–	–	+	+	+	+	+	+
CLS8	+	+	–	–	–	–	+	+	+	+
CLS9	+	+	–	–	–	+	–	+	+	+
CLS11	+	+	–	–	–	–	+	+	+	+
CLS12	+	+	–	–	–	+	–	+	+	+
CLS13	+	–	–	–	+	+	–	+	+	+
CLS15	+	–	–	+	+	+	–	+	+	+

Zinc solubilizing rhizobacterial isolates having multiple plant growth promoting characteristics; All *in vitro* tests were repeated twice for verification of results with three replicates each time; The symbol, + represents the presence of the traits while the symbol, –shows the absence of the trait.

in activity of ZnO solubilization in solid media were subjected to inoculation in minimal salt medium broth modified with ZnO to evaluate their effect on pH. Data regarding pH decrease (Table 3) showed that a large number of isolates decreased the pH of broth, but maximum drop in pH of medium up to 4.63 ± 0.02 was noted with inoculation of CLS2, followed by CLS9, CLS6, and CLS13. While isolate CLS11 displayed lowest decline in pH (Table 3). After the identification of these strains, four different strains, CLS1, CLS2, CLS3, and CLS9, were selected as highly efficient Zn solubilizers and recommended for further pot and field studies (Figure 1).

3.4 Screening of zinc-solubilizing rhizobacterial isolates

Our results for evaluating selected isolates for their potential to solubilize Zn in plate assay demonstrated that all screened rhizobacterial isolates were also positive for Zn solubilization in liquid media. Promising rhizobacterial isolates of zinc solubilization in solid media were inoculated in broth with ZnO for quantitative Zn solubilization assessment. Soluble zinc content values were noted from atomic absorption spectrophotometer. Results obtained revealed that these isolates, CLS2, CLS6, and CLS13, were statistically similar to each other, followed by CLS9 and CLS12, after 7 days of incubation (Table 4). Minimum quantity of solubilized zinc was acquired with the inoculation of isolates CLS8 and CLS11, respectively, which were non-significant with each other. The IAA generation assay revealed that eight evaluated rhizobacterial strains exhibited the ability of IAA production, while two strains, CLS8 and CLS11, did not show IAA production. In fact, with L-tryptophan, the highest production of IAA was synthesized by CLS6, followed by strains CLS2, CLS13, and CLS12, and statistically non-significant with each other. Strain CLS9 also showed remarkable IAA production without L-tryptophan. Similarly, strains CLS1 and CLS15 were non-significant with each other (Table 4). After NFM media test, all the rhizobacterial isolates were subjected to nitrogenase activity in acetylene reduction assay (ARA). Nitrogen-fixing ability in these rhizobacterial isolates was further validated by gas chromatography. The ARA results indicated that out of 10, eight bacterial isolates were excellent for nitrogenase ability. The highest value of nitrogen fixation was noticed from strains CLS2 and CLS6, which were statistically similar and non-significant with each other (Table 4). *In vitro* root colonization tests indicated that some isolates are more potent root colonizers than others. The bacterial cell counts were collected from the roots. Root colonization expressed as log cfu/mg root after dilution plating of roots on solid media after 14 days of germination. The highest root colonization was reported by strains CLS2, CLS13, and CLS6, respectively, and these isolates were statistically similar and shared the same status. However, all the tested rhizobacterial strains were well capable of colonizing canola roots (Table 4). Biofilm was produced in the form of rings in the glass tubes. All these biofilm-forming strains synthesized rough-surfaced biofilms. The strains CLS13, CLS2, and CLS6 formed particularly rigid and thick biofilms, and the adjacent bacterial cells were not disseminated when the aggregates of these strains were agitated. Furthermore, the biofilms generated by CLS9 and CLS12 were thicker and more dense than those produced by CLS1 and CLS15. Conversely, CLS8 and CLS11 produced fragile and very thin biofilms, and these biofilms were easily spread when disturbed. The biomass biofilm varied significantly between the

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TABLE 3 Zinc solubilization by rhizobacterial strains isolated from Canola (*Brassica napus* L.) rhizosphere.

Isolates	Bacterial colony diameter (mm)	Zinc halo zone diameter (mm)	Zinc solubilization efficiency (ZSE)	Zinc solubilization index (ZSI)	pH of isolates
CLS1	5.40 ± 0.26 ^{d}	12.00 ± 0.47 ^{d}	222.43 ± 2.20 ^{d}	3.21 ± 0.02 ^{d}	5.17 ± 0.05 ^{b}
CLS2	8.60 ± 0.05 ^{a}	29.63 ± 0.37 ^{a}	344.55 ± 2.32 ^{a}	4.44 ± 0.02 ^{a}	4.63 ± 0.02 ^{c}
CLS3	6.13 ± 0.08 ^{c}	12.43 ± 0.35 ^{d}	202.63 ± 2.81 ^{e}	3.02 ± 0.02 ^{e}	5.05 ± 0.04 ^{b}
CLS6	8.70 ± 0.1 ^{a}	29.80 ± 0.23 ^{a}	342.56 ± 2.10 ^{a}	4.42 ± 0.02 ^{a}	4.68 ± 0.03 ^{c}
CLS8	6.93 ± 0.14 ^{b}	7.90 ± 0.07^{e}	113.99 ± 1.33 ^{f}	2.13 ± 0.01 ^{f}	5.70 ± 0.04 ^{a}
CLS9	8.33 ± 0.14 ^{a}	23.13 ± 0.14 ^{c}	277.70 ± 3.08 ^{c}	3.77 ± 0.02 ^{c}	4.65 ± 0.04 ^{c}
CLS11	6.90 ± 0.05 ^{b}	7.93 ± 0.11 ^{e}	114.62 ± 0.71 ^{f}	2.14 ± 0.005 ^{f}	5.75 ± 0.03 ^{a}
CLS12	8.16 ± 0.12 ^{a}	25.10 ± 0.17 ^{b}	307.42 ± 2.51 ^{b}	4.07 ± 0.02 ^{b}	4.78 ± 0.04 ^{c}
CLS13	8.53 ± 0.12 ^{a}	28.80 ± 0.20 ^{a}	337.57 ± 2.90 ^{a}	4.37 ± 0.02 ^{a}	4.71 ± 0.02 ^{c}
CLS15	5.46 ± 0.17 ^{cd}	12.36 ± 0.29 ^{d}	226.36 ± 3.13 ^{d}	3.25 ± 0.03 ^{d}	5.14 ± 0.05 ^{b}
CVC	0.70	1.36	12.16	0.11	0.20

Rhizobacterial Isolates were incubated for zinc solubilization on agar media amended with ZnO for 7 days at 28 ± 2°C, Zinc halo zone diameter, ZSE and ZSI were determined using diameters of zinc halo zone and bacterial colony diameter; whereas, solubilized zinc concentration by isolates and pH drop in broth was recorded after10 days of incubation; Data presented are the mean of three replicates ± standard error and Tukey HSD test was performed at 5% ($p \leq 0.05$) probability level; CVC, critical value for comparison.

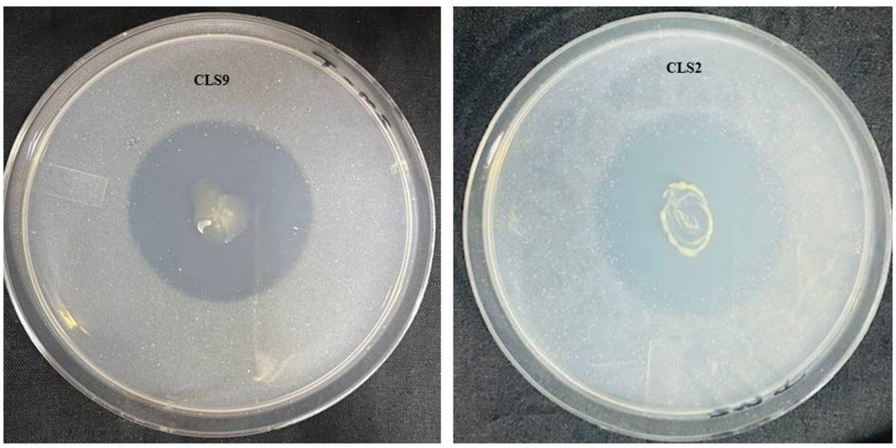


FIGURE 1
Solubilization of ZnO during *in vitro* assay by selected potent zinc-solubilizing strains after 5 days of incubation. The large clear halo zones around the colonies of *Priestia aryabhattai* (CLS2) and *Priestia megaterium* (CLS9) indicate the ZnO solubilization.

biofilm-producing strains. The highest amount of biomass biofilm was produced at OD_{660} by CLS2 (1.87 ± 0.05), followed by CLS6 (1.84 ± 0.07) and CLS13 (1.81 ± 0.04), which were statistically similar to each other, and followed by CLS9 (1.73 ± 0.04) and CLS12 (1.69 ± 0.05). The minimum quantity of biofilm biomass was built by CLS8 (1.39 ± 0.05). However, the biomass of biofilms differed not considerably between CLS1 (1.43 ± 0.07) and CLS15 (1.47 ± 0.06). CLS3 (1.63 ± 0.05) also produces a considerable amount of biofilm biomass (Table 4). Consequently, the quantity of biofilms and the bacterial numbers in these biofilms are affected by rhizobacterial strains.

3.5 Taxonomical identification of potential isolates using 16S rRNA gene amplification

Genomic DNA from 10 potent rhizobacterial isolates was subjected to their 16S rRNA gene sequencing after PCR amplification. The 16S rDNA sequences of these isolates were compared with those of known 16S rRNA sequences. The identified sequences were deposited in GenBank, and their accession numbers and strains with maximum homology are given in Table 5. Phylogenetic analysis of the 16S rRNA gene indicated that 10 isolates belonged to different genera, *Bacillus*, *Staphylococcus*, and *Priestea*. Two isolates with the ability to solubilize zinc were *Staphylococcus* sp. CLS1 and CLS15, and these isolates formed cluster with *Staphylococcus succinus* subsp. *succinus* ATCC 700337^{T} in the phylogenetic tree (Figure 2). The most potent zinc-solubilizing isolates, CLS2, CLS6, and CLS13, were found to be phylogenetically similar to type strain *Priestia aryabhattai* B8W22^{T}, showing 98.06–99.85% similarity in their 16S rRNA sequences (Table 5). The sequence analysis of the zinc-solubilizing isolates of CLS9 and CLS12 showed 98.78–99.03% homology with type strain *Priestia megaterium* strain B8W22^{T}. Three isolates belonged to the genus *Bacillus*, of which two isolates CLS8 and CLS11 clustered with

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TABLE 4 Quantitative screening of Canola (*Brassica napus* L.) associated zinc solubilizing rhizobacterial strains for different biochemical and Physiological attributes.

Isolates	Zinc solubilization (mgL^{-1} = ppm)	IAA production without L-tryptophan ($\mu g\ ml^{-1}$)	IAA production with L-tryptophan ($\mu g\ ml^{-1}$)	Nitrogen fixation ($nmol\ h^{-1}\ mg^{-1}$)	Root colonization log cfu/mg root	Biofilm production biomass at (OD_{660})
CLS1	26.06 ± 0.95^{bc}	8.92 ± 0.17^{c}	10.56 ± 0.38^{b}	71.03 ± 2.18^{d}	6.98 ± 0.14^{ab}	1.43 ± 0.07^{bcd}
CLS2	33.03 ± 0.84^{a}	12.41 ± 0.41^{ab}	16.62 ± 0.33^{a}	124.58 ± 2.96^{a}	7.13 ± 0.10^{a}	1.87 ± 0.05^{a}
CLS3	25.00 ± 0.86^{c}	6.19 ± 0.15^{d}	8.17 ± 0.47^{c}	93.90 ± 2.25^{c}	6.08 ± 0.18^{c}	1.63 ± 0.05^{abcd}
CLS6	32.43 ± 0.64^{a}	12.98 ± 0.08^{a}	16.94 ± 0.11^{a}	123.44 ± 3.73^{a}	7.12 ± 0.09^{a}	1.84 ± 0.07^{a}
CLS8	16.93 ± 0.95^{d}	–	–	71.91 ± 3.57^{d}	5.93 ± 0.19^{cd}	1.39 ± 0.05^{d}
CLS9	29.93 ± 0.86^{ab}	11.74 ± 0.36^{b}	15.57 ± 0.27^{a}	106.35 ± 4.55^{bc}	7.07 ± 0.11^{ab}	1.73 ± 0.04^{ab}
CLS11	17.40 ± 1.04^{d}	–	–	70.49 ± 3.47^{d}	5.92 ± 0.13^{d}	1.41 ± 0.05^{cd}
CLS12	29.46 ± 0.52^{ab}	12.04 ± 0.18^{ab}	15.84 ± 0.25^{a}	104.56 ± 3.46^{bc}	7.05 ± 0.17^{ab}	1.69 ± 0.05^{abc}
CLS13	32.63 ± 0.60^{a}	12.90 ± 0.14^{ab}	16.90 ± 0.15^{a}	117.80 ± 1.94^{ab}	7.13 ± 0.17^{a}	1.81 ± 0.04^{a}
CLS15	26.10 ± 0.96^{bc}	8.65 ± 0.27^{c}	10.63 ± 0.22^{b}	73.33 ± 3.16^{d}	6.94 ± 0.11^{b}	1.47 ± 0.06^{bcd}

Data presented here are the mean of three replications ± standard error; means sharing the same letters are statistically non significant and Tukey HSD test was performed at 5% ($p \leq 0.05$) probability level.

TABLE 5 Identification of Canola (*Brassica napus* L.) associated zinc solubilizing rhizobacterial strains based on 16S rRNA gene sequence analysis.

Rhizobacterial isolates	NCBI strains	Similarity	Gen Bank Accession No.
CLS1	*Staphylococcus succinus* NR028667	98.66%	ON678002
CLS2	*Priestia aryabhattai* NR115953	99.34%	ON732743
CLS3	*Bacillus subtilis* NR116017	99.73%	ON944029
CLS6	*Priestia aryabhattai* NR115953	98.06%	ON970951
CLS8	*Bacillus cereus* NR115714	99.50%	OP099855
CLS9	*Priestia megaterium* NR117473	99.03%	ON677944
CLS11	*Bacillus cereus* NR115714	99.68%	OP269542
CLS12	*Priestia megaterium* NR117473	98.78%	OP046417
CLS13	*Priestia aryabhattai* NR115953	99.85%	ON693842
CLS15	*Staphylococcus succinus* NR028667	99.65%	ON650670

Bacillus cereus ATCC 14579^{T}, while the isolate CLS3 showed sequence homology with *Bacillus subtilis* BCRC 10255^{T}.

3.6 Pathogenicity test

A blood agar assay was performed to test the isolates for their biosafety. All of the rhizobacterial isolates used for the plant experiment were confirmed to be non-pathogenic due to the lack of hemolytic activity, verifying their safe use in future studies in field experiments. The results were recorded for the appearance of clear β, greenish-brown α, and no zones γ. Positive control *Streptococcus pyogenes* strain was used for comparison (Figure 3). Clear β zone on the blood agar plate was considered as a positive result.

3.7 Pot trial for testing zinc-solubilizing rhizobacterial strains for canola growth promotion

In the present study, the potential of zinc-solubilizing rhizobacterial isolates *in vitro* to enhance canola growth in a pot

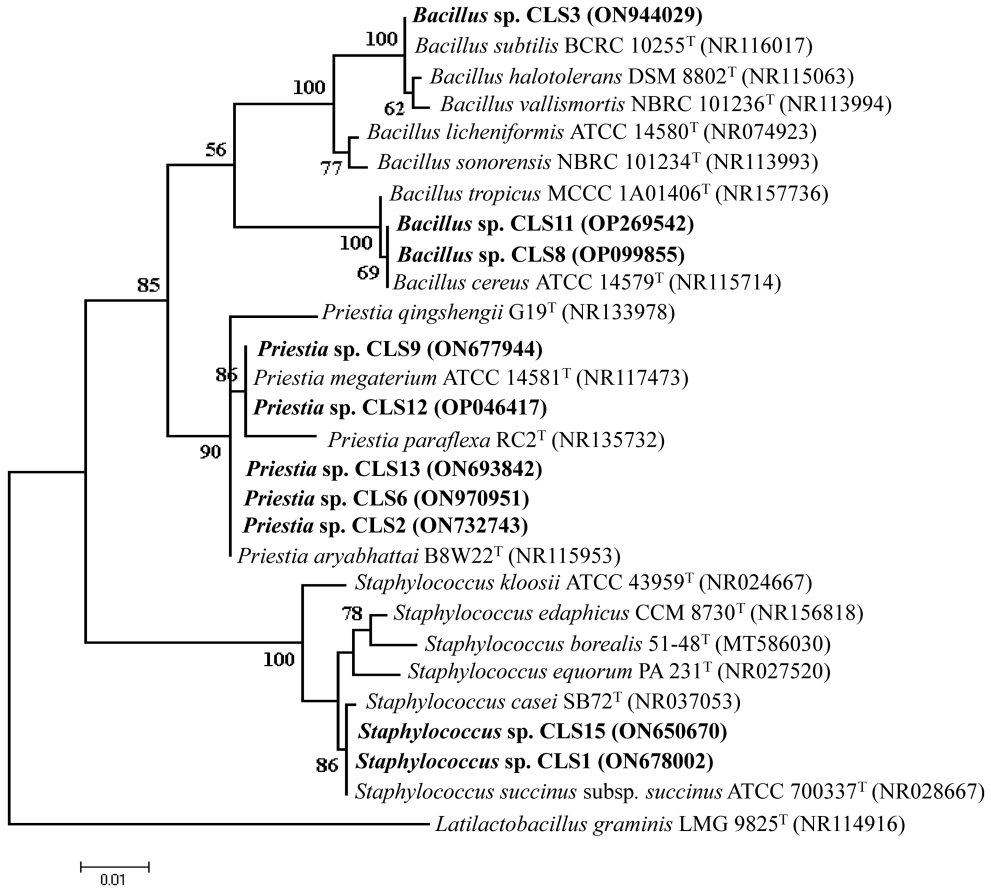


FIGURE 2
Maximum likelihood phylogenetic tree showing interrelationship of zinc-solubilizing strains (CLS1, CLS2, CLS3, CLS6, CLS8, CLS9, CLS11, CLS12, CLS13, CLS15) with closely related species of the genus Bacillus inferred from aligned sequences of the 16S rRNA gene. Tree was rooted by *Latilactobacillus graminis* (NR114916) as an outgroup. Bootstrap values expressed as a percentage of 1,000 replications are indicated at the nodes. The rhizobacterial isolates identified in the present study have been highlighted; accession number of each type strain is shown in brackets.

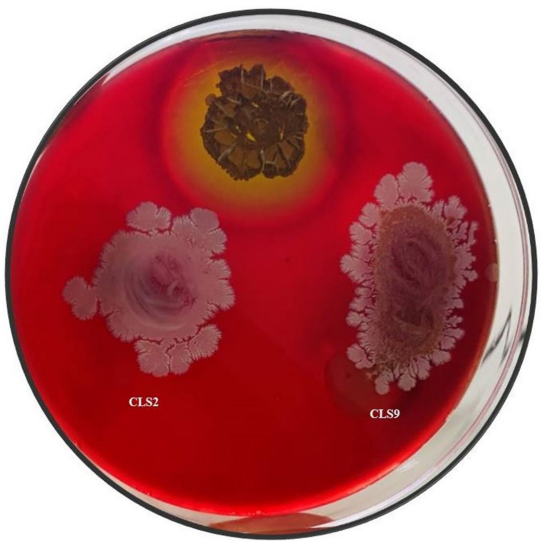


FIGURE 3
Selected rhizobacterial strains for pot and field trials showed growth on blood agar and their non-hemolytic nature as compared to the positive control (*Streptococcus pyogenes*).

experiment was tested. Seeds of two cultivars (V1 and V2) were inoculated with specified rhizobacterial strains capable of solubilizing zinc, which significantly increased canola growth. Seed priming with Zn-solubilizing isolates significantly increases the plant height, length of root, root and shoot fresh weight, root and shoot dry weight, and is statistically better than (without inoculation) control. The strain CLS2 was the best Zn solubilizer and showed a significant increase in plant height after that CLS9, which resulted in the highest increase of 2.72- and 2.50-fold, respectively, in plant height compared to control (non-inoculated) in cultivar V1. For V1, inoculation of CLS1 and CLS3 showed a notable increase in plant height by 2.36- and 2.14-fold, respectively, over non-inoculated control (Figure 4A). Comparison of treatment means revealed that maximum plant height was obtained in strain CLS2, which was followed by CLS9 and CLS1, statistically non-significant with each other in cultivar V2. Minimum plant height was recorded in non-inoculated control for cultivar V2. Whereas maximum increase of 2.53-fold in fresh weight of the shoot resulted with bacterization of CLS2, followed by CLS1 and CLS9 (2.28- and 2.18-fold, respectively) over non-inoculated control for V1 (Figure 4B). An increase of 2.14- and 1.88-fold in plant height and fresh weight of shoot, accordingly, was recorded by strain CLS3 and was non-significant to non-inoculated 100% fertilizer control. In cultivar V2, maximum increase of 2.44-fold in fresh weight of the

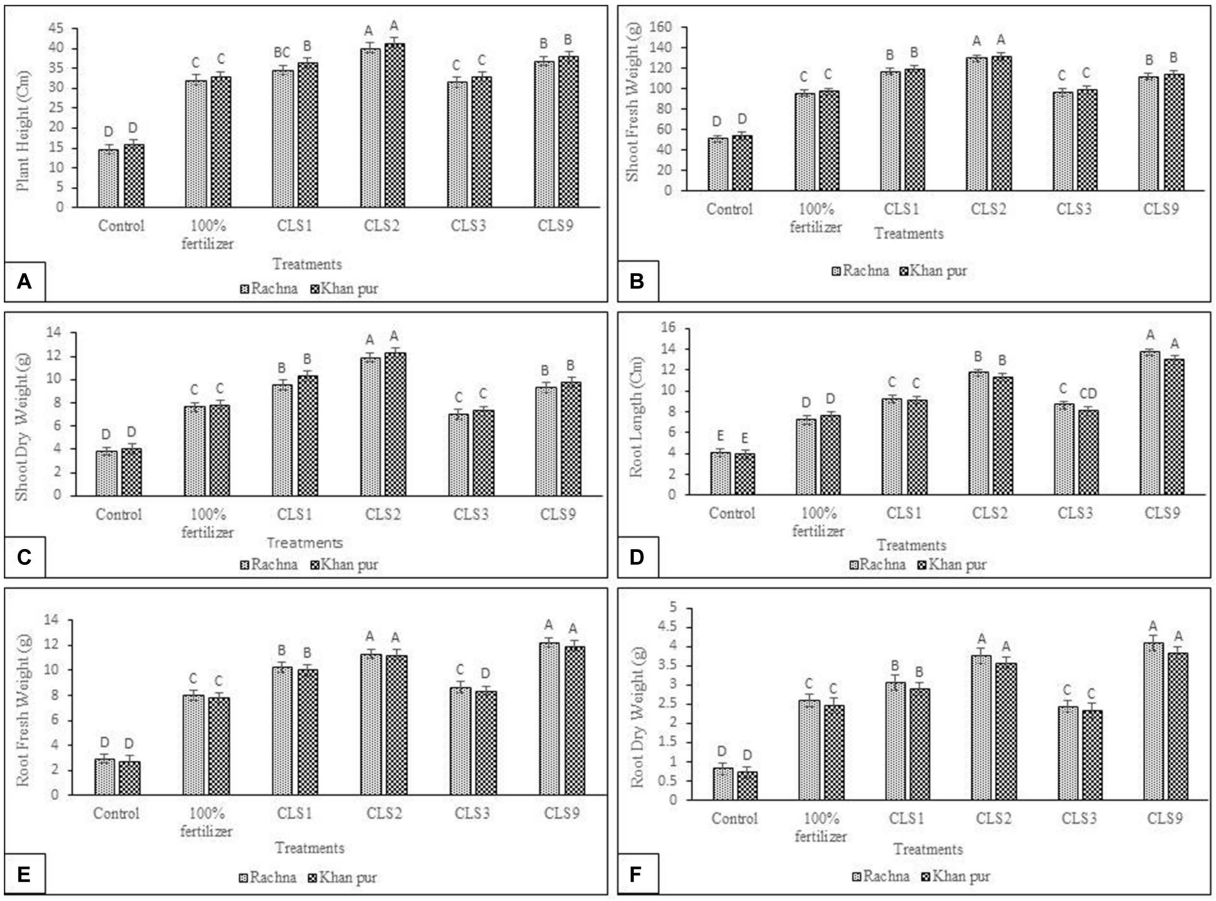


FIGURE 4
(A–F): Effect of zinc-solubilizing isolates on promotion of growth attributes **(A)** Plant Height **(B)** Shoot Fresh Weight **(C)** Shoot Dry Weight **(D)** Root Length **(E)** Root Fresh Weight **(F)** Root Dry Weight of Canola (*Brassica napus* L.) cultivars (V1 Rachna; V2 Khan Pur) in Pot trial. Data were collected at the flowering stage. The Tukey HSD test was applied at a 5% ($p < 0.05$) probability level to determine significant differences between the means of the different treatments; the same letters on the bars indicate non-significant differences among the means, and vice versa.

shoot was noticed in CLS2. In case of shoot dry weight, maximum increase of 3.08-fold was obtained with the inoculation of isolate CLS2, followed by CLS1 and CLS9 (2.47- and 2.40-fold, respectively), which did not differ significantly from each other and were succeeded by CLS3, which was non-significant with non-inoculated 100% fertilizer control in cultivar V1 (Figure 4C). Results showed that priming of Zn-solubilizing isolates significantly promoted the root length, root fresh weight, and root dry weight over non-inoculated control. Highest increase in length of root, i.e., 3.38-fold, was noted by isolate CLS9 as compared to non-inoculated control (Figure 4D). Highest fresh weight of the root was acquired with the bacterization of CLS9, which enhanced fresh weight of the root by 4.18-fold over non-inoculated control. Isolate CLS3 responded efficiently by increasing fresh weight of the root by 2.95 times, but it was non-significant to 100% fertilize treatment (Figure 4E). Inoculation of CLS1, CLS2, and CLS3 enhanced the root dry weight by 3.69-, 4.53-, and 2.92-fold, respectively, compared to control (non-inoculated). The performance of all the zinc-solubilizing rhizobacterial strains was remarkable, but in comparison between these strains, the little increase in length of root, root fresh weight, and root dry weight was reported by the inoculation of CLS3 as compared to non-inoculated control. Significant rise in root dry weight was noted by 4.93-fold with the bacterization of CLS9 over control. The isolates that solubilized Zn showed their ability to enhance total dry weight and total fresh weight of canola plants. The pot experimental results disclosed that CLS2 inoculation was significantly better among all the isolates in terms of plant height and fresh and dry weight of shoots of canola cultivars V1 and V2. Moreover, the inoculation of CLS9 proved the most effective isolates in enhancing the length of root, root fresh, and dry weight of canola in both cultivars over non-inoculated control. Lowest growth attributes in relative plant height, length of root, fresh and dry weight of shoot, and fresh and dry weight of root were observed in non-inoculated control. The results showed that both cultivars of canola were different in their response to inoculation. Cultivar V2 showed better results in inoculation.

3.8 Field trial for testing zinc-solubilizing rhizobacterial strains for canola growth and yield

The selected promising zinc rhizobacterial strains were tested to investigate their impact on growth attributes of canola cultivars, i.e.,

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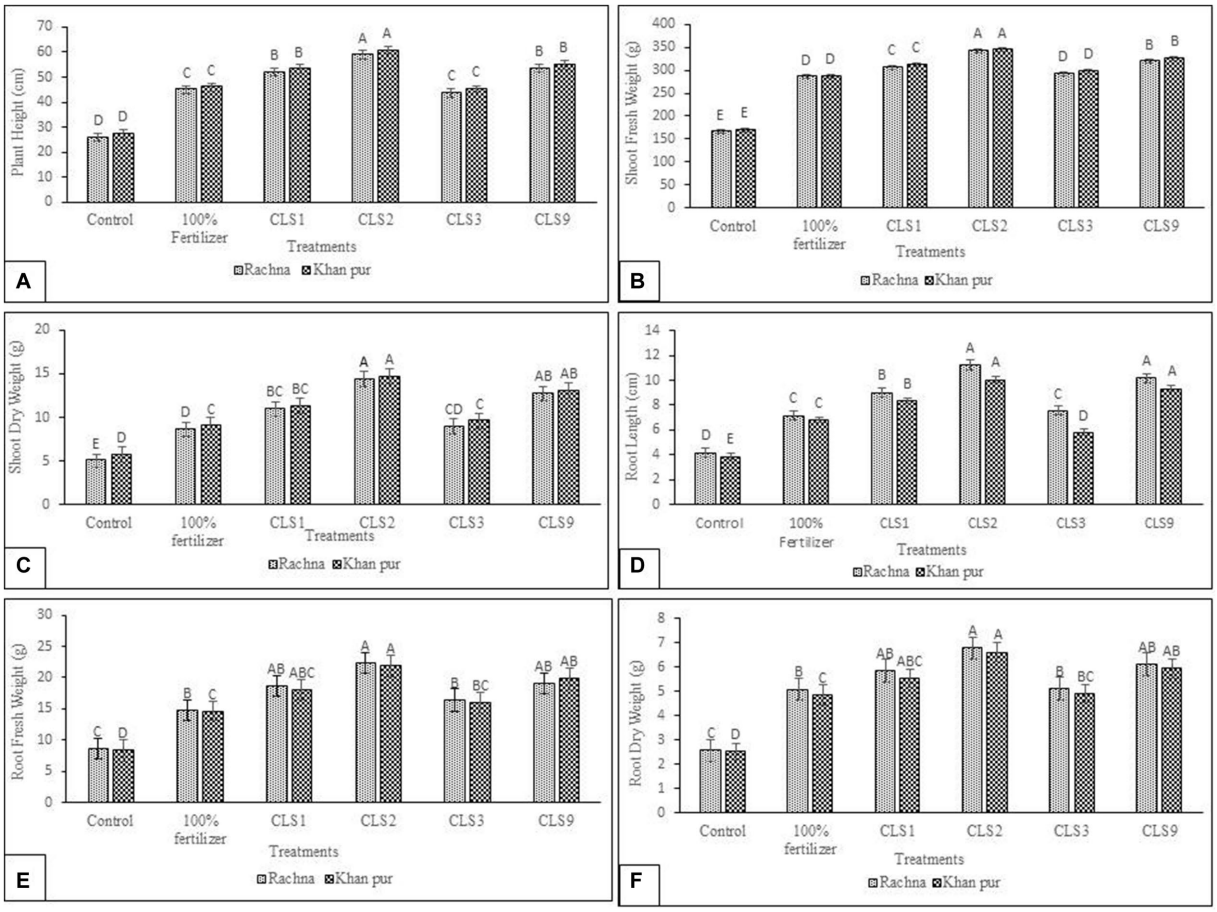


FIGURE 5
(A–F): Effect of zinc-solubilizing isolates on promotion of growth attributes **(A)** Plant Height **(B)** Shoot Fresh Weight **(C)** Shoot Dry Weight **(D)** Root Length **(E)** Root Fresh Weight **(F)** Root Dry Weight of Canola (*Brassica napus* L.) cultivars (V1 Rachna; V2 Khan Pur) in the field trial. Data were collected at the flowering stage. The Tukey HSD test was applied at a 5% ($p < 0.05$) probability level to determine significant differences between the means of the different treatments; the same letters on the bars indicate non-significant differences among the means, and vice versa.

Rachna (V1) and Khan pur (V2). The field soil was sandy loam in texture and alkaline in nature, having a pH of 8.2; EC of 2.6 dS m^{-1}; 0.65% organic carbon; 0.047% N, 4.7 mg kg^{-1}, and 197 ppm of available P and K, respectively. Total zinc concentration in the soil was 28.7 mg kg^{-1} and the available zinc concentration is 2.4 mg kg^{-1}. In cultivar V1, strain *P. aryabhattai* (CLS2) displayed the highest increase (2.26-fold) of plant height over non-inoculated control (Figure 5A). The strains *P. megaterium* (CLS9) and *S. succinus* (CLS1) also showed a significant increase in plant height (2.06- and 2.00-fold) and were statistically non-significant with each other. The strain *B. subtilis* (CLS3) increased plant height by 1.68-fold when compared to non-inoculated control and was statistically similar with 100% fertilizer treatment. In cultivar V2, maximum plant height was observed in strain CLS2 with an increase of 2.23-fold over non-inoculated control, and in order of significance, CLS2 was followed by CLS9 and CLS1, which differed non-significantly with each other. In case of plant height, the strain CLS3 varied non-significantly with 100% fertilizer treatment in cultivar V2. Both cultivars showed non-significant differences between treatment means in the case of plant height of canola. However, the cultivar means showed that V2 was significantly better than V1. A maximum (2.06-fold) increase in fresh weight of shoot over non-inoculated control was observed from CLS2, followed by CLS9 and CLS1, which reported 1.92- and 1.84-fold higher over non-inoculated control in V1 (Figure 5B). In case of inoculation minimum shoot fresh weight was recorded in CLS3 with an increase of 1.76-fold, which was statistically similar to 100% fertilizer treatment in V1.

All treatments showed the same pattern of growth in plant height and fresh weight of shoot and showed the statistically same response to inoculation in both cultivars (V1 and V2). The cultivar V2 proved better in shoot dry weight and statistically significant to V1. Maximum shoot dry weight was observed in CLS2 in both cultivars with a rise up to 2.83- and 2.55-fold compared to non-inoculated control in cultivars V1 and V2, respectively. Isolate CLS9 showed the second-best result in shoot dry weight, followed by CLS1 and CLS3, while CLS3 was almost at par with 100% fertilizer treatment in both cultivars (V1 and V2). The results showed that both cultivars are non-significant with each other in shoot dry weight (Figure 5C). The strain CLS2 recorded a maximum growth of 2.67- and 2.57-fold in root length when compared to non-inoculated control in cultivars V1 and V2, respectively (Figure 5D) and was statistically non-significant to strain CLS9 in both cultivars. CLS1 also reported a better increase of 2.15- and 2.14-fold in root length over non-inoculated control in cultivars V1 and V2, respectively. The strain CLS3 was found less effective

among the other inoculating zinc-solubilizing strains, but it was non-significant to 100% fertilizer treatments and significantly higher than non-inoculated control in cultivar V1. However, in cultivar V2, the strain CLS3 was less significant to 100% fertilizer treatments but statistically significant to non-inoculated control. The highest fresh weight of root in cultivar V1 was recorded from strain CLS2, with a rise of 2.60-fold over non-inoculated control, followed by CLS9 and CLS1, which were statistically similar to each other, increasing up to 2.22- and 2.16-fold, respectively, compared to non-inoculated control. Strain CLS3 was also better at producing root fresh weight, increasing to 1.90-fold as compared to non-inoculated control and non-significant with non-inoculated 100% fertilizer treatment in cultivar V1 (Figure 5E). In case of V2, maximum root fresh weight was obtained in CLS2, which was followed by strain CLS9 and CLS1, with an increase up to 2.36- and 2.16-fold, respectively, over non-inoculated control. Minimum values of root dry weight among the inoculated strain treatments were observed in CLS3, but it varied significantly with non-inoculated 100% fertilizer treatment as well as non-inoculated control. Both cultivars were non-significant with each other in root fresh and dry weight of canola. In cultivar V1, the inoculated plants with strain CLS2 showed a tremendous increase in root dry weight (2.61-fold) compared to non-inoculated control. The isolates CLS9 and CLS1 showed significant increase up to 2.35- and 2.25-fold in root dry weight as compared to non-inoculated control respectively, and were statistically non-significant with each other in cultivar V1 (Figure 5F). In the case of inoculation, the minimum root dry weight was shown by the strain CLS3 with an increase of 1.97-fold over non-inoculated control and statistically similar to the non-inoculated 100% fertilizer-treated plants in cultivar V1. In cultivar V2, maximum root dry weight was observed in strain CLS2, with an increase of 2.62-fold over non-inoculated control. The strain CLS9 showed the second-best result with 2.35-fold increase in dry weight of root over non-inoculated control, followed by strains CLS1 and CLS3, which were significantly higher than non-inoculated 100% fertilizer treatment and non-inoculated control as well. No significant variation in root dry weight occurred between cultivars V1 and V2 of canola. Overall, the findings of the field trials demonstrated the beneficial effects of *P. aryabhattai* (strain CLS2) and *P. megaterium* (strain CLS9) on both cultivars of canola plant. In assessment of the total effect of these bacterial inoculants, strain CLS2 was the best and manifested tremendous increase in all growth attributes, plant height, root length, fresh and dry weight of shoot, and fresh and dry weight of root. The isolate CLS9 was the second-best strain, showing remarkable growth in all growth attributes enlisted above. While non-inoculated control reported minimum plant height, length of root, fresh and dry weight of shoot, and fresh and dry weight of root. The effect of the seed-applied zinc strains on growth traits was displayed in Figures 5A–F. However, in both sand- (Pot) and soil (Field)-based experiments, CLS2 displayed the highest and most consistent rise in plant height, dry and fresh weight of shoot, and fresh and dry weight of root, followed by CLS9, and then CLS1. CLS2 displayed the highest and most consistent rise in plant height, shoot dry, and, however, strain *B. subtilis* (CLS3) was found less effective among the other inoculating zinc-solubilizing strains, but it was significantly higher than non-inoculated control in all growth attributes of canola. In the field trial investigations, the *P. aryabhattai* (CLS2) strain was the best strain for increasing canola plant growth in terms of total plant dry weight.

3.9 Potential of rhizobacterial strains on yield attributes of canola

Data pertaining to the yield attributes of canola cultivars as affected by different zinc-solubilizing isolates have been given in Figures 6A–D. The strain CLS2 demonstrated highest number of silique/plant with a 4.19- and 4.21-fold increase compared to the non-inoculated control in both cultivars. The strain CLS2 was statistically similar to strain CLS9, which had an increase of 4.08-fold in the number of siliqua of plant as compared to non-inoculated control in V1 (Figure 6A). Strain CLS1 showed remarkable increase (2.72- and 2.78-fold) in number of siliqua/plant in cultivars V1 and V2, respectively. In both cultivars, strain CLS3 was statistically identical to non-inoculated 100% fertilizer treatment, but these treatments were statistically significant from non-inoculated control in the number of siliqua/plant. On comparison of cultivars, it was revealed that higher number of siliqua was observed in cultivar V2 than V1. In cultivar V1, strain CLS2 reported a significant maximum number of seeds/silique with a 2.90-fold increase, followed by strains CLS9 and CLS1 having a 2.57- and 2.54-fold increase, respectively, over non-inoculated control and these strains were statistically similar with each other. Strain CLS3 was statistically non-significant to non-inoculated 100% fertilizer treatment, but these treatments were statistically different from non-inoculated control in cultivar V1 (Figure 6B). In cultivar V2, a comparative view of treatment means indicated that maximum increase (2.66-fold) of seeds/silique over non-inoculated control was observed in strain CLS2, followed by CLS9 and CLS1, which were non-significant with each other but significantly higher than non-inoculated control. Among the inoculation treatments, minimum values of seeds/silique were recorded by strain CLS3 varied non-significantly with non-inoculated 100% fertilizer treatment but statistically significant to non-inoculated control. All the treatment applications in the case of seeds/silique of canola showed statistically similar responses for both cultivars of canola; however, the cultivar means showed that V2 was significantly better than V1. In cultivars V1 and V2, the highest increase of 2.01 and 1.88, respectively, fold in 1,000 seed weight was recorded due to the application with strain CLS2 compared to the non-inoculated control (Figure 6C). The strain CLS2, followed by CLS9 and CLS1. These strains were statistically similar to each other up to an increase of 1.84- and 1.66-fold in 1,000 seed weight in V1. The strain CLS3 was statistically similar to non-inoculated 100% fertilizer treatment in both cultivars. Similar response of increase in 1,000 seed weight was noted by all the treatments in both cultivars; however, cultivar means indicated that V2 was significantly higher than V1. Strain CLS2 showed a maximum seed yield/plant with an increase of 1.97- and 1.83-fold over non-inoculated control in V1 and V2, respectively, and was statistically similar to strain CLS9 in both cultivars. In case of seed yield/plant, the strains CLS9, followed by CLS1 and CLS3, which were statistically similar to each other and with non-inoculated 100% fertilizer treatment but varied significantly to non-inoculated control in V1 cultivar. The strain CLS1 caused an increase of 1.54-fold in seed yield/plant, followed by CLS3, which was statistically significant to non-inoculated 100% fertilizer treatment and non-inoculated control as well in V2 (Figure 6D). The yield of canola crop was significantly enhanced by seed priming with zinc-solubilizing rhizobacteria, while the number of seeds/siliqua, number of siliqua/plant, 1,000 seed weight, and seed yield/plant increased

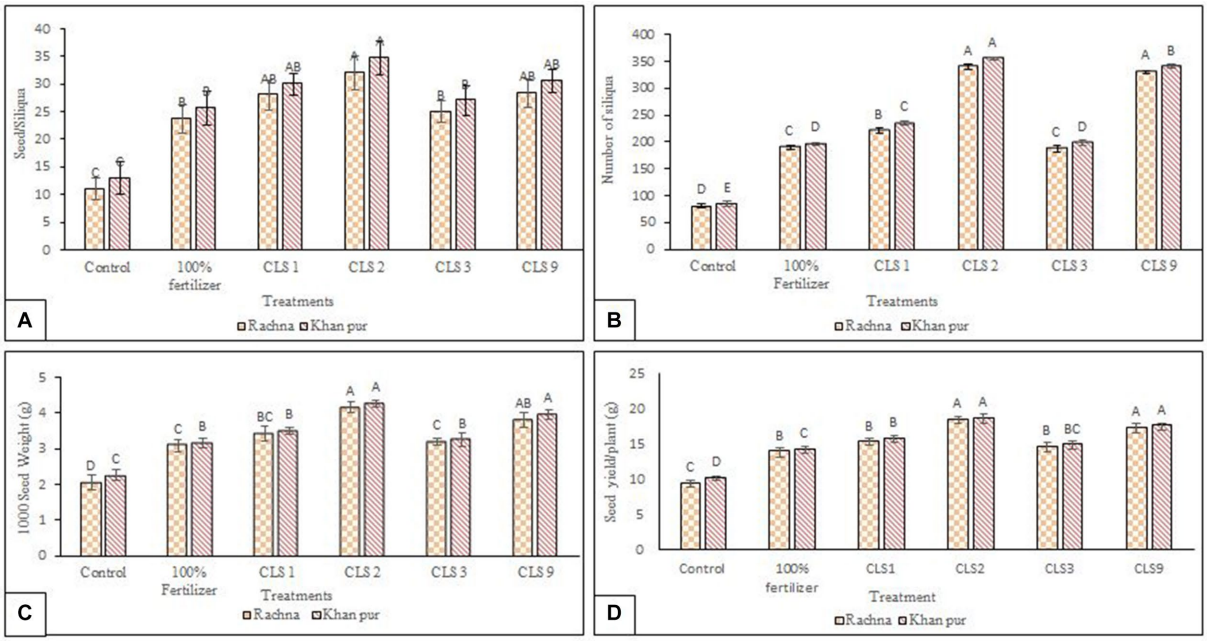


FIGURE 6
(A–D): Effect of zinc-solubilizing rhizobacteria on yield components of canola cultivars V1 (Rachna) and V2 (Khan Pur); **(A)** number of seeds/siliqua and **(B)** number of silique/plant **(C)** 1,000 seed weight and **(D)** seed yield/plant. Data for yield attributes were collected at the harvesting stage. The Tukey HSD test was applied at a 5% ($p < 0.05$) probability level to determine significant differences between the means of the different treatments; the same letters on the bars indicate non-significant differences among the means, and vice versa.

significantly with rhizobacterial treatment compared to non-inoculated control at harvest stage. Non-inoculated control reported minimum values for all the yield parameters. However, cultivar V2 was significantly better than V1 within all the yield parameters.

3.10 Effect of zinc-solubilizing isolates on oil content and fatty acid profile

Variable results of oil, protein contents, and fatty acid profile as affected by zinc-solubilizing strains among both cultivars are illustrated in Tables 6, 7. Inoculation of rhizobacterial strains significantly enhanced the oil contents of the seeds of canola. Our results indicated the significant differences between non-inoculated and inoculated seeds with rhizobacterial strains on oil and protein content. Oil content increased with non-inoculated control with 100% fertilizer. The highest oil content was observed in CLS2 and CLS9, but there was no statistical difference between the oil contents of the seeds treated with CLS2 and CLS9. The lowest oil contents belong to non-inoculated seeds (without fertilizer), followed by non-inoculated seeds with 100% fertilizer. On the other hand, linoleic acid was decreased in the following manner non-inoculated control (with 100% fertilizer) > CLS9 > CLS3 > CLS2 in contrast with non-inoculated control (without fertilizer). Just like linoleic acid, bacterial inoculation imposed a significant positive impact on linolenic acid in Table 6. Variable impact of linolenic acid in canola seeds was observed among all the inoculated treatments, where highest increase was recorded in CLS2-treated seeds in both varieties, followed by non-inoculated control (with 100% fertilizer) and CLS9, which were non-significant with each other but differed significantly to non-inoculated control. The lowest value of linolenic acid was recorded in non-inoculated control for both varieties. Highest increase in palmitic acid was observed in non-inoculated control seeds, followed by CLS1 and CLS3. However, CLS2 differed non-significantly to the CLS9 compared to the non-treated control. Bacterial inoculation imposed non-significant impact on stearic acid in variety V1 compared to non-treated control. However, in V2, highest value of stearic acid was found in seed treated with CLS3, which was non-significant with non-treated control (with 100% fertilizer), followed by CLS1, CLS9, and non-treated control, which were also non-significant with each other. Ecosenoic acid was observed to be maximum for CLS2 treated seeds, followed by CLS1, CLS3, CLS9, and non-inoculated control with 100% fertilizer, which were non-significant with each other but significantly differed with inoculated control. However, the effect of inoculated treatments on ecosenoic acid in seeds of V2 remained non-significant. The values of glucosinolates were significantly high in non-inoculated control with 100% fertilizer. Maximum values of glucosinolates were recorded in seeds treated with 100% fertilizer followed by bacterial inoculation CLS3, which was non-significant with control. Lowest values of glucosinolates were observed in seeds treated with CLS9. Both varieties displayed the same effect of all the treatments on glucosinolate accumulation. Erucic acid in the seeds of canola was substantially decreased among all the inoculated treatments as compared with the untreated control with and without fertilizer. The fatty acid profile of canola seeds of both cultivars shown in Table 7 revealed that erucic acid was determined to be 0.56–0.63% and glucosinolates 17.77–52.19 μmol g^{-1}.

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TABLE 6 Effect of zinc solubilizing isolates on oil content and fatty acid profile in seeds of Canola (*Brassica napus* L.) cultivars in field trial.

Canola cultivars	Oil contents (%)		Protein (%)		Oleic acid (%)		Linoleic acid (%)		Linolenic acid (%)	
	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2
Uninoculated control	26.89 ± 0.45^{d}	27.75 ± 0.29^{d}	20.92 ± 0.39^{a}	20.60 ± 0.48^{a}	40.12 ± 0.23^{d}	41.22 ± 0.37^{e}	13.82 ± 0.59^{d}	12.90 ± 0.40^{d}	4.57 ± 0.27^{c}	4.72 ± 0.34^{d}
100% fertilizer	35.98 ± 0.29^{c}	36.99 ± 0.44^{c}	19.64 ± 0.37^{ab}	16.57 ± 0.62^{b}	47.94 ± 0.43^{c}	49.81 ± 0.49^{cd}	20.86 ± 0.39^{a}	20.63 ± 0.64^{a}	6.26 ± 0.14^{ab}	6.50 ± 0.24^{abc}
Staphylococcus succinus (CLS1)	38.67 ± 0.65^{b}	39.43 ± 0.63^{b}	$18.34 \pm 0.71^{b-d}$	18.47 ± 0.69^{ab}	51.43 ± 0.51^{b}	52.30 ± 0.57^{bc}	14.35 ± 0.19^{cd}	14.07 ± 0.13^{cd}	4.60 ± 0.32^{c}	4.83 ± 0.33^{cd}
Priestia aryabhattai (CLS2)	42.97 ± 0.05^{a}	44.07 ± 0.30^{a}	17.02 ± 0.88^{cd}	16.91 ± 0.20^{b}	54.35 ± 0.68^{a}	56.02 ± 0.92^{a}	15.11 ± 0.38^{cd}	15.00 ± 0.37^{bc}	6.68 ± 0.25^{a}	6.91 ± 0.19^{a}
Bacillus subtilis (CLS3)	38.87 ± 0.49^{b}	40.18 ± 0.46^{c}	18.81 ± 0.14^{abc}	18.19 ± 0.68^{ab}	48.81 ± 0.44^{c}	49.08 ± 0.90^{d}	16.01 ± 0.21^{bc}	15.70 ± 0.29^{bc}	4.97 ± 0.39^{bc}	5.18 ± 0.47^{bcd}
Priestia megaterium (CLS9)	42.09 ± 0.30^{a}	42.92 ± 0.42^{a}	15.92 ± 0.31^{d}	17.17 ± 0.40^{b}	53.26 ± 0.79^{ab}	53.69 ± 0.56^{ab}	17.00 ± 0.40^{b}	16.49 ± 0.38^{b}	6.28 ± 0.48^{ab}	6.70 ± 0.45^{ab}
Cultivar means	37.58^{b}	38.56^{a}	18.44^{a}	17.98^{a}	49.32^{b}	50.35^{a}	16.19^{a}	15.80^{a}	5.56^{a}	5.81^{a}

Effect of zinc solubilizing rhizobacteria on oil contents and fatty acid profile of canola cultivars presented in this table are the three replications ± standard error; Canola cultivars are V1 (Rachna) and V2 (Khan Pur). Data for measurement of fatty acid profile of seeds was collected at maturity of canola crop. The Tukey HSD test was applied at a 5% ($p \leq 0.05$) probability level to determine significant differences between the means of the different treatments; the same letters on the bars indicate non-significant differences among the means, and vice versa; CVC, critical value for comparison.

4 Discussion

Keeping in view the role of zinc-solubilizing rhizobacterial isolates on the growth and production of canola plants, the present study was aimed to isolate and identify the promising strains which were potential candidates to solubilize zinc and other nutrients. Ten isolated Zn-solubilizing bacteria associated with canola roots, rhizosphere, and soil were cataloged from 84 samples, and the identification of these rhizobacterial strains was used to verify the diversity of isolates in different sampling sites. Overall, our results indicated that bacteria belonging to the *Bacillus* genera were the dominant root-associated bacteria of canola, identified as *B. cereus*, *B. subtilus*, *P. aryabhattai*, and *P. megaterium* from the canola rhizosphere of all three locations. *P. aryabhattai* was the dominant strain in all the rhizospheric samples collected from three different locations. *Priestia* members were originally categorized as *Bacillus* species, a genus having extensive polyphyly among its members. *P. megaterium* also showed good association with canola plants that were isolated from two locations. While the strain *S. succinus* was also isolated from two sites. In our study, similar isolates identified from three different sampling sites of canola plants indicated that these strains were host-specific. Our results are similar to the findings of Rani et al. (2023) who also found that the crop-specific Zn-solubilizing bacteria from several locations throughout the globe. This research is an update about the role of *Bacillus* and *Staphylococcus* spp. as zinc solubilizers in canola crops as inexpensive economic agricultural inputs. At different sampling sites, the availability of same bacterial strains associated with host plants (canola) is highly valuable for planning future investigations due to the important role of these rhizospheric bacteria in oil seed crop enhancement studies. Zinc is one of the most requisite micronutrients required for effective growth and development of plants. It ameliorates canola productivity in addition to providing nutritional security. Its deficiency not only affects nutritional quality, but also growth and yield of canola. Since Pakistani soil is alkaline and calcareous in nature, the pH is found around 6.8–9.2 (Samreen et al., 2019). Due to high pH and low organic matter of Pakistani soil, it is zinc-deficient. Soil-applied inorganic zinc becomes unavailable soon after its application. By decreasing the soil pH, zinc-solubilizing rhizobacteria secrete organic acids and transform the insoluble forms of inorganic zinc into plant-soluble forms of zinc, so it becomes available in soil for plant uptake. Similar findings have also been documented in previous work of Masood et al. (2022) and Ali et al. (2023). To date, little is known about interventions such as the use of soil bacteria to solubilize unavailable zinc and improve zinc uptake in plants. Its well-known zinc mobilization abilities appear to be prevalent among bacterial taxa, *Bacillus*, which is one of the most extensively researched genera because it is found to be abundant in nature and has many growth-enhancing characteristics (Zhao et al., 2011; Naseer et al., 2020). In our investigation, we identified rhizobacterial strains with unique characteristics, such as improved plant growth through mitigation of zinc. Among the identified strains, the genus *Bacillus* was the most dominant in terms of solubilizing non-labile zinc in soil and promoting canola growth, yield, and nutrient accumulation in grains. All of these Zn-solubilizing *Bacillus* strains are Gram-positive plant-associated bacteria that secrete growth-promoting metabolites that enhance plant growth, nutrient acquisition, and suppress soil-borne plant pathogens (Meena et al., 2017). Among 10 zinc-solubilizing strains, four zinc-solubilizing bacterial strains were screened *in vitro* for their plant

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TABLE 7 Effect of zinc solubilizing isolates on oil content and fatty acid profile in seeds of Canola (*Brassica napus* L.) cultivars in field trial.

	Palmitic acid (%)		Stearic acid (%)		Ecosenoic acid (%)		Erucic acid (%)		Glucosinolates (μmol/g)	
Canola cultivars	V1	V2	V1	V2	V1	V2	V1	V2	V1	V2
Uninoculated control	6.23 ± 0.18^{a}	6.03 ± 0.18^{a}	2.28 ± 0.09^{a}	2.45 ± 0.13^{ab}	3.98 ± 0.22^{b}	4.29 ± 0.21^{a}	2.64 ± 0.26^{a}	2.58 ± 0.18^{a}	33.62 ± 0.76^{b}	33.10 ± 0.64^{b}
100% fertilizer	5.77 ± 0.22^{ab}	5.66 ± 0.18^{a}	2.43 ± 0.16^{a}	2.62 ± 0.21^{a}	4.31 ± 0.18^{ab}	4.45 ± 0.21^{a}	1.35 ± 0.11^{b}	1.25 ± 0.07^{b}	52.19 ± 1.34^{a}	50.75 ± 1.09^{a}
Staphylococcus succinus (CLS1)	5.66 ± 0.25^{ab}	5.40 ± 0.17^{ab}	2.09 ± 0.32^{a}	2.28 ± 0.21^{ab}	4.03 ± 0.38^{ab}	4.19 ± 0.40^{a}	0.73 ± 0.04^{bc}	0.63 ± 0.02^{c}	22.98 ± 0.94^{c}	23.90 ± 0.70^{c}
Priestia aryabhattai (CLS2)	4.57 ± 0.19^{c}	4.36 ± 0.16^{b}	1.61 ± 0.05^{a}	1.73 ± 0.04^{b}	5.52 ± 0.35^{a}	5.71 ± 0.32^{a}	0.71 ± 0.08^{c}	0.56 ± 0.08^{c}	23.61 ± 1.17^{c}	25.10 ± 1.24^{c}
Bacillus subtilis (CLS3)	4.81 ± 0.30^{bc}	5.07 ± 0.37^{ab}	2.40 ± 0.18^{a}	2.60 ± 0.15^{a}	4.20 ± 0.23^{ab}	4.39 ± 0.23^{a}	0.87 ± 0.04^{bc}	0.84 ± 0.04^{bc}	29.90 ± 0.96^{b}	30.92 ± 1.12^{b}
Priestia megaterium (CLS9)	4.23 ± 0.11^{c}	4.38 ± 0.15^{b}	2.02 ± 0.10^{a}	2.19 ± 0.15^{ab}	4.39 ± 0.43^{ab}	4.59 ± 0.49^{a}	0.79 ± 0.08^{bc}	0.63 ± 0.09^{c}	17.77 ± 0.68^{d}	18.45 ± 0.32^{d}
Cultivar means	5.21^{a}	5.15^{a}	2.14^{a}	2.31^{a}	4.40^{a}	4.60^{a}	1.18^{a}	1.08^{a}	30.01^{a}	30.37^{a}

Effect of zinc solubilizing rhizobacteria on fatty acid profile of canola cultivars presented in this table are the three replications ± standard error; Canola cultivars are V1 (Rachna) and V2 (Khan Pur). Data for measurement of fatty acid profile of seeds was collected at maturity of canola crop. To calculate significant differences among the means of different treatments, Tukey HSD test was applied at 5% ($p \leq 0.05$) probability level. The same letters on the bars show the non-significant differences between the mean, and vice versa.

growth-promoting abilities based on their Zn solubilization efficiency and Zn solubilization index. The zinc-solubilizing bacteria produce a variety of secondary metabolites that enhance Zn availability and plant growth (Yadav et al., 2022). The results are similar to those stated by Upadhyay et al. (2021), who found that inoculation of zinc-solubilizing bacteria notably increased plant height and root length of maize plants. Bacterial solubilization of Zn-mediated organic acid secretion may result in a drop in pH, which is important for enhancing their Zn solubility and uptake (Ramesh et al., 2014). Our findings were also supported by earlier reports, where increase in zinc availability was achieved by drop of pH of the medium (Mumtaz et al., 2019). Previously, various zinc-solubilizing *Bacillus* strains *viz. B. aryabhattai* (Siddikee et al., 2010; Mumtaz et al., 2017; Naseer et al., 2020), *B. subtilis* (Liu et al., 2018; Abbaszadeh-Dahaji et al., 2020; Ahmad et al., 2021), *B. cereus* (Khande et al., 2017; Mumtaz et al., 2019) and *B. megaterium* (Dinesh et al., 2018; Bhatt and Maheshwari, 2020; Rezaeiniko et al., 2022) were reported as ideal candidates for biofortification of Zn in several crops. Among these isolated strains, CLS2 showed a remarkable solubilization potential for zinc oxide by producing large clear halo zones and expressed highest potential as zinc solubilizer plant growth-promoting isolate throughout the pot and field experiments. Therefore, meticulous use of this isolated strain CLS2 could help in providing a considerable amount of soluble zinc along with augmented nutrient uptake, plant growth, and yield in sustainable manner (Bhatt and Maheshwari, 2020). Strain CLS2 produced siderophore, hydrogen cyanide, and protease, which was in line with the findings of (Mumtaz et al., 2017) who also declared the positive outcomes for similar characteristics in *B. aryabhattai* strains. The plant experiment results also show that canola seeds inoculated with *B. megaterium* (CLS9) performed second best among all other treatments, resulting in increased root and shoot length and fresh and dry weight of root and shoot. In our study, inoculation of *P. aryabhattai*, followed by *P. megaterium*, *S. succinus*, and *B. subtilis* performed better in terms of growth and production of canola. Castiglione et al. (2021) highlighted the plant growth-promoting features (IAA and siderophore production and solubilization of phosphate) of *S. succinus* in different crops (quinoa and maize), while Orhan and Demirci (2020) observed the ability of nitrogen fixation exhibited by *S. succinus*. According to Oliva et al. (2023), *S. succinus* promotes the production of fine roots and an increase in root fresh and dry weight of maize plants. However, to the best of our knowledge, this is the first report demonstrating the significance of *S. succinus* (CLS1) as an efficient zinc solubilizer, nutrient enhancer, and plant growth promoter isolated from canola rhizosphere. As a result, it is recommended as an alternative to chemical fertilizers. Maximum values for production of auxins, root colonization, and phosphate and zinc solubilization ability were found in these four selected strains, which could be used to improve canola growth and yield. The same results were presented by Pandey et al. (2018), who found that the *B. pumilus* and *B. subtilis* improved *Amaranthus* growth parameters. Previous reports also demonstrated that PGB inoculation increases the growth of canola (Nadeem et al., 2014). Increased canola growth could be related to the potential of Zn-solubilizing rhizobacterial isolates to increase nutrient availability through nitrogen fixation, solubilizing the insoluble phosphate, starch hydrolysis, zinc solubilization, and the production of phytohormones and siderophores. Several studies have been reported, where a range of microbial genera capable of solubilizing different zinc sources have been procured from canola (Dabrowska et al., 2017; Masood et al., 2022). In comparison to the non-inoculated control, the inoculation of Zn-solubilizing rhizobacterial isolates extensively improved growth parameters. Our results are also similar to those of Ramesh et al. (2014) who stated that inoculation of zinc-solubilizing strain *P. aryabhattai* increased the plant height, shoot, and root dry weight in soybeans and wheat. The second-best zinc solubilizer strain,

P. megaterium, is a nutrient enhancer and plant growth promoter, suggesting that it may be employed as a substitute for nutritional shortfalls in the host plant by converting insoluble zinc to the soluble one and as an alternative to synthetic chemical fertilizers. Inoculation of *B. subtilis* as zinc-solubilizing rhizobacterial strain significantly increased dry matter and grain yield, which was followed by *B. megaterium* in soybean plant (Danhorn and Fuqua, 2007). Biofilm formation is crucial for efficient root colonization, provides the bacteria an advantage to compete with other microorganisms, and increases chances of bacterial survival in hostile environments (Ramey et al., 2004; Yuan et al., 2015; Altaf and Ahmad, 2016). *P. aryabhattai* (CLS2) showed the highest rate of ability to form biofilm. Therefore, biofilm production could be relevant to the isolates to assess their behavior in field trials. Biofilm formation is crucial for efficient root colonization, provides the bacteria an advantage to compete with other microorganisms, and increases chances of bacterial survival in hostile environments (Ramey et al., 2004; Yuan et al., 2015; Altaf and Ahmad, 2016). *P. aryabhattai* (CLS2) showed the highest rate of ability to form biofilm. Therefore, biofilm production could be relevant for the isolates to assess their behavior in field trials. In addition to this, our study goes well with Ansari et al. (2023), who also reported the multifunctional PGP traits as well as rhizosphere colonization and biofilm formation by *B. subtilis*, which enhanced the growth of wheat plants. Similar findings have also been documented in the previous work of Bach et al. (2022) who isolated *B. aryabhattai* strains from canola rhizosphere, and these strains formed the biofilm and showed the synergic action by the production of siderophore. Soil porosity and water contents were also influenced by rhizobacterial EPS secretions (Shahzad, 2020). Thus, production of EPS by microbial isolates in the plant rhizosphere stabilized the soil aggregates by improving porosity, root proliferation, water contents, and their gummy exudations (Deka et al., 2019; Khan and Bano, 2019; Cheng et al., 2020). To delay soil drying locally and related adverse effects, *Bacillus subtilis* and plants modify their surroundings by releasing EPS (Benard et al., 2023). Significant root colonization is required for rhizobacteria to develop themselves adequately in the rhizosphere, and PGP rhizobacteria are an effective way of enhancing agricultural output by suppressing plant pathogens (Zhou et al., 2016; Kalam et al., 2020; Jiao et al., 2021). Compared to other test isolates, CLS2 and CLS9 were stronger root colonizers. Our results also revealed that zinc-solubilizing isolates *B. megaterium* and *B. cereus* were also capable of solubilizing inorganic as well as organic phosphate. Furthermore, our findings are consistent with those of Dubey and Maheshwari (2011), who discovered that *B. subtilis* solubilizes zinc and promotes canola production. Previously, it was demonstrated that these Zn-solubilizing *Bacillus* strains had multiple plant growth-promoting characteristics and the ability to promote maize growth, yield, and nutrient uptake (Mumtaz et al., 2017; Mumtaz et al., 2018; Mumtaz et al., 2022). The Zn-solubilizing *Bacillus* strains have been well documented for the production of IAA, which plays an essential role in plant microbe associations that increase and promote crop growth and production (Saxena et al., 2020). Our results showed that all the tested isolates in pot and field experiments have the ability to produce IAA both in the presence and absence of L-tryptophan. Highest IAA produced by isolates CLS2 and CLS9 might play a role in enhancing the growth of canola plants. Our results are also in line with those of Iqbal et al. (2023) who also claimed the increased canola crop growth and yield with the introduction of IAA synthesizing *Bacillus* strains. Furthermore, a recent study on Zn-solubilizing bacterial strains was revealed to enhance rice crop growth parameters (Shakeel et al., 2023). Previously, Asghar et al. (2002) suggested the production of IAA by bacterial strains isolated from the rhizosphere of *Brassica* species to improve growth yield and oil content of canola. Our results supported the findings of Hussain et al. (2015) who presented that the seed inoculation of zinc-solubilizing isolates (*B. subtilis* and *B. megaterium*) enhanced zinc contents in wheat grains, which ultimately resulted in improved growth in both pot and field trials. Canola oil has the lowest saturated fat content of any vegetable oil; hence, it is preferred by diet-conscious consumers. Erucic acid and glucosinolates are harmful to both human and animal health, and they impart a bitter taste. The safe limits for these compounds are 30 $\mu mol\, g^{-1}$ of glucosinolates and < 2% erucic acid in oil in oil-free diets (Moghadam et al., 2011). Our findings are consistent with those of Asghar et al. (2004), who similarly noticed a significant increase in oil content of *Brassica napus* L. by seed inoculation. However, in our study, the strains CLS2 and CLS9 demonstrated encouraging outcomes in terms of erucic acid and glucosinolate accumulation. There were low values of erucic acid in seeds inoculated with these strains. The above-mentioned results displayed the supportive role of inoculated strains in increasing oil and protein contents. Moreover, inoculation assisted the plants to maintain nutrition balance by reducing erucic acid and glucosinolate accumulations. One of the most limiting causes for low agricultural output is soil-borne pathogens. One of these mechanisms is the synthesis of siderophores, and these microbial compounds can lock iron away from pathogens (Kramer et al., 2020; Dar et al., 2021). As a result, cultivating region-specific microbial strains for the formation of most effective bioinoculum is required to maximize crop output and nutrient content of particular crop (Afzal et al., 2019). The use of harmless, ecologically safe, and beneficial PGPR as bioformulations has been suggested to be very beneficial in increasing agricultural productivity in a sustainable manner (Verma et al., 2019). It can also help us to cope with the problem of malnutrition by increasing Zn concentration in canola grains. These rhizobacteria could be used as biofertilizers to substitute agrochemicals in order to increase canola production, and this is in conformity with the findings of Zainab et al. (2021). Currently, limited reports are available on microorganisms that can transform insoluble forms of zinc into accessible forms. As a result, efforts were conducted to identify and evaluate the Zn-solubilizing bacteria from the canola rhizosphere for the purposes of growth stimulation and zinc biofortification.

5 Conclusion

Biofortification by inoculation of PGPR is one of the recently established alternatives for addressing agricultural issues brought on by a growing population, minimizing the use of chemical fertilizers, and contributing to development of environmentally friendly agriculture on a global level. The objective of this research was to isolate the zinc-solubilizing bacterial isolates from canola fields. The molecular identification of these isolates offers an advantage in obtaining a larger number of microorganisms from natural environments. As a result, these multi-trait isolates may be desirable inoculants for increasing canola yield and nutrient quality, hence reducing malnutrition. The isolated Zn-solubilizing bacteria improve nutrient utilization efficiency through a variety of mechanisms, including increased surface area accessible by plant roots and the synthesis of siderophores and

exopolysaccharides. The majority of these bacteria could fix nitrogen, synthesize siderophores, IAA and solubilize zinc and phosphate. This research promotes the development of biotechnological techniques, such as inoculation with Zn-solubilizing bacteria that improves canola growth and yield. In the current study, selected rhizobacterial strains, *S. succinus* (CLS1), *P. aryabhattai* (CLS2), *B. subtilis* (CLS3), and *P. megaterium* (CLS9), have been demonstrated to be extremely effective zinc-solubilizing strains for seed inoculation, along with improvements in growth and yield attributes of canola in controlled as well as field conditions. This is the first study that reveals *S. succinus* as a Zn-solubilizing associated bacteria of canola plant. Most of the strains used in this study are not yet reported for zinc-solubilizing ability and their effect on canola growth and yield. These inoculants would be useful in improving oil quality and lowering the application of synthetic fertilizers in agriculture. The efficient rhizobacterial isolates identified in this study could be exploited to alleviate zinc deficiency, leading to a potential key for a sustainable canola crop production strategy.

Data availability statement

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

Author contributions

SJ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. NE: Project administration, Resources, Supervision, Writing – review & editing, Validation. FM: Methodology, Project administration, Resources, Supervision, Writing – review & editing, Validation.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1446064/full#supplementary-material

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Effects of fertilizer application on the bacterial community and weathering characteristics of typical purple parent rocks

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Introduction: Rock weathering is a fundamental process that shapes Earth's topography, soil formation, and other surface processes. However, the mechanisms underlying the influence of fertilizer application on weathering remain poorly understood, especially with respect to bacterial intervention.

Methods: In this study, purple parent rocks from Shaximiao Group (J_2s) and Penglaizhen Group (J_3p) were selected to investigate the effects of fertilizer application on the bacterial community and weathering characteristics of these rock by leaching experiment.

Results: The results revealed that: fertilizer application, especially when at high levels, greatly altered the abundance, diversity and composition of the bacterial community in weathered products. Through redundancy analysis, a decrease in pH and increases in available nutrients (AN and AP) resulting from fertilizer application were identified as the key factors driving changes of bacterial community composition in weathered products. Moreover, fertilizer application promotes the physical and chemical weathering of the parent rocks to some extent. This is especially true for the chemical weathering of J_2s. Structural equation model indicated that fertilizer application affects weathering through multiple pathways by affecting the chemical properties (pH, C:N and AP), specific bacterial genera (IMCC26256, *Ramlibacter*, and *Nitrosospira*), and bacterial community composition of weathered products.

Discussion: Our study links weathering characteristics with chemical properties and bacterial community changes of weathered products after fertilizer application, which plays a key role in controlling and predicting dynamic changes of rock weathering in space and time. It is helpful to further understand the law of human activities affecting the surface processes.

KEYWORDS

bacterial community composition, bioweathering, fertilization, rock weathering, weathered products

1 Introduction

Weathering is a fundamental process of the land surface, which directly affects the topography, soil formation, material circulation and other surface processes (Balland et al., 2010; Eppes et al., 2002; Xi et al., 2018). Moreover, the consumption of atmospheric CO_2 over geological timescales plays a crucial role in global climate change (Goldsmith et al., 2010; Them et al., 2017). In this

process, substances (rocks, minerals, etc.) disintegrate (physical weathering), decompose (chemical weathering) and form new substances (Fang et al., 2023), which are influenced by various natural and anthropogenic factors (Jin and Gan, 2013; Pawlik, 2013). However, since the middle of the 20th century, anthropogenic activities have caused unprecedented and rapid changes (Lewis and Maslin, 2015) in land use (Song et al., 2018), biodiversity (Isbell et al., 2017), environmental conditions (Marvel et al., 2019), and material cycling (Austin et al., 2013). Notably, recent studies have estimated that anthropogenic activities contribute between 16 and 40% of weathering processes on the basis of mass balance calculations (Huang et al., 2016; Yu et al., 2016).

Fertilizer application is an important activity in agricultural production, and with the continuous increase in the global population, agricultural activities have intensified, which has led to increased fertilizer usage. According to the official website of the Food and Agriculture Organization of the United Nations, the global consumption of N, P_2O_5, and K_2O fertilizers reached a staggering 201 million tons in 2020. This figure reflects a remarkable 49% increase compared with 2000. Long-term and excessive fertilizer application affects the soil nutrient content, pH, cation exchange capacity and other properties. Changes in soil chemical properties lead to changes in the process and rate of weathering. For example, the introduction of organic matter through livestock organic fertilizers has been shown to inhibit the weathering of carbonate rocks by increasing CO_2 concentrations and increasing the pH (Song et al., 2017b). Conversely, the soil acidification caused by the application of chemical fertilizers, especially ammonium nitrogen fertilizer (Guo et al., 2010; Bibi et al., 2014; Raza et al., 2020), leads to the leaching of salt group ions and the activation of aluminum (Bibi et al., 2014; Zhao et al., 2022), while causing a decrease in rock strength and disruption of the rock structure. This process contributes to the acceleration of rock physicochemical weathering. Biological weathering occurs when physicochemical weathering is assisted by biological processes. Bacterial weathering, as a significant pathway in bioweathering, plays a vital role in rock weathering (Sel and Binal, 2021). Bacteria can accelerate mineral weathering reactions through various mechanisms, including the production of organic acids and metal–ligand carriers, alteration of redox conditions, and the formation of biofilms (Wu, 2007; Mao et al., 2017; Chen et al., 2022). Increasing evidence supports the notion that bacteria profoundly mediate mineral weathering processes. Fertilizer application is known to cause the changes of bacterial abundance, diversity and community composition in soil (Yuan et al., 2013; Leff et al., 2015; Zeng et al., 2016; Ling et al., 2017; Yu et al., 2019). This change is closely related to the soil chemical properties affected by fertilizer application. Consequently, studying bacteria under different fertilization conditions is pivotal for understanding the complexities of weathering processes.

Weathering is a slow process that is difficult to observe. But owing to their easy weathering characteristics, purple parent rocks are prone to disintegration under the influence of environmental factors and anthropogenic activities. Therefore, they represent an ideal material for studying weathering processes. Through field observations and simulation experiments, scholars have reported that the weathering disintegration thickness of purple parent rocks can reach 11.2 ~ 19.6 $mm.yr.^{-1}$ on average (Zhu et al., 2008). In recent years, we have determined the rates of physical weathering of a variety of purple parent rocks under different influencing factors through a series of indoor and outdoor experiments (Zhang et al., 2016; Zhao et al., 2018). These studies mainly addressed natural factors such as water, heat and acid deposition. There are few studies concerned with anthropogenic activity influence-fertilizer application. Therefore, the objective of this study was to comprehensively examine the degree of weathering and the mechanisms underlying the effects of fertilization on typical purple parent rocks under various fertilization conditions. The elucidation of these relationships will contribute to the development of sustainable strategies for purple soil regions, ensuring their long-term productivity and environmental integrity.

2 Materials and methods

2.1 Field sites

The experimental site is located at the Yanting Purple Soil Agroecology Experimental Station of the Chinese Academy of Sciences (N 31°16′52″ E105°27′20″), at an altitude of 420 m. The average annual temperature in the experimental area is 17.3°C. The average annual rainfall is 880.7 mm. The predominant cropping system involves winter wheat and summer corn cultivation.

2.2 Rock sample preparation and experiment description

Two representative purple parent rocks, namely, Shaximiao Group (J_2s) (N 29°46′04″ E 104°48′42″) and Penglaizhen Group (J_3p) (N 31°16′52″ E 105°27′20″), which account for the largest exposed area in the Sichuan Basin, were selected as the experimental materials. To ensure the reliability and comparability of the experimental data, parent rocks of the same lithological type were sourced from identical sources and exhibited consistent coloration and degrees of weathering. The parent rock samples were subsequently naturally dried, followed by crushing and sieving to obtain rock fragments with five distinct particle size fractions: > 60 mm, 40–60 mm, 20–40 mm, 10–20 mm, and 5–10 mm. The mineral composition (Supplementary Table S1), major elemental content (Supplementary Table S2) and chemical properties (Supplementary Table S3) of the original parent rocks were determined.

Leaching columns with a diameter of 160 mm and a height of 400 mm were prepared for the experiment. The columns were filled with 600 g of rock fragments of each particle size fraction, totaling 3,000 g. Larger rock fragments were positioned at the bottom of the column, whereas smaller fragments were placed at the top. The experiment was set up with two variables: the application of fertilizer type and level. Nitrogen fertilizer (NH_4HCO_3) applied alone and combined application of nitrogen, phosphorus and potassium fertilizers (NH_4HCO_3, $NH_4H_2PO_4$ and KCl) were selected as the two types of fertilizer. The amount of fertilizer was determined according to the conventional fertilization amount (for the wheat planting period, N-P_2O_5-K_2O = 130-90-36 $kg \cdot ha^{-1} \cdot a^{-1}$; for the corn planting period, N-P_2O_5-K_2O = 150-90-36 $kg \cdot ha^{-1} \cdot a^{-1}$). The conventional fertilization amount was considered the 100% fertilizer level, and the experiment included four levels: 0, 100, 200 and 300%. Therefore, the experiment consisted of seven treatments: (i) control group without fertilizer (CK); (ii) 100% N fertilizer treatment (N1); (iii) 200% N fertilizer treatment; (iv) 300% N fertilizer treatment (N1); (v) 100% NPK fertilizer treatment (NPK1); (vi) 200% NPK fertilizer treatment; and (vii) 300% NPK fertilizer treatment. The fertilizers were dissolved

in 50 mL of deionized water and uniformly applied to the surface layer of the leaching columns. The leaching columns were installed in a field in June 2021 for the natural rainfall leaching experiments. The timing of fertilization was based on the local fertilization schedule in the months of June and October each year. After each rainfall event, the leached solutions were collected from the leaching columns. The weathered products of the parent rock were collected in September 2023. The samples were subjected to bacterial community analysis at −4°C, and physical and chemical properties were assessed after the samples were subjected to room temperature drying.

2.3 Chemical properties of weathered products

The weight of the <2 mm particles in the weathered products was measured via dry sieving, and the chemical characteristics of these particles were determined after drying at 65°C. The pH was determined via a potentiometric method at a water-to-soil ratio of 2.5:1. The cation exchange capacity (CEC) was determined via cobalt hexachloride leaching spectrophotometry (Renault et al., 2009). The total nitrogen (TN) and soil organic carbon (SOC) contents were determined via a combustion elemental analyzer. The contents of total phosphorus (TP), total potassium (TK), and other conserved elements were determined via X-ray fluorescence spectroscopy (XRF). Available nitrogen (AN) was determined via the alkaline hydrolysis diffusion method. Available phosphorus (AP) was determined via the sodium bicarbonate extraction molybdenum antimony colorimetric method. Available potassium (AK) was determined via ammonium acetate extraction flame photometry. The sample mineral composition was determined via X-ray diffraction (XRD) analysis (Sanchez and Gunter, 2006).

2.4 DNA extraction, PCR amplification, and high-throughput sequencing

The total genomic DNA of weathered product samples was extracted via an OMEGA Soil DNA Kit (OmegaBio-Tek, Norcross, GA, United States). Bacterial 16S rRNAV3-V4 region-specific primers were selected for PCR amplification, and according to the selected 16S V4 region, PCR amplification was carried out via the primers 338F (5′-barcode+ACTCCTACGGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The amplification products were subjected to 2% agarose gel electrophoresis, and the target fragments were excised and then recovered via an Axygen Gel Recovery Kit. The PCR products were quantified on a microplate reader (BioTek, FLx800) via the Quant-iT PicoGreen dsDNA Assay Kit. After the quantification step, the samples were mixed according to the amount of data required for each sample. Sequencing was performed via the NovaSeq-PE250 pattern of the Illumina MiSeq platform at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China).

2.5 Evaluation indices of weathering

The degree of chemical weathering is often determined through the analysis of geochemical parameters. The chemical index of alteration (*CIA*), which effectively captures the extent of weathering by considering various elements involved in the weathering process and exhibiting a monotonic response to silicate weathering (Nesbitt et al., 1996), has been widely used to assess the degree of rock weathering. The *CIA* is calculated as follows:

$$\mathrm{CIA} = \frac{\mathrm{Al_2O_3}}{\mathrm{Al_2O_3} + \mathrm{Na_2O} + \mathrm{K_2O} + \mathrm{CaO^*}} \times 100 \quad (1)$$

where oxides are presented in molar units. CaO* in Equation 1 is the CaO content in silicate; if n (Na_2O) > n (CaO), then n (CaO) = n (CaO*), if n (Na_2O) < n (CaO), then n (Na_2O) = n (CaO*).

A particle size of <2 mm is considered as the soil-forming standard for purple parent rock. The mass fraction of weathered products of <2 mm particle size was used as the physical weathering index to determine the soil formation rate (*SFR*) (Soil Research Office of Chinese Academy of Sciences Chengdu Branch, 1991).

The calculation of the *SFR* is performed as follows:

$$SFR = \frac{m - m_1}{m} \times 100\% \quad (2)$$

where: m in Equation 2 is the total mass of the parent rock in the lysimetric column (3,000 g), and where m_1 is the mass of weathered products with >2 mm grain size at the end of the experiment.

2.6 Statistical analysis

Analysis of variance (ANOVA) and Pearson's correlation analysis were performed in SPSS 26 to statistically analyze the experimental results. Microbiome bioinformatics was performed with QIIME 2019.4 according to official tutorials. Permutational multivariate analysis of variance (PERMANOVA) was performed with R 4.3.2 to evaluate the differences in bacterial community composition. Redundancy analysis (RDA) was performed by CANOCO 5 to evaluate the effects of the chemical properties of the weathered products on the total bacterial community composition. The above data were visualized in Origin 2021 and R 4.3.2. Structural equation models (SEMs) of the relationships between fertilization conditions, chemical properties, bacteria and degree of weathering were constructed via SMARTPLS 3 (Armonk, United States).

3 Results

3.1 Chemical properties of weathered products

As shown in Table 1, the chemical properties of the weathered products changed to different degrees under the different fertilizer treatments. For J_2s, the pH, CEC and SOC content of all of the fertilizer treatments were significantly lower than those of the CK treatment (except for the SOC content under the NPK1 fertilizer treatment) ($p < 0.05$). The pH decreased with increasing fertilizer level under the same fertilizer type. The order of magnitude of pH was NPK < N < CK. Conversely, the AN and AK contents in all of the

TABLE 1 Weathered product chemical properties under different treatments.

Parent rock	Treatment	pH	CEC	TN	SOC	TP	TK	AN	AP	AK
J_2s	CK	9.79 ± 0.03a	20.08 ± 0.18a	0.03 ± 0.00a	0.22 ± 0.09a	0.09 ± 0.01c	3.20 ± 0.01ab	15.53 ± 3.39d	16.19 ± 8.00d	99.92 ± 0.42d
	N1	9.33 ± 0.06b	19.90 ± 0.30b	0.03 ± 0.00a	0.15 ± 0.03b	0.09 ± 0.01c	3.21 ± 0.02ab	25.26 ± 0.33c	11.06 ± 2.68d	102.58 ± 7.84d
	N2	9.27 ± 0.05bc	19.94 ± 0.23ab	0.03 ± 0.00a	0.14 ± 0.02b	0.08 ± 0.00c	3.19 ± 0.02b	27.38 ± 3.34c	9.28 ± 3.84d	103.60 ± 0.73d
	N3	9.11 ± 0.05c	19.20 ± 0.20b	0.03 ± 0.00a	0.15 ± 0.04b	0.10 ± 0.02c	3.25 ± 0.03a	60.61 ± 7.68b	14.03 ± 8.49d	111.88 ± 21.99d
	NPK1	8.79 ± 0.09d	18.88 ± 0.51b	0.04 ± 0.00a	0.23 ± 0.01a	0.19 ± 0.02b	3.17 ± 0.02b	58.70 ± 6.41b	38.70 ± 2.01c	174.47 ± 7.07c
	NPK2	8.55 ± 0.16e	19.11 ± 0.51b	0.03 ± 0.00a	0.14 ± 0.01b	0.24 ± 0.02a	3.24 ± 0.05ab	68.21 ± 7.60b	55.17 ± 3.57b	245.00 ± 16.08b
	NPK3	8.52 ± 0.15e	19.35 ± 0.22b	0.03 ± 0.00a	0.14 ± 0.01b	0.24 ± 0.01a	3.24 ± 0.02ab	96.26 ± 5.03a	67.60 ± 4.66a	309.57 ± 18.44a
J_3p	CK	9.05 ± 0.01ab	19.21 ± 0.48a	0.04 ± 0.00a	0.20 ± 0.06a	0.08 ± 0.00d	2.89 ± 0.03a	19.59 ± 1.07d	7.58 ± 1.27d	115.40 ± 3.00d
	N1	9.04 ± 0.03ab	18.57 ± 0.24a	0.03 ± 0.00a	0.12 ± 0.01a	0.08 ± 0.00d	2.95 ± 0.08a	19.58 ± 5.34d	6.31 ± 1.13d	110.00 ± 6.10d
	N2	9.06 ± 0.02ab	18.82 ± 0.80a	0.04 ± 0.01a	0.15 ± 0.03a	0.07 ± 0.00d	2.91 ± 0.07a	21.39 ± 3.57 cd	5.40 ± 0.85d	106.75 ± 5.75d
	N3	9.02 ± 0.01ab	17.71 ± 0.30a	0.03 ± 0.00a	0.14 ± 0.03a	0.08 ± 0.00d	2.83 ± 0.04a	24.88 ± 3.53 cd	7.72 ± 2.33d	98.25 ± 1.52d
	NPK1	9.08 ± 0.01a	17.59 ± 0.61a	0.03 ± 0.00a	0.17 ± 0.03a	0.15 ± 0.00c	2.85 ± 0.05a	30.06 ± 3.94c	35.15 ± 1.33c	177.07 ± 7.97c
	NPK2	9.06 ± 0.05ab	17.32 ± 1.13a	0.04 ± 0.00a	0.17 ± 0.02a	0.25 ± 0.01b	2.86 ± 0.09a	41.04 ± 3.90b	60.98 ± 6.01b	275.4 ± 13.31b
	NPK3	8.99 ± 0.03b	17.56 ± 0.85a	0.04 ± 0.00a	0.15 ± 0.02a	0.40 ± 0.02a	2.99 ± 0.04a	52.12 ± 4.40a	96.78 ± 12.52a	350.7 ± 10.54a

CK=No fertilizer; N1 = 280 kg · ha^{-1} · a^{-1} N; N2 = 560 kg · ha^{-1} · a^{-1} N; N3 = 840 kg · ha^{-1} · a^{-1} N; NPK1 = N-P_2O_5-K_2O: 280-180-72 kg · ha^{-1} · a^{-1}; NPK2 = N-P_2O_5-K_2O: 560-360-144 kg · ha^{-1} · a^{-1}; NPK3 = N-P_2O_5-K_2O:840-540-216 kg · ha^{-1} · a^{-1}; CEC($cmol.kg^{-1}$)—Cation exchange capacity; TN(%)—Total nitrogen; SOC(%)—Soil organic carbon; TP(%)—Total phosphorus; TK(%)—Total potassium; AN(%)—Available nitrogen; AP(%)—Available phosphorus; AK(%)—Available potassium. Numbers depict means ± standard deviations ($n = 3$), and different lowercase letters indicate that there were significant differences among all treatments ($p < 0.05$).

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fertilizer treatments were greater than those in the CK treatment, with corresponding increases as the fertilizer level increased. The order of magnitude of the AN and AK contents was CK < N < NPK. The AN content under all the fertilizer treatments and the AK content under the NPK fertilizer treatments were significantly greater than those under the CK treatment ($p < 0.05$). The contents of TP and AP in the NPK fertilizer treatments were significantly greater than those in the CK treatment ($p < 0.05$), with corresponding increases as the fertilizer level increased. The content of TP in the N fertilizer treatment was not significantly different from that in the CK treatment ($p > 0.05$), but the AP content was significantly lower than that of the CK treatment ($p < 0.05$). The contents of TN and TK did not significantly differ among the treatments ($p > 0.05$). The chemical properties of J_3p were less sensitive to fertilizer treatments than those of J_2s were. There were no significant changes in the chemical properties ($p > 0.05$), except for the contents of TP and available nutrients, which were significantly greater under the NPK fertilizer treatments than under the CK treatment ($p < 0.05$).

3.2 Bacterial abundance, diversity and community composition of weathered products

A total of 3,923,050 sequences were identified from all samples via Illumina® MiSeq sequencing, and 3,293,394 valid sequences were retained after quality filtering. The sequences were analyzed and identified as belonging to 39 phyla, 129 classes, 337 orders, 559 families, 1,164 genera and 2,780 species. Among them, the dominant phyla were Proteobacteria, Actinobacteriota, Gemmatimonadota, Bacteroidota, Chloroflexi and Acidobacteriots, which collectively accounted for 73.90 to 90.95% of the entire bacterial community. Sixteen genera were identified as abundant (with relative abundances greater than 0.05% in all of the treatments and mean values exceeding 1%). The most abundant genera were *Longimicrobiaceae, Sphingomonas, Blastococcus, Longimicrobium* and JG30-KF-CM45, and the number of sequences of these genera accounted for 23.93% ~ 54.81% of the total number of sequenced genera (Figure 1).

For subsequent analysis, rarefaction curves were plotted, which revealed that the sequencing results sufficiently reflected the diversity contained in the sample (Supplementary Figure S1). To assess the alpha diversity of the bacterial community of weathered products, abundance was characterized by the Chao1, coverage was represented by Good's coverage index, and diversity was determined by the Shannon. The Good's coverage indices were all greater than 0.99, indicating that the sequencing capability covered most of the characteristics of the bacterial community (Figure 2).

For J_2s, the Chao1 of the 100% N and NPK fertilizer treatments was greater than that of the CK treatment, although the difference was not statistically significant ($p > 0.05$). The Chao1 of the 200 and 300% N and NPK fertilizer treatments were lower than those of the CK treatment, with a significant decrease observed at the 300% N fertilizer treatment level ($p < 0.05$). Under the same type of fertilizer, the Chao1 tended to decrease with increasing fertilizer level. The pattern of change in the Shannon was the same as that in the Chao1, but there was no significant difference between the fertilizer treatments and the CK treatment. For J_3p, the Chao1 was consistently lower than that of the CK treatment for the fertilizer treatments (except for the 100% NPK fertilizer treatment), where the Chao1 was significantly lower under the 200% N and 300% NPK fertilizer treatments ($p < 0.05$). Like

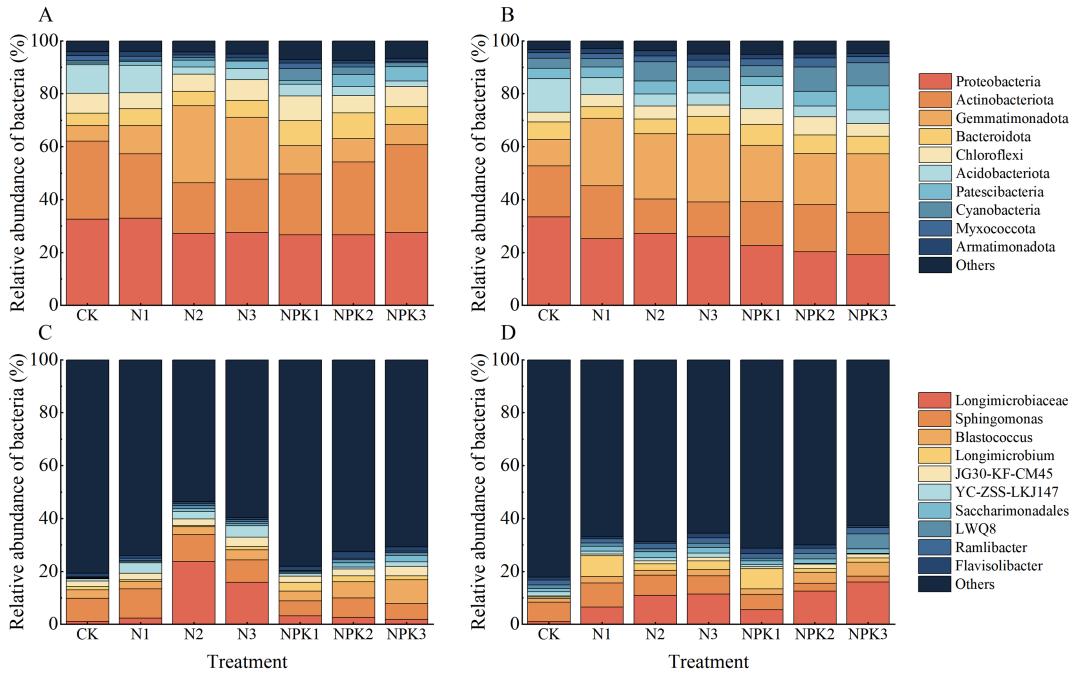


FIGURE 1
Distribution of bacteria in weathered products under different treatments. **(A)** The dominant phyla of the purple parent rock of J_2s. **(B)** The dominant phyla of the J_3p purple parent rock. **(C)** The dominant genus of the purple parent rock of J_2s. **(D)** The dominant genus of the J_3p purple parent rock.

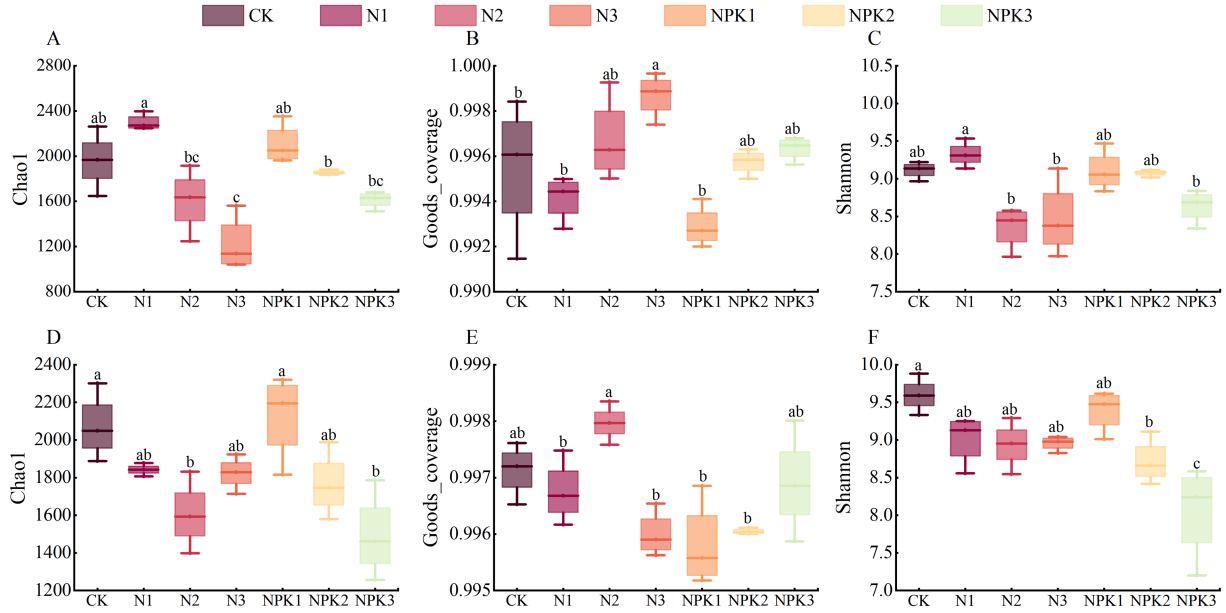


FIGURE 2
The α diversity index of the bacteria in the weathered products under different fertilization treatments. **(A)** Chao1 of the J_2s purple parent rock. **(B)** Goods_coverage index of the J_2s purple parent rock. **(C)** Shannon of J_2s the purple parent rock. **(D)** Chao1 of the J_3p purple parent rock. **(E)** Goods_coverage index of the J_3p purple parent rock. **(F)** Shannon index of the J_3p purple parent rock. Different lowercase letters indicate that there were significant differences among the treatments ($p < 0.05$).

in J_2s, the Chao1 decreased with increasing fertilizer level in the NPK fertilizer treatment. The pattern of change in the Shannon was comparable to that in the Chao1.

Nonmetric multidimensional scaling (NMDS) analysis based on the bray–curtis distance algorithm was conducted to assess the beta diversity of the bacterial community of weathered products. The results revealed distinct differences in the bacterial community among the different fertilizer treatments (Figure 3). The N and NPK fertilizer treatments were distinguished from the CK treatment along NMDS1, whereas the N and NPK treatments were differentiated along NMDS2. Furthermore, clusters representing different fertilizer levels within the same fertilizer types were separated from each other along NMDS1. Overall, the results of PERMANOVA revealed significant differences in community composition among the treatments. Similar patterns were observed for both parent rocks.

Redundancy analysis (RDA) revealed that environmental factors (pH, AP and AN content) played important roles in bacterial community composition of weathered products under the different fertilizer treatments (Figure 4), with AP having the most pronounced effect (Supplementary Table S4).

3.3 Effects of fertilizer application on weathering

The *CIA* revealed that fertilizer application generally promoted chemical weathering of the parent rocks (Figure 5). Specifically, for J_2s, compared with the CK treatment, all the fertilizer treatments significantly increased the *CIA*, with increases ranging from 0.89 to 1.40% ($p < 0.05$). In contrast, compared with J_2s, J_3p presented a lower degree of chemical weathering and a lower sensitivity to fertilizer. Compared with that under the CK treatment, the CIA of the weathered products under the 300% N fertilizer treatment decreased by 0.04%. The *CIA* of the other fertilizer treatments was greater than that of the CK treatment, increasing by 0.13% ~ 0.67%, but there was no significant difference between the treatments ($p > 0.05$). Within the same type of fertilizer, the *CIA* of weathered products tended to decrease with increasing level of fertilizer.

The *SFR* was calculated as an index of the degree of physical weathering (Figure 6), and the *SFR* of J_2s was 6.82% ~ 10.54% under each treatment. Compared with those in the CK treatment, the *SFR* in the N and 200% NPK fertilizer treatments increased by 19.97% ~ 45.78%, with a significant increase under 100 and 200% N fertilizer treatments ($p < 0.05$). However, the *SFR* decreased by 2.15% ~ 5.68% under the 100 and 300% NPK fertilizer treatments, but the differences were not statistically significant ($p > 0.05$).

Compared with the CK treatment, all of the fertilizer treatments (except for the 100% N fertilizer treatment) resulted in an increase in the *SFR*, ranging from 0.71 to 45.24%, in J_3p. The 200 and 300% NPK fertilizer treatments significantly increased the *SFR* ($p < 0.05$). In contrast, the *SFR* of the 100% N fertilizer treatment decreased by 5.71%, but the difference was not statistically significant ($p > 0.05$). Overall, except for the N fertilizer treatments in J_3p, the *SFR* of all the treatments for both parent rocks reached their peak values at the 200% fertilizer level.

Multivariate analysis of variance (MANOVA) was conducted to examine the influence of parent rock type and fertilizer conditions on the weathering indices (Table 2). The results revealed a significant effect of parent rock type on the *CIA* ($p < 0.05$). Both parent rock type and fertilizer type had a significant effect on the *SFR* ($p < 0.05$), and their interaction also had a significant effect ($p < 0.05$).

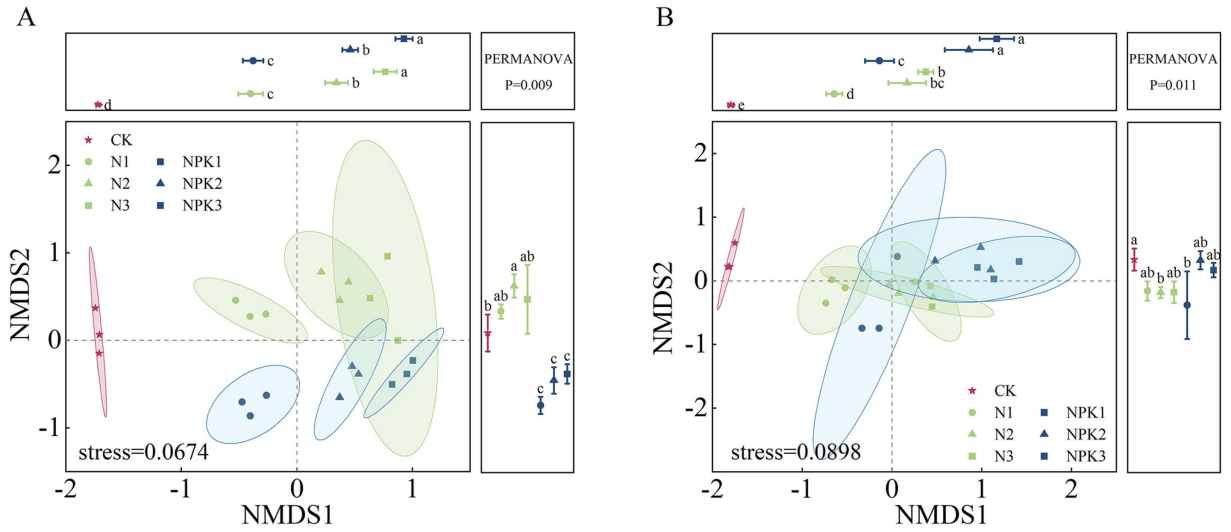


FIGURE 3
Nonmetric multidimensional scales (NMDS) beta diversity analysis of the bacterial community composition in the weathered products under different treatments. **(A)** J_2s purple parent rock. **(B)** J_3p purple parent rock.

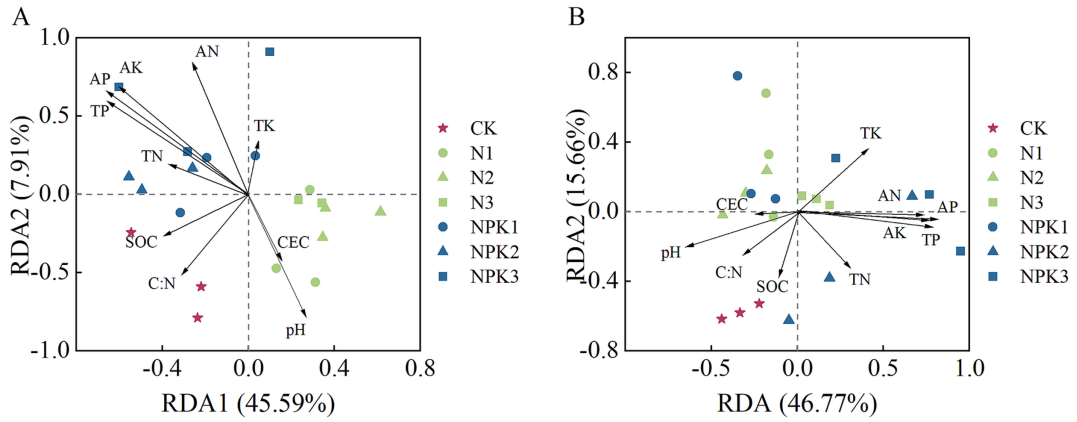


FIGURE 4
Redundancy analysis (RDA) of the effect of different chemical properties of the bacterial community composition in the weathered products under different treatments. **(A)** J_2s purple parent rock. **(B)** J_3p purple parent rock.

3.4 Effects of fertilizer-driven biochemical properties on weathering

Correlation analyses were conducted to investigate the relationships between chemical properties, bacterial diversity indicators, bacterial community composition (BCC), dominant phyla, dominant genera, and weathering indices. In J_2s, the *CIA* was significantly negatively correlated with pH, the SOC content, the C:N, and the relative abundance of Acidobacteriota and IMCC26256, and was significantly positively correlated with the relative abundance of *Nitrosospira*. Moreover, a correlation was observed between the bacterial community composition and the *CIA*. The *SFR* was significantly negatively correlated with the contents of TN and AP and the relative abundances of Actinobacteria and *Flavisolibacter* and significantly positively correlated with the relative abundances of *Sphingomonas*, YC-ZSS-LKJ147, *Ramlibacter*, and *Nitrosospira*. The bacterial community composition was also correlated with the *SFR*. Among the factors affecting J_3p, only the relative abundance of Subgroup_7 was significantly correlated with the *CIA*. The *SFR* was significantly negatively correlated with the CEC and the relative abundances of Proteobacteria and *Sphingomonas* and significantly positively correlated with the contents of TP, AN, AP and AK and the relative abundances of *Blastococcus*, JG30-KF-CM45, LWQ8, *Ramlibacter*, and *Parviterribacter*. Like that in J_2s, bacterial community composition in J_3p was also related to the *SFR* (Figure 7). A comparison of the two weathering indices revealed that physical weathering was more sensitive to each factor than chemical weathering.

4 Discussion

Fertilizer application profoundly influences the chemical properties of weathered products. The pH decreased after fertilizer application (Table 1). This finding is consistent with the results of previous soil-related studies (Guo et al., 2010; Zhang et al., 2024). Nitrate leaching

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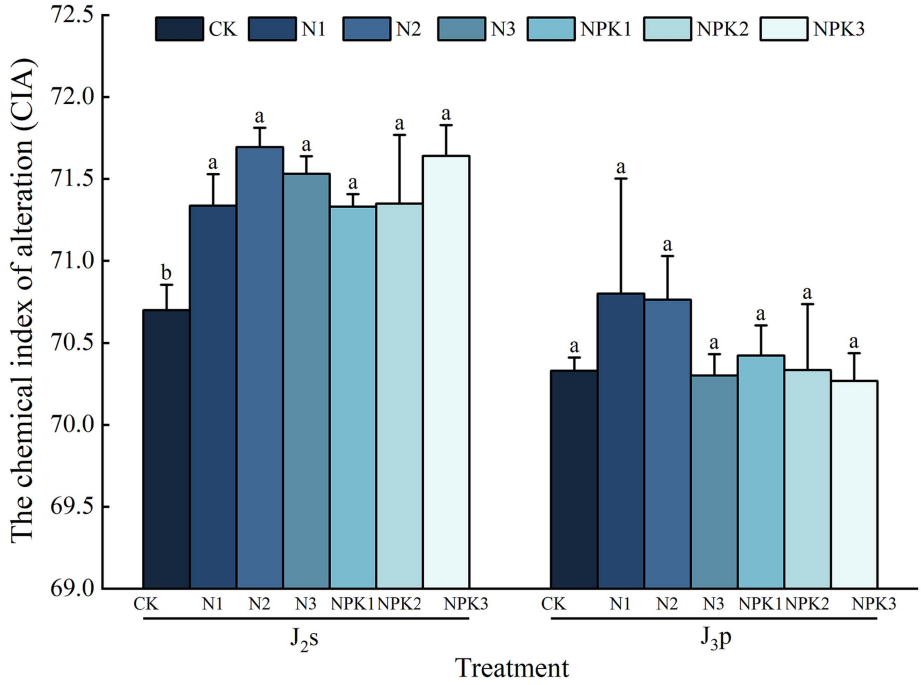


FIGURE 5
Chemical index of alteration (*CIA*) of the weathered products under different treatments. Values are the means with standard deviations shown by vertical bars (n = 3). Different lowercase letters indicate that there were significant differences among all treatments ($p < 0.05$).

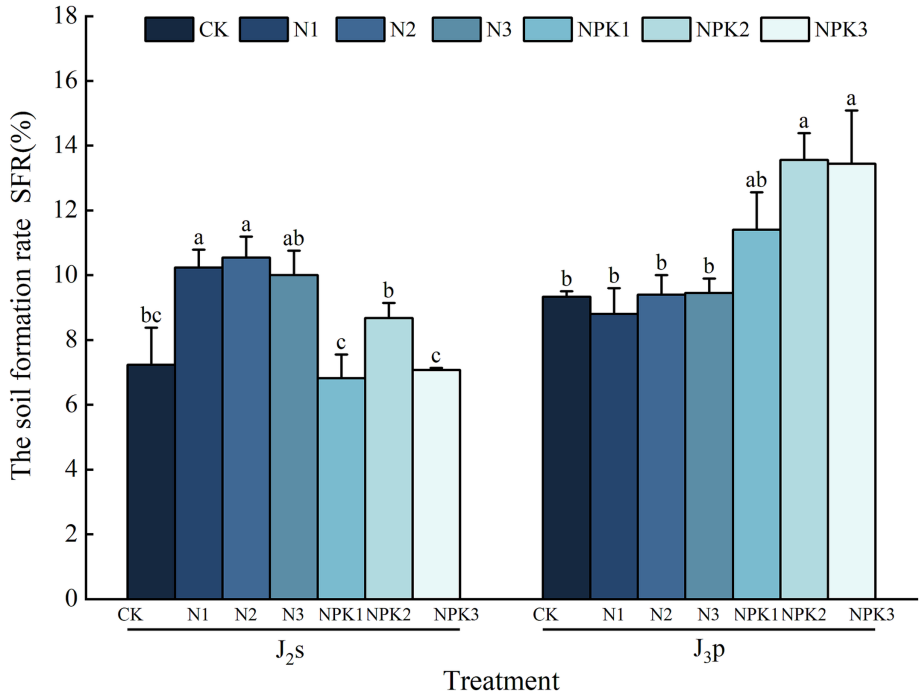


FIGURE 6
The soil formation rate (*SFR*) of the weathered products under different treatments. Values are means with standard deviations shown by vertical bars (n = 3). Different lowercase letters indicate that there were significant differences among all treatments ($p < 0.05$).

TABLE 2 Multivariate analysis of variance (MANOVA) of rock type, fertilizer type and fertilizer level.

Weathering index	Treatment	DF	MS	F	*p*_value
CIA	Rock type	1	8.980	66.208	0.000
	Fertilizer type	1	0.292	2.150	0.156
	Fertilizer level	2	0.031	0.229	0.797
	Rock × fertilizer type	1	0.090	0.664	0.423
	Rock × fertilizer level	2	0.254	1.871	0.176
	Fertilizer type × fertilizer level	2	0.136	1.001	0.382
	Rock × fertilizer type × fertilizer level	2	0.017	0.124	0.884
SFR	Rock type	1	0.004	33.080	0.000
	Fertilizer type	1	0.000	3.743	0.041
	Fertilizer level	2	0.000	1.335	0.261
	Rock type × fertilizer type	1	0.008	73.714	0.000
	Rock type × fertilizer level	2	0.000	1.214	0.317
	Fertilizer type × fertilizer level	2	0.000	1.502	0.246
	Rock × fertilizer type × fertilizer level	2	0.000	0.166	0.848

caused by fertilizer application accounted for 59% ~ 66% of the total acidity in semiarid regions of the U.S.A (Tarkalson et al., 2006). The pH reduction is attributed primarily to the production of protons during the process of ammonium oxidation to nitrite and further to nitrate. The loss of nitrate removes alkaline ions such as calcium and magnesium, consequently causing soil acidification (Raza et al., 2020). Notably, the pH decrease in J_3p was not significant compared with that in J_2s because of its higher content of $CaCO_3$, which acts as a neutralizing agent (Zhao et al., 2022). Compared with those in the CK treatment, some nutrients significantly increased under the fertilizer treatments, which resulted from the exogenous nutrient inputs from fertilizer application. However, except for TP (under the NPK fertilizer treatments), the contents of the total nutrients did not significantly increase, and even the SOC content was lower than that in the CK treatment. Available nutrients can be adsorbed by clay minerals, such as illite, which adsorbs ammonium nitrogen, and with time, its lattice ion exchange stabilizes available nitrogen into total nitrogen (Heleen et al., 2021). In this study, we used rock as the material, as the specific surface area and clay mineral content are much lower than those of soil, making it difficult to fix the available nutrients, and there were no plant-mediated nutrients, so the total nutrients cannot be increased. In addition, the sole application of chemical fertilizers leads to a decline in the content of SOC, due to its inability to provide organic carbon sources and its ability to alter the composition and activity of the microbial community (Demoling et al., 2007). Specifically, the application of nitrogen fertilizer alone intensifies the activities of ammonia-oxidizing bacteria and nitrifying bacteria (Han et al., 2023), and these microorganisms consume SOC during their metabolic procedures, thus reducing the content of SOC and the C:N (Zhong et al., 2016; Mori et al., 2018).

The characteristics of the bacterial community of weathered products also changed concomitantly with the changes in chemical properties caused by fertilizer application. Consistent with previous research, the bacterial abundance and diversity of weathered products significantly decreased with increasing levels of fertilizer (Yuan et al., 2013; Burns et al., 2015; Zeng et al., 2016; Yu et al., 2019). Correlation analysis revealed that pH, contents of TP and available nutrients play pivotal roles in shaping bacterial diversity (Figure 7), which is consistent with the findings of previous studies (Zhang et al., 2017; Cheng et al., 2020). The bacterial diversity exhibited a consistent association with the pH gradient, which can be attributed to the narrower pH optima of specific bacteria (Lagos et al., 2016; Rousk et al., 2010). Moreover, the increased availability of nutrients resulting from fertilizer stimulated bacterial growth and contributed to increased bacterial diversity (Cheng et al., 2020). However, the low SOC content resulting from high-level fertilizer constrains bacterial growth (Demoling et al., 2007).

According to NMDS (Figure 3), differences in the composition of bacterial communities have also been observed under different fertilizer treatments, which has been confirmed in previous studies (Yuan et al., 2013; Leff et al., 2015; Cheng et al., 2020). The dominant phyla with relative abundances greater than 1% for all samples were Proteobacteria, Actinobacteriota, Gemmatimonadota, Bacteroidota, Chloroflexi, and Acidobacteriota. The relative abundance of these dominant phyla varied under the different fertilizer treatments (Figure 1). Fertilization-induced changes in bacterial community composition can be explained by the co-nutrient hypothesis: nutrient enrichment favors copiotrophic bacteria colonization, while oligotrophic ones may be unaffected or negatively affected (Dai et al., 2017; Fierer et al., 2007). An examination of the relative abundances of the dominant phyla (Figure 8) revealed that Gemmatimonadota and Bacteroidota (copiotrophic groups) generally tended to increase under fertilizer application. Conversely, Actinobacteriota and Acidobacteriota (oligotrophic groups) displayed an overall decreasing trend under fertilizer application, whereas the relative abundance of Chloroflexi (oligotrophic group) did not significantly differ among the treatments. Surprisingly, the relative abundance of Proteobacteria (copiotrophic group) decreased under fertilizer application, particularly in response to NPK fertilizer treatments. Proteobacteria are known to thrive in nutrient-rich environments, primarily consuming labile organic matter (Demoling et al., 2007). Therefore, despite the significant increase in nutrient availability resulting from fertilizer application, the concurrent decrease in the SOC content renders the environmental conditions unfavorable for the growth and reproduction of Proteobacteria. This finding suggested that, in addition to nutrient preferences, different bacteria have distinct limiting conditions for each nutrient.

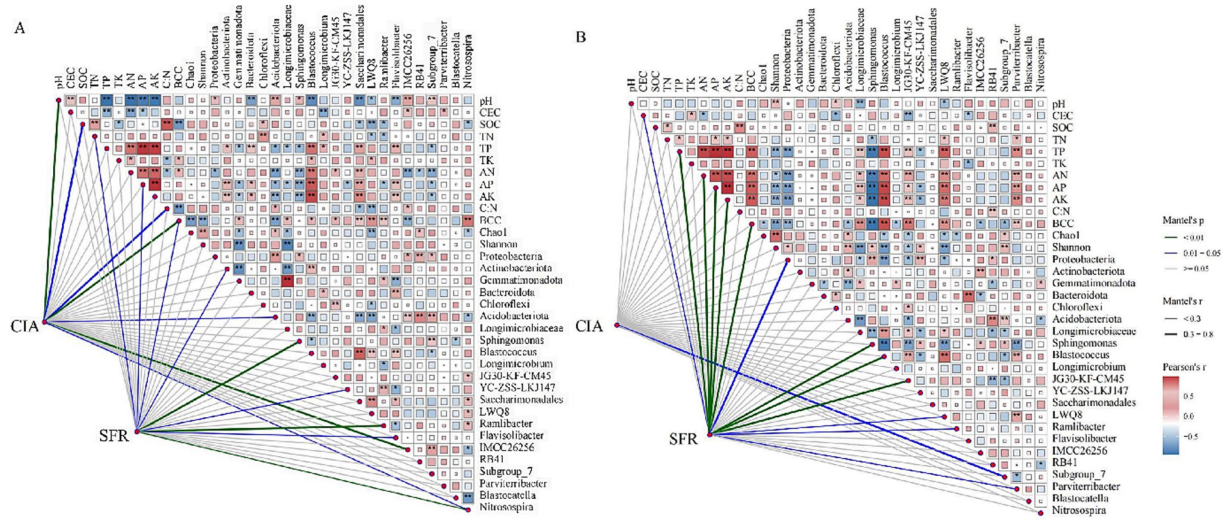


FIGURE 7
Correlations among weathering evaluation indices (the chemical index of alteration and the soil formation rate), chemical properties, dominant phyla, dominant genera, bacterial abundance, diversity and community composition. **(A)** J_2s purple parent rock. **(B)** J_3p purple parent rock. * and ** indicate significances at $p < 0.05$ and $p < 0.01$.

Redundancy analysis (RDA) of the dominant genera (Supplementary Table S4) revealed that the contents of AP and AN and the pH of the weathered products were critical factors influencing bacterial growth under fertilization. Notably, the AP content had a stronger effect on the bacterial community composition than did the other chemical factors. These findings suggest that fertilizer application mitigates bacterial phosphorus limitation and influences bacterial community composition by increasing phosphorus effectiveness (Liu et al., 2020). This finding was further validated via SEM (Figure 9).

These alterations in biochemical properties that are consequent to fertilizer application affect the weathering processes and velocity of purple parent rocks through multiple pathways. Compared with the CK treatment, fertilizer application stimulated chemical weathering in J_2s (Figure 5). The *CIA* of J_2s was correlated with pH, SOC content, C:N, bacterial community composition, and specific bacteria (Figure 7). SEM revealed that the pH, C:N, AP content, relative abundance of IMCC26256, and relative abundance of *Ramlibacter* were key factors influencing the chemical weathering of J_2s (Figure 9). Specifically, the pH and C:N of weathered products under different fertilizer conditions were the most important drivers that interactively controlled chemical weathering through the influence of the relative abundance of IMCC26256. IMCC26256 is significantly and positively correlated with the N_2O index (Sui et al., 2020), indicating the involvement of IMCC26256 in the nitrogen cycling process of weathered products, which is proven to be correlated with weathering processes. Additionally, AP regulated J_2s chemical weathering through *Ramlibacter*. *Ramlibacter* possesses genes associated with the phosphorus scavenging pathway, which are involved in high-affinity phosphate acquisition and storage of polyphosphate particles and play crucial roles in residual phosphorus mineralization (Props et al., 2019; Zhang et al., 2021). The phosphorus solubilization process can facilitate the release of Ca and Mg, thereby impacting mineral weathering (Li et al., 2023). The input of AP resulting from fertilizer application disrupts the P balance regulated by *Ramlibacter* and inhibits the growth and reproduction of *Ramlibacter*, consequently affecting the weathering process.

Fertilizer application enhanced the physical weathering of J_2s, except for the NPK fertilizer treatments at the 100 and 200% levels (Figure 6). Correlation analysis revealed that the contents of TN and AP, the bacterial community composition, and specific bacteria were correlated with the *SFR* (Figure 7). SEM revealed that the level of fertilizer had a positive effect on *Nitrosospira*, which plays a role in physical weathering processes (Figure 9). *Nitrosospira* belongs to the group of ammonia-oxidizing bacteria (AOB), and its ammonia oxidation activity represents the rate-limiting step in nitrification (Huang et al., 2023). Previous studies have demonstrated that the H^+ produced during nitrification under fertilization undergoes ion exchange reactions with minerals within rock crystals, leading to cavity enlargement, pore unclogging, and enhanced rock disintegration and destruction (Li et al., 2021; Song et al., 2017a). Furthermore, fertilizer type impacts the AP content, significantly influencing the physical weathering of J_2s. Similar to the mediation mechanism by which *Ramlibacter* mediates *CIA* through AP, the process of P dissolution promotes mineral weathering. However, the introduction of excessive amounts of AP can inhibit this process. This observation helps explain the disparity in the degree of physical weathering between the two different types of fertilization.

Compared with that of J_2s, the chemical weathering of J_3p was not sensitive to fertilizer application. Under the same fertilizer type, chemical weathering degree of J_3p tended to decrease with increasing fertilizer level. This finding aligns precisely with the results derived from the incubation experiments focused on the influence of nitrogen fertilizer on weathering (Li et al., 2023). The *CIA* was not correlated with any of the factors except the relative abundance of Subgroup_7. According to the SEM (Figure 9), an effective pathway to influence chemical weathering was not formed by the given factors, which may have occurred because the influence of fertilization on chemical weathering factors was much lower than the influences of external environmental factors, such as rainfall and temperature. These changes did not reach the threshold for causing changes in chemical weathering.

In contrast, the NPK fertilization treatments significantly enhanced the physical weathering of J_3p (Figure 6). Correlation

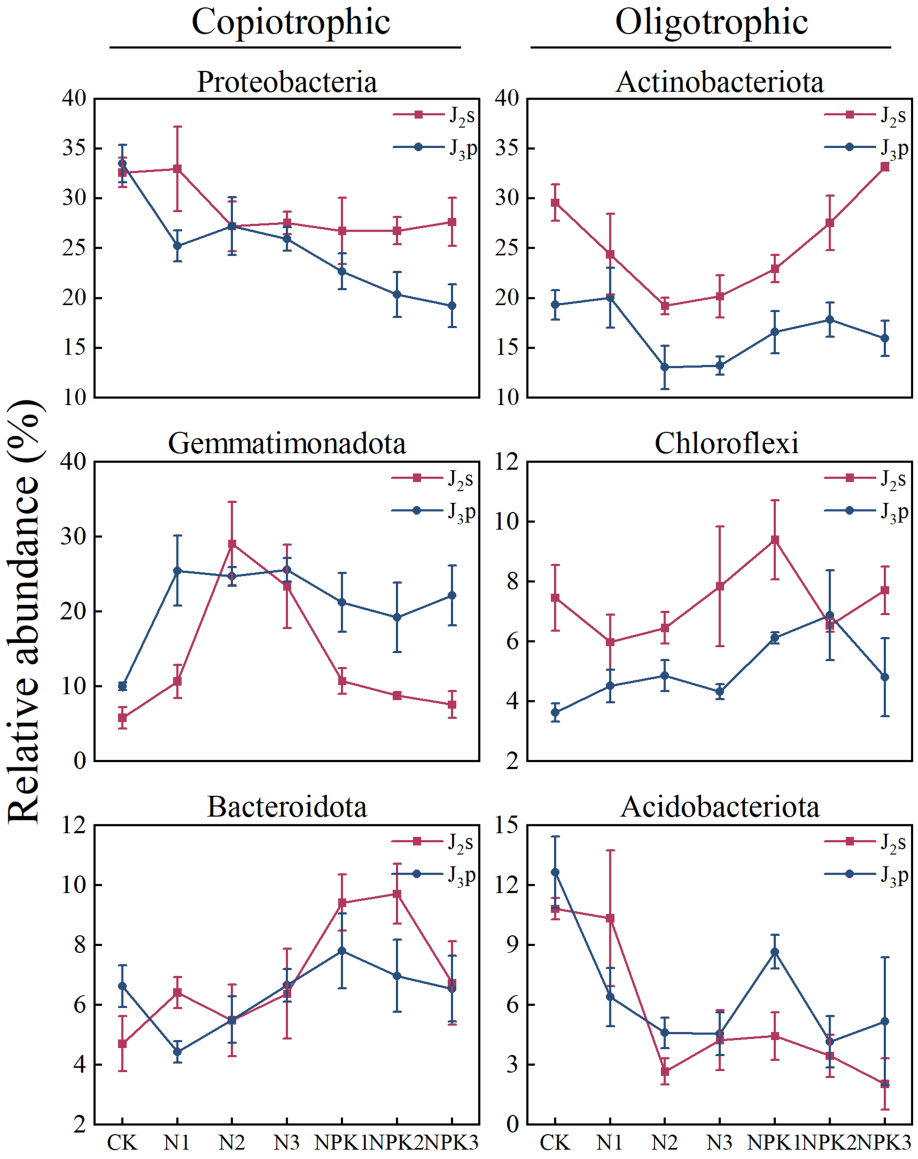


FIGURE 8
The relative abundance of bacteria classified into copiotrophoc and oligotrophic groups at the phylum level under different treatments.

analysis revealed that the *SFR* of J_3p was associated with the CEC, the contents of TP and available nutrients, the bacterial community composition, and the abundances of specific bacteria (Figure 7). SEM revealed that the impact of fertilizer application on the physical weathering of J_3p can be divided into two pathways (Figure 9). First, the level of fertilizer had a negative effect on the pH, directly influencing physical weathering. Previous studies have demonstrated that H^+ input accelerates weathering. However, J_3p exhibited the opposite trend due to its high pH value and the neutralizing effect of its large amount of $CaCO_3$, which resulted in minimal influence of fertilizer application on the pH. Consequently, although the SEM indicated a positive feedback effect on physical weathering, the actual path coefficient was small. Second, fertilizer application indirectly affected the bacterial community composition through the AP and directly affected the bacterial community composition through the fertilization level, thereby influencing physical weathering in J_3p. RDA revealed that AP was the primary driver of bacterial community composition Multiple studies have shown that nutrient inputs and associated environmental changes can alter bacterial community composition (Leff et al., 2015; Cheng et al., 2020; Zi et al., 2023). Recent findings from functional gene screening studies further support these results, indicating that the distribution and function of weathering-associated bacteria are strongly influenced by external factors such as pH, nutrient effectiveness, and carbon and nitrogen sources (Chen et al., 2022; Epihov et al., 2021). These findings align with the results obtained in this study.

In addition, the intrinsic properties of the parent rock (mineral composition and resistance to weathering) are major factors in the selection bias of weathering bacteria. For example, *Burkholderia*, which has an excess of negatively charged surface groups and phosphate groups, is more inclined to adsorb to orthoclase, which has a less negatively charged surface (Uroz et al., 2015). This explains the

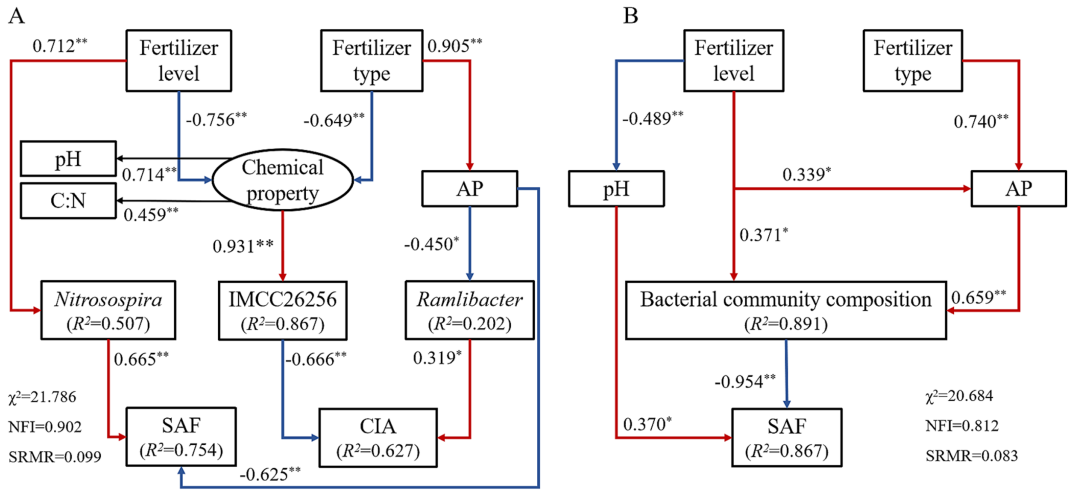


FIGURE 9
Structure equation models (SEMs) of the relationships between the chemical properties, relative abundance of dominant genus, bacterial abundance, diversity and community composition. **(A)** J_2s purple parent rock. **(B)** J_3p purple parent rock. Lines in red and blue color with arrows indicate significant positive and negative paths, respectively. Numbers on the lines indicate the standard path coefficients, * and ** indicate significances at $p < 0.05$ and $p < 0.01$. R^2 values represent the proportions of variance explained by relationships with other variables.

difference in bacterial weathering between the two parent rock types under fertilizer application.

5 Conclusion

Fertilizer application altered the bacterial community, with increasing fertilizer levels leading to decreased bacterial diversity and abundance and changes in community composition of weathered products. The two types of parent rock have different weathering characteristics and sensitivities to fertilizer types. J_2s manifested more potent chemical weathering, whereas J_3p exhibited relatively preponderant physical weathering. Overall, fertilizer application enhanced weathering of the purple parent rock.

Through correlation analysis and structural equation models, the mechanisms of fertilization impacting the weathering of purple parent rocks were revealed: (1) Fertilizer application exerts indirect effects on physicochemical weathering through the modulation of chemical properties (pH, C:N and AP content), which are mediated by specific bacteria (IMCC26256 and *Ramlibacter*), and the overall bacterial community composition of weathered products. (2) Fertilizer application directly influences physical weathering by directly modifying chemical properties (pH and AP content). (3) Fertilizer application directly impacts physical weathering by altering the relative abundance of specific bacteria (*Nitrosospira*).

This study explored the effects of fertilizer application on bacteria and weathering. However, in reality, the influence of plants on rock weathering under fertilization cannot be ignored. Moreover, plants and microorganisms' metabolic activities interact. Future studies should comprehensively consider their combined effect on rock weathering to dissect fertilization's impact. Consequently, it can provide a novel approach for the research on the sustainable development, carbon cycling, and climate regulation of agricultural areas.

Data availability statement

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

Author contributions

XW: Conceptualization, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing. JZ: Funding acquisition, Methodology, Writing – review & editing. CL: Formal analysis, Validation, Writing – review & editing. LD: Validation, Writing – review & editing. RC: Investigation, Writing – review & editing. TZ: Resources, Writing – review & editing. ZH: Writing – review & editing. GL: Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Supplementary material

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Metagenomic analysis revealed the bioremediation mechanism of lead and cadmium contamination by modified biochar synergized with *Bacillus cereus* PSB-2 in phosphate mining wasteland

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Introduction: Phosphate mining wasteland is contaminated with heavy metals, such as lead (Pb) and cadmium (Cd), which pose significant environmental risks. Ecological restoration of these lands is crucial, but limited research has focused on the remediation of heavy metal-contaminated soils using modified biochar and functional microorganisms.

Methods: In this study, we investigated the bioremediation of phosphate mining wasteland soil using modified biochar in combination with the phosphate-solubilizing bacterium *Bacillus cereus*. The effects of this synergistic approach on soil nutrient content, heavy metal immobilization, and microbial community structure were assessed.

Results and discussion: The results indicated that the available phosphate content in the soil increased by 59.32%. The content of extractable state Pb^{2+} and Cd^{2+} decreased by 65.06 and 71.26%, respectively. And the soil nutrient conditions were significantly improved. Synergistic remediation can significantly increase the diversity and abundance of soil microbial communities ($p < 0.05$). *Janibacter*, *Lysobacter*, *Ornithinimicrobium*, *Bacillus*, and *Salinimicrobium* were the main functional flora during soil remediation, with significant correlations for the promotion of Pb^{2+} and Cd^{2+} immobilization and the increase of available phosphate and organic matter. *ZitB*, *czcD*, *zntA*, and *cmtR* are the major heavy metal resistance genes and regulate metabolic pathways to make microbial community function more stable after soil remediation in phosphate mining wasteland.

KEYWORDS

phosphate mining wasteland, heavy metals, modified biochar, phosphate solubilizing bacteria, bioremediation

1 Introduction

Mining is a critical activity in mineral processing, energy development, metallurgical engineering, etc., which is essential for the strategic development of humankind (Chen J. et al., 2020; Sonter et al., 2020). Extensive phosphate mining activities form a large amount of solid waste, leading to the formation of more and more phosphate mining wasteland (Guo et al., 2021). Phosphate mining wasteland not only wastes valuable soil resources but also poses serious environmental challenges (Sun et al., 2018; Yuan et al., 2019). Among the contaminants, cadmium and lead have attracted considerable attention due to their high concentrations, persistence, and complex remediation processes (Peng et al., 2021). Both cadmium and lead can accumulate in the human body through the food chain as a result of long-term rainwater erosion, leaching, and diffusion into the soil (Qu et al., 2024; Qiu et al., 2021). Lead is a highly toxic neurotoxin that can cause severe health issues, including cancer and anemia, upon entry into the body (Yu et al., 2021; Marshall et al., 2020). Excessive cadmium exposure leads to accumulation in the liver and kidneys, resulting in hepatic damage and renal insufficiency (Howard et al., 2024). Consequently, there is an urgent need for efficient and environmentally friendly technologies to address the dual challenges of lead and cadmium contamination in soil. Such solutions are essential to safeguard human health and promote sustainable land use practices.

Various remediation technologies, such as soil leaching and phytoremediation, have been employed to remove heavy metals from contaminated soils (Tu et al., 2020). However, these methods have notable drawbacks, including high costs, the potential for secondary pollution, and lengthy remediation times, which limit their applicability (Cheng et al., 2024; Deng et al., 2024). Therefore, identifying efficient, cost-effective, and environmentally friendly remediation strategies is essential for addressing heavy metal pollution in mining areas. In recent years, microbial remediation has emerged as a promising approach due to its environmental benefits and lower costs (Che et al., 2024). Phosphorus-solubilizing bacteria (PSB), one of the most widely utilized functional strains, play a crucial role in mitigating heavy metal contamination (Zhao et al., 2023). Furthermore, these bacteria are capable of transforming insoluble phosphates derived from phosphate mining by-products into forms that are readily accessible for biological uptake through the secretion of organic acids (Chen et al., 2023a; Chen et al., 2023b). However, high heavy metal concentrations, such as lead and cadmium, can severely affect microbial cells, causing cell death through mechanisms like membrane disruption and DNA damage (Chen et al., 2023b; Shao et al., 2019; Jiang et al., 2020). Furthermore, the severe pollution and poor nutrient conditions prevalent in phosphate mining wasteland can weaken the remediation capacity of PSB and hinder their long-term colonization (Qi et al., 2023). Consequently, when faced with high concentrations of toxic cadmium and lead contamination from phosphate mining wasteland sites, microbial survival and reproduction often depend on complementary protective technologies.

Biochar, a carbon-rich material produced from organic waste through pyrolysis under limited oxygen, is a promising soil amendment that enhances carbon sequestration and soil fertility (Sun et al., 2020; Zhang et al., 2021). Its extensive surface area and porous structure make it an ideal habitat for microorganisms, enhancing microbial colonization (Singh et al., 2022; Luo et al., 2022). Studies have demonstrated that the combined application of biochar and PSB can address nutrient deficiencies and improve remediation efficiency (Lin et al., 2023). For instance, Qi et al. (2021) showed that bacterial-carrying biochar effectively immobilized uranium and cadmium, improving soil properties and microbial activity. Similarly, Chen et al. (2023b) found that swine manure biochar helped PSB better manage heavy metal stress, leading to higher removal rates of lead and cadmium compared to PSB alone. Despite these benefits, separating and recycling biochar after remediation remains a challenge (Reguyal et al., 2017). Magnetisation may be a good solution, as magnetizing biochar with iron oxides may not only effectively overcome its drawbacks but also improve its ability to remove pollutants (Yi et al., 2019). For example, Wang et al. (2015) found that the maximum adsorption capacity of magnetic biochar for arsenic was 428.7 mg/kg, approximately twice that of Biochar. Duan et al. (2022) achieved the recovery (100%) of iron-based modified biochar from soil and a 5.4% removal of Pb using dry magnetic separation. Therefore, the combination of biochar and heavy-metal-resistant phosphate-solubilizing bacteria can not only significantly enhance the remediation efficiency of heavy metal pollution in phosphate mining wasteland but also facilitate the recycling of biochar, offering an innovative and effective strategy for the treatment of contaminated sites.

In this study, a novel biochar-based adsorbent, BC-1, was developed using corn cob biochar (BC-0) as the raw material by combining phosphate-solubilizing bacteria (PSB) with the loading of Fe3O4. A comprehensive assessment was conducted on the combined remediation effect of BC-1 and heavy-metal-resistant phosphate-solubilizing bacterium PSB-2 (*Bacillus cereus*) on lead- and cadmium-contaminated soils in phosphate mining wasteland. The focus was on key parameters such as the immobilization of heavy metals, enhancement of soil nutrient profiles, and alterations in microbial community composition, gene expression, and functional potential. These aspects were further explored through metagenomic sequencing to provide a detailed understanding of the underlying microbial dynamics. The aim of this study was to provide a theoretical basis and technical support for the bioremediation of heavy metal pollution in phosphate mining wasteland.

2 Materials and methods

2.1 Soil, biochar, and PSB

The experimental soil was extracted from a phosphate mining wasteland in Yichang City, Hubei Province (111°1056″–111°1217′ E, 31°1730″- 31°20 00″ N). It was air-dried, ground, and passed through a 2-mm sieve. The soil was then stabilized in a ventilated dry place for two weeks to assess its physicochemical properties for subsequent experiments. Soil physicochemical properties were

TABLE 1 Physicochemical properties of soil and biochar before and after modification.

Property	Soil	BC-0	BC-1
pH	7.79 ± 0.02	8.69 ± 0.26	9.03 ± 0.14
Carbon content (%)	–	55.23 ± 1.37	39.54 ± 1.24
Fe (%)	–	0.12 ± 0.02	22.78 ± 0.76
Specific surface area (m^2/g)	–	9.25 ± 0.65	54.32 ± 1.27
Total phosphate (g/kg)	24.70 ± 0.59	2.38 ± 0.13	4.95 ± 0.46
Available phosphate (mg/kg)	220.19 ± 19.62	17.41 ± 0.17	8.79 ± 0.39
Available Pb (g/kg)	110.97 ± 3.96	0.06 ± 0.01	0.03 ± 0.01
Available Cd (g/kg)	34.58 ± 0.21	0.03 ± 0.01	0.02 ± 0.01
Organic matter (g/kg)	22.13 ± 2.36	48.63 ± 2.41	39.29 ± 1.86
Cation exchange capacity (c mol/kg)	9.17 ± 0.06	35.65 ± 2.68	58.65 ± 1.63

"BC-0" refers to pretreated unmodified biochar, and "BC-1" refers to the composite modified biochar loaded with Fe_3O_4 after microbiological modification.

determined after stabilization and the results are shown in Table 1. The corn cob biochar was prepared by holding at 500°C for 2.5 h under oxygen-limited conditions (Sha et al., 2023). The strain PSB-2, which was isolated and screened, has good phosphate solubilizing capacity as well as heavy metal Pb^{2+} and Cd^{2+} tolerance (Supplementary Figure 1). The strain had high homology (100%) with *Bacillus cereus*, with the accession number CP050183.1. PSB-2 was inoculated in sterilized LB medium (tryptic protein 10.0 g/L, sodium chloride 10.0 g/L, yeast infusion powder 5.0 g/L, and pH 7.0), activated and cultured to logarithmic growth stage (Chen and Achal, 2019).

2.2 Preparation and characterization of modified biochar

After grinding and sieving, the corn cob biochar was washed with 0.1 mol/L HNO_3 solution to remove ash from the pore structure. It was washed again with deionized water, then filtered and dried to get pretreated unmodified biochar (BC-0). Pretreated corn cob biochar BC-0 was incorporated into *Bacillus cereus* PSB-2 liquid LB medium cultured to logarithmic phase (biochar: medium = 1 g: 5 mL). It was then incubated at 30°C and 180 *r*/min for 48–72 h, filtered, washed, dried and ground. Microbiologically modified corn cob biochar was compositely modified by co-precipitation method loaded with Fe_3O_4 (Wang et al., 2021). Finally, it was filtered and washed with deionization, dried and milled to produce the composite modified biochar BC-1.

The surface morphology of BC-0 and BC-1 was examined using a field emission scanning electron microscope (Gemini SEM 300, Zeiss, Germany). Functional group characterization of the biochar before and after modification was performed using a Fourier-transform infrared spectrometer (NICOLET 6700, Thermo Fisher, USA). Additionally, lattice characteristics were analyzed using an X-ray diffractometer (D8 ADVANCE, Bruker, Germany).

2.3 Exploration of Pb^{2+} and Cd^{2+} adsorption in solution

Take 50 mL of mixed solution containing 200 mg/L Pb^{2+} and 50 mg/L Cd^{2+}, add 0.10 g of the modified biochar BC-1 to the mixed solution, and oscillate at room temperature for 24 h to reach adsorption equilibrium. Then, the adsorbent that completed the adsorption was recovered with filter separation method, dried and ground. BC-1 before and after adsorption was subjected to X-ray photoelectron spectroscopy (XPS; ESCALAB XI+, Thermo Fisher Scientific, USA) to study the changes in elemental and bonding energies before and after adsorption on the adsorbent, as well as the adsorption mechanism. Besides, the recovered adsorbent particles were subjected to adsorbent desorption with 0.05 mol/L HCl solution and separated by filtration. The desorbed BC-1 was washed with deionized water, dried, ground and sieved. The adsorption-desorption operation was repeated for five cycles. The sorbent after five cycles of adsorption was then magnetically performed with a vibrating sample magnetometer (VSM; 8604, Lake Shore, USA) to analyze its magnetic stability.

2.4 Soil remediation experiments

Stabilized contaminated soil was divided into 11 cm pots with 500 g of soil per portion, and the concentration of adsorbent material or PSB-2 added to the soil uniformly was set at 2%, which was determined based on previous studies and further confirmed through preliminary experiments (Lahori et al., 2024; Li et al., 2023). Different treatments were set up, control (CK), PSB-2 (M), unmodified biochar (BC-0), modified biochar (BC-1), and PSB-2/modified biochar composite (MBC-1), with three parallel replications for each treatment. The soil remediation experiments were conducted by placing the soil in a naturally ventilated area at room temperature. After microbial colonization, the soil was replenished with distilled water every 5 days, maintaining the soil at 40% humidity. And 15 g soil sample was taken periodically from each experimental pot, dried, ground, and passed through a sieve for physicochemical determination. Continuous soil culture experiments were conducted for 55 days.

2.5 Determination of soil properties and heavy metal concentrations

The pH, total phosphate (TP), available phosphate (AP), extractable state Pb^{2+} and Cd^{2+} as well as soil organic matter (OM) and soil cation exchange capacity (CEC) of the soil samples were determined during the remediation process. The pH was measured using a pH meter (Zhang et al., 2020). TP was measured by ultraviolet spectrophotometry (UV-3600, Shimadzu, Japan) after digestion of soil samples with concentrated sulfuric acid and potassium persulfate, respectively (Afzal et al., 2020). AP was determined using ammonium vanadium molybdate colorimetric method after extraction with $NaHCO_3$ (0.5 mol/L, pH 8.5) solution (Teng et al., 2019). Extractable state Pb^{2+} and Cd^{2+} were extracted by diethylenetriaminepentaacetic acid (DTPA)

method (Tu et al., 2020). Then, they were determined using a flame atomic absorption spectrophotometer (ICE-3500, Thermo Fisher Scientific, Massachusetts, USA). OM and CEC were also determined for the remediated soil (Qi et al., 2021).

2.6 Soil macro-genomics analysis

After the completion of soil remediation at the phosphate mining wasteland, 10 g of fresh soil samples from each of the CK, M and MBC-1 were taken to extract microbial whole DNA from the soil using a soil DNA kit. Compared and analyzed in the cloud platform of Shanghai Major-bio Technology Co. The NR database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were used for species, functional and genetic correlation analysis (Buchfink et al., 2015).

2.7 Data analysis

Phylogenetic analysis of the PSB-2 strain was conducted using the MEGA11 software to construct phylogenetic relationships. X-ray photoelectron spectroscopy (XPS) data were calibrated and peak fitting was performed using Avantage software (Thermo Scientific^{TM}). X-ray diffraction (XRD) data were analyzed with JADE 6.0. Each experimental set was repeated three times, and data were expressed as mean values with standard deviations. One-way ANOVA, utilizing Tukey's method, was employed to assess the significance of differences in the data, using SPSS 26.0 for statistical analysis. All experimental data were visualized using Origin software.

3 Results and discussion

3.1 Physicochemical properties and characterization of modified biochar

The physicochemical properties of corn cob biochar before and after modification are shown in Table 1. After microbial modification and Fe_3O_4 loading modification, the pH of BC-1 was slightly increased to 9.03. The significant reduction of elemental C content and available phosphate content in biochar may be attributed to the growth and metabolism of phosphate solubilizing microorganisms, which played the role of microbial modification and released part of the available phosphate in the biochar (Chen et al., 2019). The Fe content in the modified biochar was significantly higher, accounting for 22.78%, indicating successful loading of Fe_3O_4. The larger the specific surface area, the better the adsorption effect of biochar (Zhao et al., 2020). The specific surface area of modified BC-1 was increased from 9.25 to 54.32 m^2/g, which is a 5.87-times increase in specific surface area. Biochar has high organic matter content and cation exchange before and after modification, and can act as a soil conditioner, which is important for improving soil nutrient conditions.

The scanning electron microscopy (SEM) results, illustrated in Figures 1A, B, unveil the intricate porous structure of biochar. This distinctive morphology not only provides convenient channels for the efficient transport of substances but also serves as a protective microenvironment that safeguards the reproductive processes of microorganisms, as previously highlighted by Quilliam et al. (2013). Moreover, Jiang et al. (2014) demonstrated that the high porosity of biochar enables robust interactions with metal ions through functional groups such as carbonyl, carboxyl, and hydroxyl groups, thereby enhancing its adsorptive capacity. Following microbial and loading modifications, a significant accumulation of fine particles was observed on the surface and within the pore structure of BC-1. This finding confirms the successful incorporation of Fe3O4 nanoparticles onto the modified biochar, which induces a notably rougher surface texture compared to its unmodified counterpart. This enhanced surface roughness significantly increases the number of available adsorption sites, thereby markedly improving the immobilization efficiency of Pb (II) and Cd (II) ions.

The functional group structure of biochar is closely related to its adsorption capacity, and FTIR was always used to qualitatively identify the characteristic functional groups of biochar materials (Yang et al., 2021). As shown in Figure 1C, the characteristic peak at 3,440 cm^{-1} corresponds to the -OH functional group, and the characteristic peak at 1,640 cm^{-1} corresponds to the C = O (Xiao et al., 2019; Sha et al., 2023). Other characteristic peaks near 1,440, 1,030 and 880 cm^{-1} correspond to C = C, C-O and C-H, respectively (Dai et al., 2020; Qi et al., 2021). The change in peak area after biochar modification proves that the increase in the type and number of functional groups can provide sufficient adsorption sites for heavy metal pollutants. A new characteristic peak appeared in BC-1 at 577 cm^{-1}, which is attributed to the Fe-O and it is characteristic of Fe_3O_4 (Dong et al., 2018; Omer et al., 2023). In the XRD analysis results (Figure 1D), the diffraction peaks of BC-1 at 2θ = 35.4° (311), 43.5° (400), 57.1° (511) and 63.1° (440) are also attributed to Fe_3O_4 (Omer et al., 2023; Wang et al., 2016). The characterization results are all sufficient evidence of successful Fe_3O_4 loading in composite modified biochar.

3.2 Solution adsorption probing

To further investigate the adsorption effect and mechanism of BC-1 on Pb^{2+} and Cd^{2+}, the changes in the binding energy of BC-1 before and after adsorption were analyzed with XPS, and the magnetic stability of BC-1 before and after adsorption were investigated with VSM (Figure 2). In the full spectrum (Figure 2A), the major elements of modified biochar BC-1 before adsorption were C, N, O and Fe. After the adsorption experiments in solution, there were obviously more elemental absorption peaks of Pb and Cd in the spectrum, which proved that the modified biochar successfully adsorbed the heavy metals Pb^{2+} and Cd^{2+} in solution. As shown in Figures 2B, C, after adsorption, the binding energy of BC-1 at 139.07 eV corresponds to the Pb 4f7 orbital and at 144.02 eV is attributed to the Pb 4f5 orbital, respectively. The binding energies at 405.08 eV and 411.18 eV are attributed to the Cd 3d5 and Cd 3d3 orbitals, respectively.

In the C1s spectrum (Figure 2D), the peaks appearing at 284.80, 285.50, 286.90, and 289.20 eV in the BC-1 sample before adsorption represent C-C, C-O, C = C, and -O-C = O bonds,

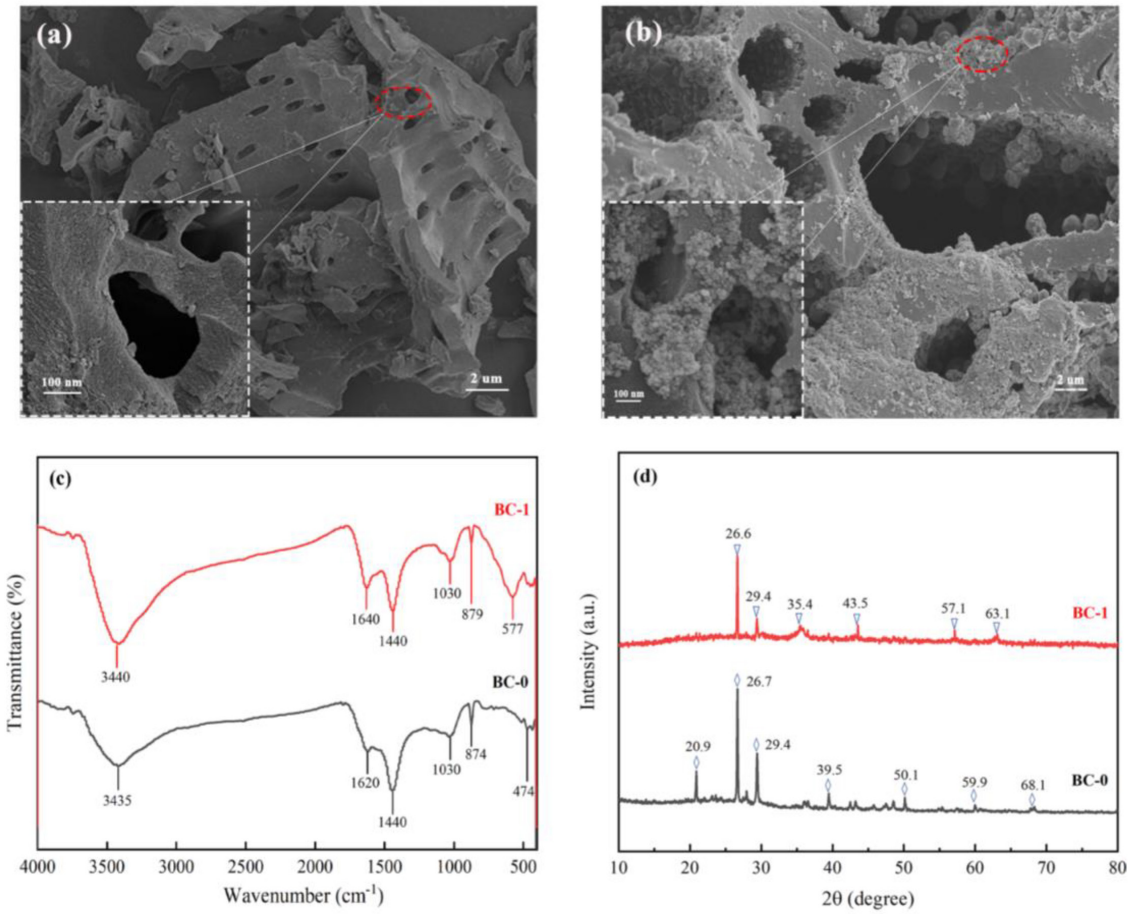


FIGURE 1
Characterization results of biochar before and after modification. SEM results of BC-0 **(A)** and BC-1 **(B)**. **(C)** FTIR results. **(D)** XRD results.

respectively (Huong et al., 2017; Gao et al., 2019). After adsorption, the binding energies of C-O and C = O appeared at 286.10 eV and 288.90 eV, respectively. After adsorption and immobilization of heavy metal ions, the relative peak areas of C-O and C = O decreased significantly and the binding energy of the corresponding bond increases, suggesting that the complexation of -OH and -COOH in the adsorbent plays an important role in adsorption (Sha et al., 2023). Besides that, -O-C = O disappeared from the peak area after the adsorption was completed, proving that π–π interaction also plays an important role in the adsorption of heavy metal ions (Liu et al., 2021). In the O1s spectrum (Figure 2E), the binding energies appearing at 530.34, 532.00, and 532.98 eV in BC-1 before adsorption are attributed to Fe-O, C-O, and C = O, respectively (Omer et al., 2023; Ji et al., 2022). After adsorption is complete, the binding energies corresponding to these bonds appear at 530.40, 532.22 and 533.40 eV. The change in the peak area and the shift in the binding energy of the bonds also proved the presence of complexation and ion exchange of oxygen-containing functional groups during the immobilization of heavy metal ions (Zheng et al., 2021).

The magnetic stability of BC-1 was evaluated by fitting the hysteresis return lines of BC-1 before and after adsorption, and the results are illustrated in Figure 2F. The saturated magnetization strength (Ms) of BC-1 was 17.05 emu/g before adsorption, and after one round of adsorption equilibrium, its saturated magnetization strength decreased to 14.89 emu/g. The results of the five cycles of adsorption testing demonstrated that the saturated magnetization strength of BC-1 remained at 11.10 emu/g, indicating that BC-1 exhibited excellent magnetic stability. Based on which, the magnetic adsorbent can be efficiently recovered in subsequent soil experiments in the presence of an applied magnetic field.

3.3 Soil remediation experiments

The changes of physicochemical properties in soil remediation experiments are shown in Figure 3. From Figure 3A, the pH of BC-0 and BC-1 were increased compared to CK. It is due to the dissolution of alkaline substances in biochar into the soil, which regulated the soil pH (Tu et al., 2020). The soil pH decreased slightly in M and MBC–1, which is consistent with the study of Tu et al. (2020). Interestingly, the pH of the MBC–1 group dropped sharply on the 15th day. This could be attributed to the fact that after PSB–2 adapted to the new environment and stably colonized the soil, it produced a significant number of organic acids, resulting in changes in soil pH. This is consistent with the research of Xie et al. (2021). These organic acids, such as Gluconic acid, oxalic acid, malonic acid, citric acid and succinic acid, are common metabolites

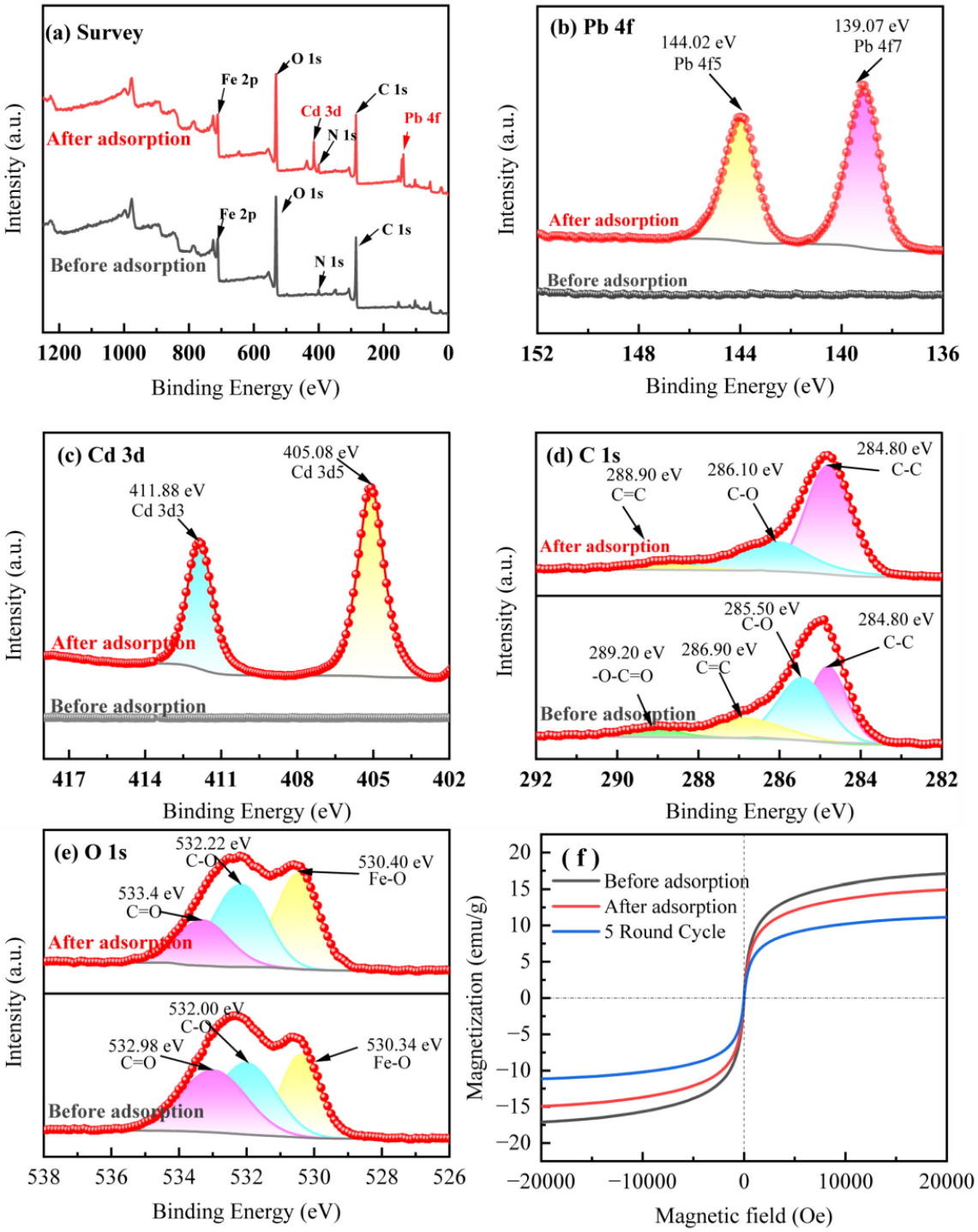


FIGURE 2
XPS results and hysteresis return lines before and after adsorption of BC-1. **(A)** Survey of the sample. **(B)** Pb 4f. **(C)** Cd 3d. **(D)** C 1s. **(E)** O 1s. **(F)** Hysteresis return line.

of PSB, which can dissolve insoluble phosphates, thereby reducing the pH value (Teng et al., 2019). Subsequently, on the 25th day, the pH value of the MBC–1 group increased rapidly, and then it decreased gradually. Yuan et al. (2011) reported a similar fluctuation in soil pH within the incubation with biochar from crop residues. They suggested the quick increase of soil pH was due to the dissolution of alkaline substances (such as inorganic carbonate) in the biochar, and then the pH was slightly changed after these readily released alkaline substances were depleted.

On the 55th day of the restoration, as shown in Figure 3B, the available phosphorus content in the soil of the M and MBC–1 groups reached 404.76 mg/kg and 447.62 mg/kg, respectively, an increase of 44.07 and 59.32% compared to the control group (CK). Numerous studies have demonstrated that secretion of organic

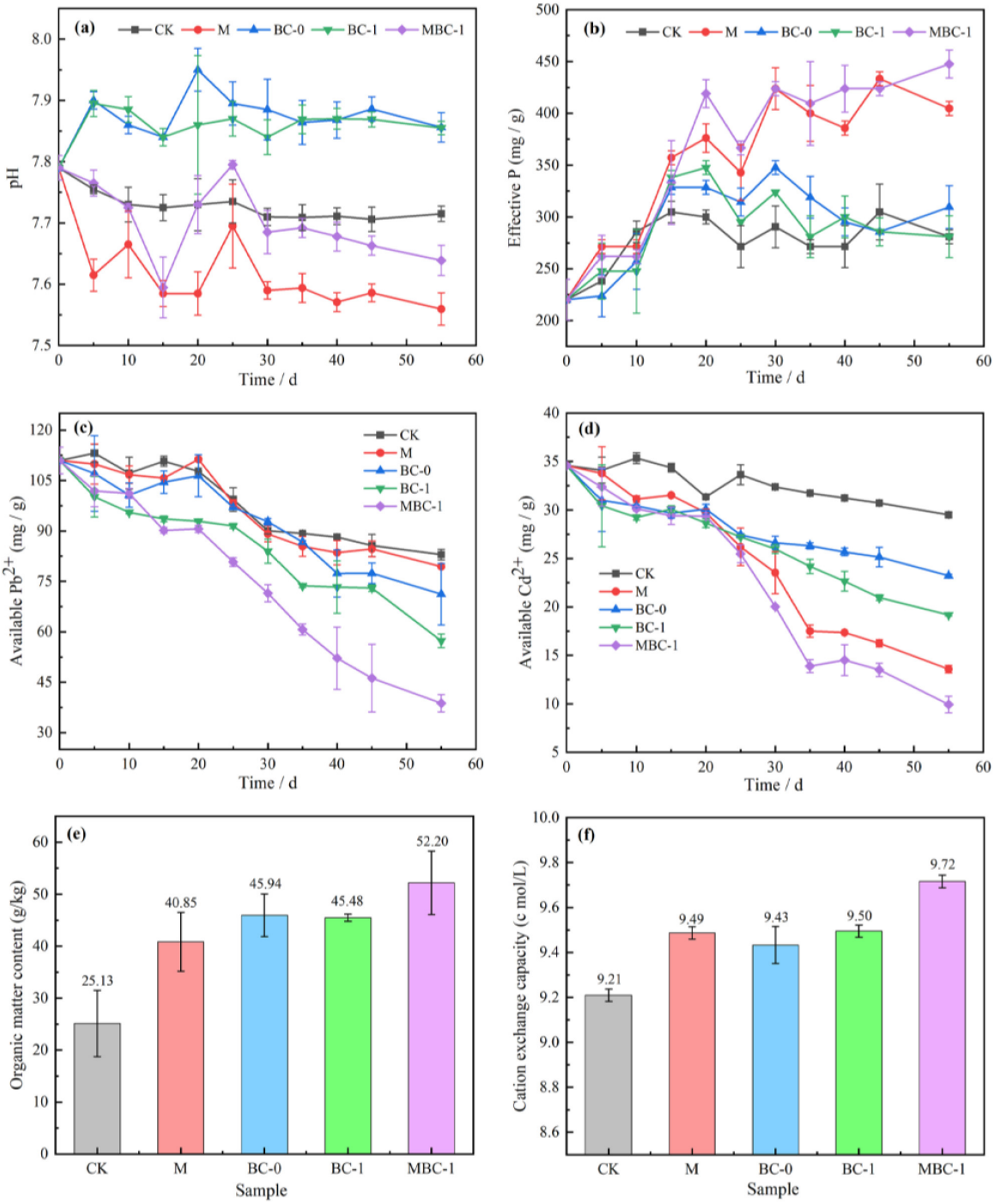


FIGURE 3
Physicochemical characterization during soil remediation. **(A)** pH change. **(B)** Available phosphate. Extractable state Pb^{2+} **(C)** and Cd^{2+} **(D)**. Soil organic matter content **(E)** and cation exchange capacity **(F)**.

acids by PSB is a critical microbial process that promotes phosphate solubilization (Gupta and Kumar, 2017). During bioremediation, the PSB-2 produces and releases small molecule organic acids by growing and metabolizing. The dissolution of insoluble phosphate in the soil into soluble phosphate promoted the release of phosphate. These findings are consistent with the results of the majority of previous studies (Lazo et al., 2017; Oteino et al., 2015). The fact that the available phosphorus content in the MBC–1 group is higher than that in the M group also indicates that biochar contributes to the increase in soluble phosphorus, which is consistent with the findings of Chen H. et al. (2020).

Changes in extractable state Pb^{2+} and Cd^{2+} content in soil remediation of phosphate mining wasteland are shown in Figures 3C, D. The contents of extractable state heavy metals Pb^{2+} and Cd^{2+} in MBC-1 were significantly reduced compared with CK. The concentrations of extractable state Pb^{2+} and Cd^{2+} in the soil

of MBC-1 after remediation were 38.77 mg/kg and 9.94 mg/kg, which were reduced by 65.06 and 71.26%, respectively. The MBC-1 demonstrated the most effective remediation effect. It is proved that the synergistic remediation of modified biochar and PSB is an effective method to manage Pb and Cd pollution in soil.

The remediation of soil heavy metal contamination by modified biochar synergized with PSB involves a variety of mechanisms. Modified biochar can effectively reduce the mobility and bioavailability of heavy metal ions by physical adsorption, chemical complexation, ion exchange and precipitation (Gao et al., 2022). Its pore structure and elements (C, N, S, O, P, and Ca) provide habitat and nutrients for PSB growth (Beesley et al., 2011). PSB, in turn, can induce phosphate precipitation, and the released available phosphate directly immobilizes heavy metal ions in the soil, reducing their bioavailability (Ji et al., 2022; Li et al., 2021a). For example, Chen et al. (2016) isolated a strain of PSB *Bacillus cereus* 12-2 from lead and zinc smelting sites, which converted Pb into $Ca_{2.5}Pb_{7.5}(OH)_2(PO_4)_6$ nanocrystals, confirming the biomineralization of Pb as hydroxyapatite. Besides, PSB can promote the release of available phosphate from biochar and dissolves both organic and inorganic phosphate by secreting enzymes and small-molecule organic acids (Li et al., 2018). The OH^-, CO_3^{2-} and PO_4^{3-} ions produced during this process can form stable precipitates with heavy metal ions (Inyang et al., 2012). Phosphate precipitation, particularly metal-phosphate precipitation, is recognized as a key mechanism for heavy metal immobilization (Yang et al., 2021). However, PSB needs to be effectively protected in the complex soil environment to obtain the maximum effect of remediating heavy metal pollution. Biochar, by virtue of its large pores and strong adsorb ability, can reduce the loss of available phosphorus dissolved by phosphate solubilizing bacteria, which will help PSB to effectively fix Pb (II) and Cd (II) for a long time. Therefore, the synergistic effect of biochar with high phosphate content and PSB has a superior heavy metal stabilization ability in phosphate soil remediation, and the heavy metal elements will be immobilized in the biochar by forming complexes with phosphate (Álvarez-Rogel et al., 2018).

Soil organic matter content (OM) and soil cation exchange (CEC) are important indicators of soil fertility. Phosphate mining wasteland is impoverished and undernourished, making it difficult for other plants to survive. After the experiment, the OM as well as CEC was determined to assess the soil fertility improvement, and the results are shown in Figures 3E, F. The best soil fertility improvement was achieved in MBC-1, where soil nutrient conditions were greatly improved. Its soil organic matter content reached 52.20 g/kg, which was 107.72% higher than that of CK (25.13 g/kg), and the CEC was also significantly higher. Numerous studies have demonstrated that one of the most consistent responses after applying biochar (BC) is the increase in OM. This is mainly because biochar reduces the cycling rate of organic matter, or the organic matter is directly incorporated into the biochar (Lu et al., 2014; Qi et al., 2021). In addition, it has been revealed that the negatively charged functional group structure on the surface of biochar adsorbs cations, thus promoting an increase in soil cation exchange (Oliveira et al., 2017). Heavy metal cations in the phosphate mining wasteland soil are exchanged with cations in the biochar and immobilized as complexes in the biochar or precipitated in the soil, resulting in a decrease in the bioavailability of heavy metal ions (Jain et al., 2020; Qi et al., 2021). Therefore, the addition of biochar can also compensate for the soil infertility caused by heavy metal pollution, improve the soil microbial environment, and thus affect the stability of Pb and Cd in the soil.

3.4 Analysis of microbial communities

Alpha diversity of restored microbial communities was analyzed in the phosphate mining wasteland (Supplementary Table 1), while the microbial communities of M and MBC-1 changed considerably in comparison with CK. The diversity index (Shannon and Simpson), richness index (Chao 1) and evenness index (Pielou_e) gradually increased in CK, M and MBC-1, respectively. It was due to the fact that, not only *Bacillus cereus* was able to increase the diversity of microbial functions (Qin et al., 2015), but also biochar had a positive impact on microbial diversity (Xu et al., 2023). The increasing Pielou_e index reflected the increasingly even distribution of the community, while an excellent community coverage index (Coverage = 1) ensured the reliability of this sequencing result (Chen and Achal, 2019). The Venn diagram visualized the statistics of species unique or shared among the samples (Figure 4D). A total of 3,138 species were shared by CK, M and MBC-1, indicating that the microbial communities were extremely similar. More importantly, M and MBC-1 had extremely high similarity with 878 shared species. Moreover, the unique species of CK, M and MBC-1 were 114, 102, and 104, respectively, demonstrating the changes in microbial structure through different restorations.

Figure 4A showed the changes in the relative abundance of microbial phyla levels during the ecological restoration process in the phosphate mining wasteland. The results indicate that the M and MBC-1 groups significantly altered the composition of the microbial community. A total of 178 phyla were detected in soil samples. Among them, *Actinobacteria*, *Proteobacteria*, and *Acidobacteria* were the three phyla with the highest abundance in all samples. As studied in Wang et al. (2023), *Proteobacteria* and *Actinobacteria* were the dominant phylum in the Cd and Pb contaminated soils. Compared to CK, the relative abundances of *Bacteroidota*, *Deinococcus-Thermus*, *Firmicutes*, *Candidatus Cloacimonetes*, and *Planctomycetota* significantly increased in M and MBC-1, consistent with findings by Wu et al. (2019). Studies have shown that *Bacteroidota* can not only enhance the soil's metal–fixing ability by regulating the physical and chemical properties of the soil (Li et al., 2024), but also increase the contents of available phosphorus and carbon in the soil through improving phosphorus dissolution and organic mineralization (Duan et al., 2020; Qin et al., 2020). Kruczynska et al. (2023) also pointed out that an increase in the abundance of *Bacteroidota* may indicate an improvement in soil quality. *Deinococcus-Thermus* is known to dominate the transformation of Cd fractions and regulate Pb mobility (Cui et al., 2021; Kavehei et al., 2022; Ren et al., 2021). *Firmicutes*, which contain a cluster of heavy metal tolerance genes, are commonly found in mining soils (Zhao et al., 2019). These phyla changes are closely linked to the application of biochar, which provides a favorable environment for microbial colonization, significantly influencing the abundance, diversity, composition, structure, and function of soil microorganisms (Zheng et al., 2022).

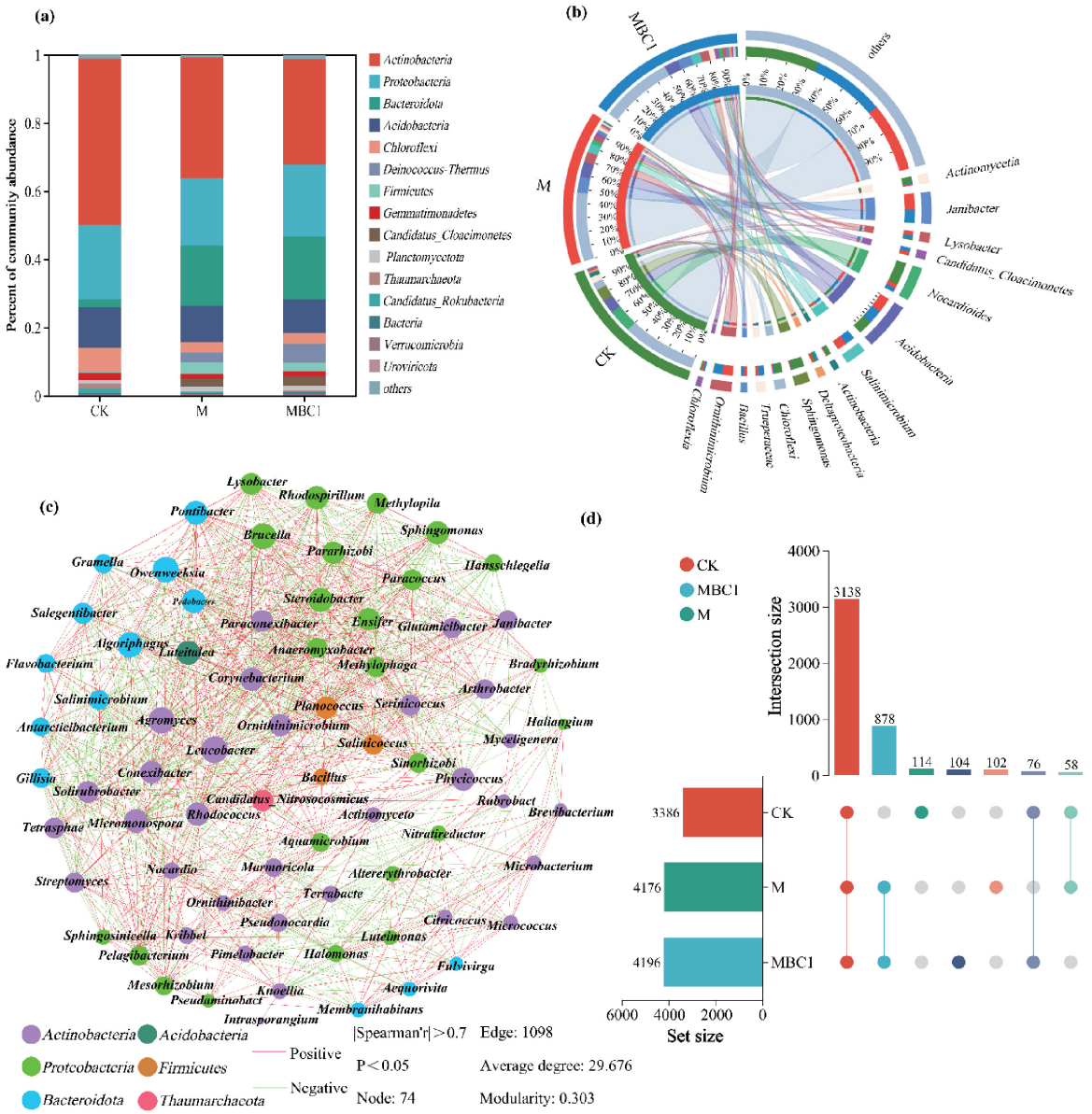


FIGURE 4
Analysis of soil microbial community. **(A)** Relative abundance analysis of microorganisms in different treatments (CK, M and MBC-1) (top 15 phyla). **(B)** Relative abundance analysis of microorganisms in different treatments (CK, M and MBC-1) (top 15 genera). **(C)** Microbial correlation network (genus level, abundance > 1%) and **(D)** Venn diagram analysis.

Additionally, the MBC-1 group showed significantly higher numbers of *Bacteroidota* and *Deinococcus-Thermus* compared to CK and M, indicating that the combined remediation of PSB-2 and BC-1 not only enhanced the capacity to remediate cadmium and lead contamination but also improved soil quality.

Figure 4B presents the relative abundance of the top 15 microbial genera. Following remediation, the reduction of Pb and Cd concentrations led to a significant decrease in the relative abundances of sensitive and tolerant bacteria, such as Nocardia and *Sphingomonas*, which are key heavy metal-tolerant genera. In M and MBC-1, the relative abundances of Nocardia and *Sphingomonas* were significantly lower compared to CK. Conversely, the abundances of *Janibacter*, *Ornithinimicrobium*, *Salinimicrobium*, *Lysobacter*, and *Bacillus* increased in M and MBC-1. *Janibacter* (Vetrovsky and Baldrian, 2015) and *Lysobacter* (Hu et al., 2021) are particularly noteworthy, as they possess well-documented capabilities for remediating heavy metal-contaminated soils through various mechanisms, such as biosorption and biotransformation. The increase in these genera suggests a positive shift in the microbial community toward those that can actively contribute to soil decontamination efforts. Additionally, the elevated abundance of *Bacillus* is significant, as it indicates successful colonization by PSB-2.

Microbial network analysis showed the correlation between species (Figure 4C), which could obtain the coexistence of species in environmental samples and was significant for understanding the potential interactions between microorganisms within a community (Wood et al., 2017). Apparently, 74 nodes

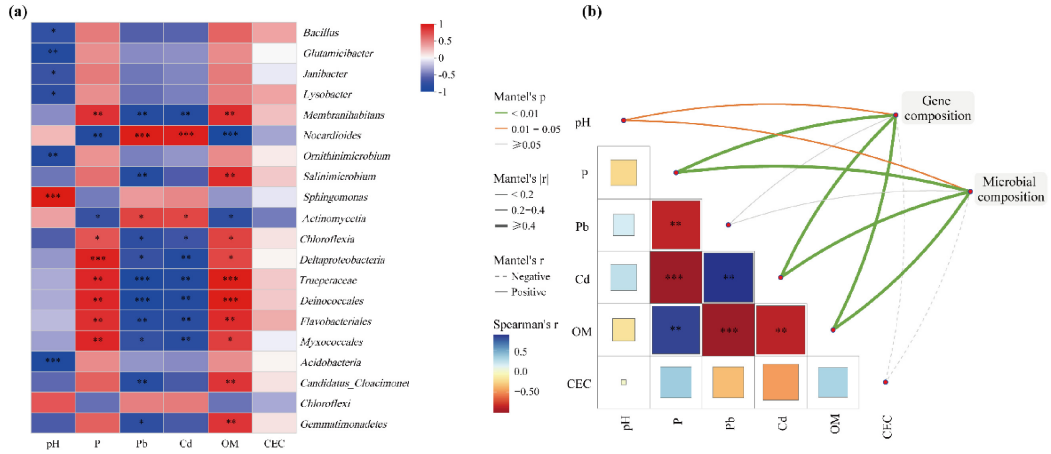


FIGURE 5
Correlation analysis between microorganisms and environmental factors. **(A)** Heatmap of correlations with environmental factors and microorganisms (top 20 genera). **(B)** Correlation of gene composition and microbial composition with environmental factors based on Mantel tests. Significance levels are denoted with $*p < 0.1$, $**p < 0.01$, $***p < 0.001$.

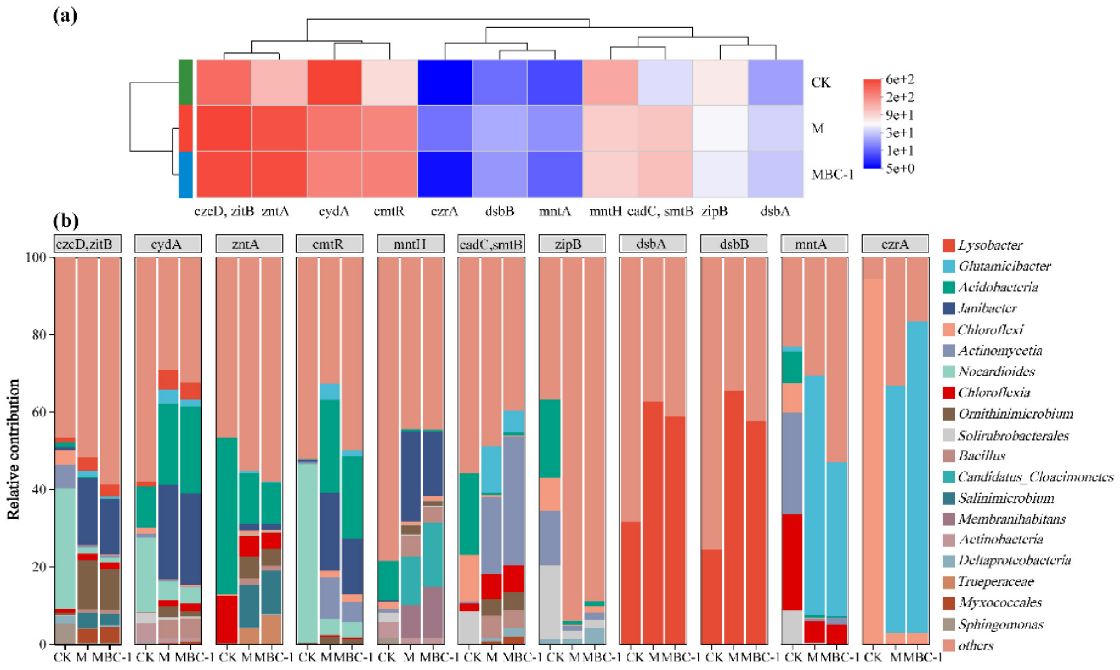


FIGURE 6
Analysis of heavy metal resistance genes in different treatments (CK, M and MBC-1). **(A)** Heatmap showing changes in the relative abundance of heavy metal resistance genes. **(B)** Species contribution of heavy metal resistance genes.

belonged to six different phyla, *Actinobacteria* (43.24%), *Proteobacteria* (32.42%), *Bacteroidota* (17.57%), *Firmicutes* (4.05%), *Thaumarchaeota* (1.35%), and *Acidobacteria* (1.35%). Furthermore, there were 1,098 edges, of which 53.73% were positive. *Bacillus*, which was added to the soil to participate in Pb and Cd remediation, showed significant positive correlation with *Planococcus*, *Salinicoccus*, *Leucobacter*, *Rhodococcus*, *Pelagibacterium*, and *Mesorhizobium*. *Bacillus* was already shown to be used in the remediation of heavy metal pollution with its ability to solubilize insoluble phosphate and excellent heavy metal resistance (Wani et al., 2019).

3.5 Correlation analysis of environmental factors

According to the correlation analysis between microorganisms and environmental factors (Figure 5A), pH, AP, available Pb^{2+} and available Cd^{2+} showed high correlation with microorganisms. However, the correlation between CEC and microorganisms was low. As studied in Li et al. (2021b), OM, Cd, Pb, TP, and pH had significant effects on bacterial community composition and distribution. Soil pH was significantly positively correlated

with *Sphingomonas* while negatively correlated with *Bacillus*, *Glutamicibacter*, *Janibacter*, *Lysobacter*, and *Ornithinimicrobium*. AP and OM showed significant positive correlation with *Membranihabitans*, but significant negative correlation with *Nocardioides*. Available Pb^{2+} and available Cd^{2+} had significant positive correlation with *Nocardioides* but significant negative correlation with *Membranihabitans*. Phosphate solubilizing microorganisms produced large amounts of organic acids, which led to the reduction of pH in the soil and the dissolution of insoluble phosphate, thus releasing soluble phosphate (da Silva et al., 2023).

There were correlations between environmental factors (Figure 5B). AP and OM significantly negatively correlated with available Pb^{2+} and available Cd^{2+}; AP was significantly and positively correlated with OM; available Pb^{2+} and available Cd^{2+} showed significant positive correlation. These findings were highly consistent with the study of Jing et al. (2020). From the correlation of gene composition and microbial composition with environmental factors (Figure 5B), it was observed that the gene composition and microbial composition are significantly correlated with pH, AP, available Pb^{2+}, available Cd^{2+}, OM and CEC. OM affected the bacterial community structure and promoted the production of AP (Mohamed et al., 2022), which led to the precipitation of available heavy metal ions (Miretzky and Fernandez-Cirelli, 2008).

3.6 Species contribution to heavy metal resistance genes

Figure 6 showed the relative abundance of different heavy metal resistance genes in different treatments as well as the species contribution. The relative abundance of *zitB*, *czcD*, *zntA*, *cmtR*, *cadC*, *smtB*, and *dsbA* in MBC1 was higher than that in CK compared to groups CK and M (Figure 6A). *zitB* and *czcD* transporters belong to the cation diffusion facilitator (CDF) family, which can transport Cd^{2+} (Chi et al., 2020). *zntA* is considered to be a Pb and Zn transporter protein ATPase with Cd and Pb resistance (Lee et al., 2001). *cmtR*, *cadC*, and *smtB* are not only transcriptional regulators of the ArsR family, but also Cd/Pb-responsive transcriptional repressors (Salam et al., 2020; Wang et al., 2005). *dsbA* is mainly involved in dithiol formation, and *dsbA* is mainly involved in dithiol formation, and *dsbA* is mainly involved in the formation of dithiols. *dsbA* is mainly involved in the formation of dithiols, which form thiol groups with high affinity for Cd (Stafford et al., 1999). The reduction of Cd and Pb concentrations in the MBC1 group was attributed to the high expression of these genes.

According to the species contribution of heavy metal resistance genes (Figure 6B), the species contribution of the same gene varied across treatments. *zitB*, *czcD*, *cydA*, *cmtR* in CK were the main contributors of *Nocardioides*. *Janibacter* was the main contributor of *zitB, czcD*, *cydA*, *cmtR*, *mntH* in M and MBC-1. *Janibacter* is the major contributor to *zitB*, *czcD, cydA*, *cmtR*, and *mntH* in M and MBC-1. *Lysobacter* is the major contributor of *dsbA* and *dsbB*. In M and MBC-1, *Corynebacterium glutamicum* was the major contributor of *mntA* and *czrA*. *Bacillus cereus* was involved in contributing *zitB*, *czcD*, *cydA*, *zntA*, *mntH*, *cadC*, *smtB*.

After remediation, the evolution of community structure also caused changes in the function of species, and microorganisms adapted to the changes in the growing environment by regulating various metabolic functions (Zhang et al., 2024). The function of the soil microbial community was predicted by PICRUSt (Supplementary Figure 2; Liu J. et al., 2018). Based on the secondary metabolic pathways, the relative abundance of amino acid metabolism, carbohydrate metabolism in treatments were higher, which were the foundational metabolic pathways necessary for microbial survival (Liu T. et al., 2018). Moreover, the exposure of heavy metals in soil caused an increase in amino acid metabolism (Qian et al., 2023). It has been demonstrated that most of the pathways of microorganisms would be reduced with increasing concentrations of heavy metals (Ma et al., 2022).

4 Conclusion

This study developed a novel biochar-based adsorbent, BC-1, from corn cob biochar (BC-0) through the synergistic combination of phosphate-solubilizing bacteria (PSB) and Fe_3O_4 loading. This modification significantly enhanced BC-1's adsorption capacity for Pb^{2+} and Cd^{2+}, while maintaining magnetic stability. Oxygen-containing functional groups (-OH, -COOH) and Fe-O bonds on the surface, along with π-π interactions, played a key role in ion exchange and complexation during adsorption. In combination with *Bacillus cereus*, BC-1 effectively remediated phosphate mining wasteland soil, increasing effective phosphate content (447.62 mg/kg) and reducing extractable Pb^{2+} (65.06%) and Cd^{2+} (71.26%). This treatment also improved soil organic matter and cation exchange capacity, enhancing soil health. Additionally, the remediation increased microbial community diversity and abundance, with *Janibacter*, *Lysobacter*, *Ornithinimicrobium*, *Bacillus*, and *Salinimicrobium* as the dominant groups. The upregulation of heavy metal resistance genes (*ZitB*, *czcD*, *zntA*, and *cmtR*) highlighted the microbial community's robust response to Pb^{2+} and Cd^{2+} stress. Moreover, the stabilization of microbial function, especially in genetic processing, environmental processing, and metabolic pathways, supports the long-term efficacy of this remediation approach. This study suggests that combining PSB with modified biochar offers a promising, green, and sustainable solution for remediating heavy metal-contaminated soils in phosphate mining wastelands.

Data availability statement

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

Author contributions

YZ: Data curation, Investigation, Writing – original draft. JP: Data curation, Investigation, Writing – original draft. ZW: Methodology, Validation, Visualization, Writing – original draft.

FZ: Formal analysis, Methodology, Software, Writing – review and editing. JY: Investigation, Validation, Visualization, Writing – review and editing. RC: Conceptualization, Project administration, Supervision, Writing – review and editing. CX: Conceptualization, Funding acquisition, Investigation, Writing – review and editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1529784/full#supplementary-material

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Phosphate-solubilizing microorganisms for soil health and ecosystem sustainability: a forty-year scientometric analysis (1984–2024)

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Phosphate-solubilizing microorganisms (PSM) play a crucial role in promoting crop growth by enhancing phosphorus supply and reducing phosphorus loss in soil. However, a comprehensive bibliometric overview of the research landscape on PSM in agricultural applications has been lacking. This study conducts a bibliometric analysis to explore global research trends, key contributors, and collaborative networks in the application of PSM in ecological restoration, providing valuable insights for future research. A total of 1,662 documents from the Web of Science Core Collection, spanning from 1984 to 2024, were extracted and analyzed using Bibliometrix and CiteSpace software. The findings reveal a period of rapid growth in this field since 2018. Initially, research focused on microbial soil nutrients, such as phosphate rock and *Azospirillum brasilense*. Current research hotspots have shifted towards topics like drought and salt stress, as well as productivity, reflecting an increasing emphasis on mitigating the impacts of global warming and environmental changes. China and India lead in research output, contributing 36.67% of the total articles. The Indian Council of Agricultural Research published the highest number of articles. Future research on PSM should emphasize their role in enhancing nutrient uptake, improving soil health, and mitigating environmental stresses, supporting sustainable agriculture and ecological restoration. This bibliometric analysis of 1,162 articles by 7,454 authors from 101 countries highlights critical advances at the intersection of soil microbiology, sustainable land management, and climate change adaptation. These findings provide a foundation for addressing global challenges like soil degradation, nutrient cycling, and food security, aligning with the Sustainable Development Goals.

KEYWORDS

phosphorus availability, ecological restoration, crop growth, global research trends, sustainable agriculture, microbial soil nutrients

1 Introduction

Phosphorus is a critical macronutrient essential for plant growth and development, playing a pivotal role in various processes including energy transfer, photosynthesis, and nutrient cycling (Kamerlin et al., 2013; Majeed et al., 2023). Despite its significance, phosphorus often acts as a limiting factor in numerous terrestrial ecosystems, primarily due to its existence in

forms that are not readily available to plants (Syers et al., 2008; Rawat et al., 2021). Global estimates indicate that approximately 40% of the world's agricultural soils suffer from phosphorus deficiency, which negatively impacts both food production and ecosystem health (Vance et al., 2003). This limitation severely hampers soil productivity and the capacity for ecosystem recovery, posing a significant challenge to sustainable land management and ecological restoration efforts (Xiao et al., 2021; Wang et al., 2023). In regions where phosphorus depletion is most acute, soil fertility often declines by over 50%, thereby greatly restricting the potential for ecosystem recovery (Huang et al., 2012; Qin et al., 2023). A promising approach to address phosphorus deficiency in soils involves the utilization of phosphate-solubilizing microorganisms (PSM) (Richardson and Simpson, 2011). As a crucial component of soil microbiota, PSM can convert insoluble soil phosphorus into plant-accessible forms through their metabolites (organic acids, phosphatases) or via synergistic interactions within microbial communities (Richardson et al., 2009; Alori et al., 2017; Rawat et al., 2021). This not only improves phosphorus uptake by plants, but also accelerates nutrient cycling, enhances soil structure, and supports broader ecosystem functions by accelerating nutrient cycling and improving the bioavailability of critical minerals, making it a key strategy for sustainable ecosystem management (Rajwar et al., 2018).

The role of PSM in ecological restoration has gained prominence due to their capacity to enhance nutrient cycling, stimulate plant growth, and aid biodiversity recovery in degraded ecosystems (Liang et al., 2020; Chen et al., 2023). By improving phosphorus availability in the soil, By increasing phosphorus bioavailability, PSM contribute to critical ecosystem services, such as improving soil stability, supporting vegetation regrowth, and enhancing carbon sequestration (Sun et al., 2023; Chen et al., 2024). These attributes align closely with global efforts to address land degradation and promote sustainable land management (Hussain et al., 2019; Silva et al., 2023; Gurav et al., 2024). Furthermore, In the context of global warming, PSM have shown significant potential to help ecosystems adapt to changing climatic conditions by enhancing nutrient cycling under stress conditions, such as drought and heat (Aqeel et al., 2023). This ability to maintain or restore ecosystem functions under climate change is crucial for increasing ecosystem resilience, ensuring food security, and mitigating climate-related impacts on ecosystems (Rawat et al., 2021).

While the ecological and agricultural importance of PSM has been extensively studied (Lambers, 2022; Iftikhar et al., 2024), a systematic and comprehensive global analysis of this research domain remains largely unexplored (Alori et al., 2017; Ampese et al., 2022). Existing studies often neglect the evolving research priorities and their implications for land management and ecosystem restoration, limiting a holistic understanding of PSM's role in sustainability. Bibliometrics involves the quantitative analysis of academic publications and their citation data to comprehend patterns and developments in scientific knowledge dissemination (Garfield, 1972; Aria and Cuccurullo, 2017; Ampese et al., 2022; Montazeri et al., 2023). Its primary objectives include measuring research output impact, evaluating academic contributions of institutions or individuals, identifying research trends, and monitoring the evolution of scholarly domains (Glänzel, 2003; Wang et al., 2024). A fundamental tool in bibliometrics is citation analysis, which assesses the significance of research based on citation frequency (Van Raan, 1997). Collaboration network analysis elucidates relationships among scholars, facilitating understanding of scientific cooperation and knowledge dissemination patterns (Aria and Cuccurullo, 2017). Beyond identifying research hotspots, bibliometrics tracks disciplinary development, providing empirical support for research evaluation, academic management, and policy formulation, ultimately contributing to the optimal allocation of research resources (Egghe and Rousseau, 1990; Hirsch, 2010).

This study aims to conduct a bibliometric analysis on the application of PSM in ecological restoration, utilizing data from the Web of Science Core Collection, covering literature from 1984 to 2024. Through an examination of publication trends, author collaborations, keyword co-occurrence, and geographic distribution, this research endeavors to map the development of PSM research within the context of ecological restoration. The findings aim to provide a comprehensive landscape of PSM research, identifying significant contributors, uncovering emerging themes, and pinpointing underexplored areas that can guide future ecological restoration strategies. This analysis intends to provide researchers and practitioners with insights for more effective utilization of PSM in ecological restoration efforts.

2 Materials and methods

2.1 Data sources and preprocessing

As shown in Figure 1, This study was conducted according to the PRISMA 2020 guideline (Page et al., 2021). The search was conducted in "Web of Science Core Collection," with "All" selected in "Editions." The search query focused primarily on "Phosphate-solubilizing microorganisms," and the data retrieval strategies were designed as follows: TS = ("phosphor-releas*" OR "phosphor-solubili*" OR "phosphate-releas*" OR "phosphate-solubili*" OR "phosphor-mobiliz*" OR "phosphate-mobiliz*" OR "phosphor releas*" OR "phosphor solubili*" OR "phosphate releas*" OR "phosphate solubili*" OR "phosphor mobiliz*" OR "phosphate mobiliz*") AND TS = (microorganism* OR microb* OR bacteria* OR Fungi OR Fungus) AND TS = (ecolog* OR ecosystem* OR environment* OR nutrient* OR fertilit*) AND TS = (rehabilit* OR restor* OR improve* OR remedia* OR bioremedia* OR sustainab* OR manage*). The data used in this study can be accessed through Mendeley Data (Mendeley Data, V2, doi:10.17632/xrp4hp58gs.2).

This initial query yielded 1,378 English publications on December 31, 2023, covering the period from January 1, 1994, to December 31, 2023. An additional search was conducted on December 9, 2024, extending the dataset to 1,662 publications. After screening the records (Figure 1), 47 records were excluded due to incomplete bibliographic information, such as missing author names, publication dates, or other essential details necessary for proper analysis. Following full-text review, 15 records were excluded because they were not relevant to the content of the study.

2.2 Data analysis

To analyze the retrieved data comprehensively, this study utilized CiteSpace (6.3.R1 Advanced), Bibliometrix R-package (Aria and Cuccurullo, 2017), and Microsoft Excel 2021. These tools were selected based on their established effectiveness in conducting

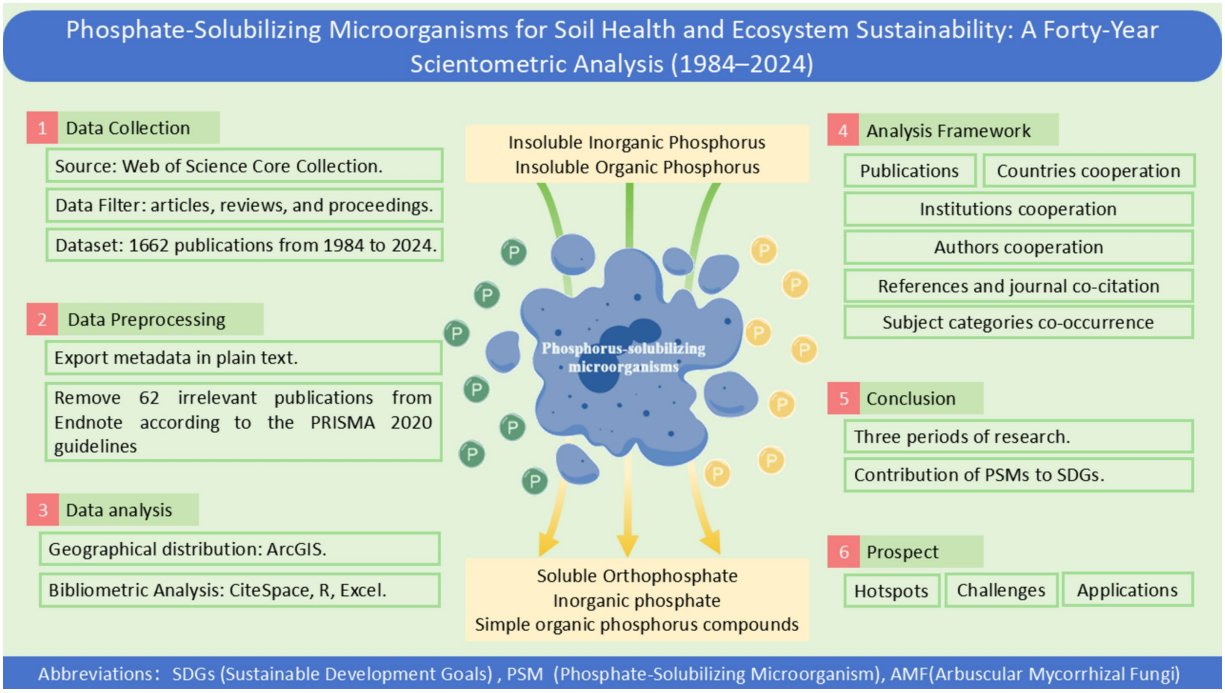


FIGURE 1
Bibliometric analysis flow of this study. Abbreviation in this study: SDGs (Sustainable Development Goals), PSM (Propensity Score Matching) and AMF (*Arbuscular Mycorrhizal* Fungi).

co-occurrence analysis, collaboration network mapping, and visualizing research trends. CiteSpace was used for co-citation and keyword co-occurrence analyses, identifying research hotspots and trends over time (Chen and Leydesdorff, 2014; Chen, 2017). These analyses revealed correlations between literature, emerging frontiers, and thematic developments (Chen, 2006; Montazeri et al., 2023; Du and Li, 2024). CiteSpace's algorithms were also applied to assess keyword characteristics, where node size indicated frequency, and link thickness reflected co-occurrence strength (Li et al., 2023). Key nodes (centrality >0.1) were identified as critical to connecting research themes, and the burstiness indicator highlighted emerging research trends and hotspots (Guo and Yao, 2024). Pajek complemented this by processing large-scale network data, enabling visualization of collaboration and citation networks (Batagelj and Mrvar, 2004). ArcGIS facilitated spatial analyses to map research distributions and academic cooperation patterns (Borge-Holthoefer and Moreno, 2012). Bibliometrix provided a robust platform for bibliometric analysis, integrating data transformation, analysis, and visualization (Aria and Cuccurullo, 2017).

However, bibliometric methods may introduce certain biases. Data source selection can lead to sample bias, as reliance on databases like Web of Science or Scopus may overlook non-English and regional studies (Alois, 2024). Additionally, keyword extraction and classification involve subjectivity, where handling synonyms and polysemes can affect results (Liu et al., 2021). Overemphasis on highly cited papers may also overshadow emerging or niche research areas and these biases can impact the accurate identification of research hotspots and trends (Larivière, 2010). In this study, all the literature data have been optimized to avoid the above problems.

3 Results

3.1 Publication analysis

The number of publications indicates the total count of papers published within a specific research field over a designated period, and fluctuations in this number can signify development trends within that field (Pan et al., 2024). The corpus of publications, as shown in Figure 2 (a), primarily comprised articles (1367), followed by reviews (233), and proceedings papers (17). The publication trend can be divided into three stages (1984–2008, 2009–2017, 2018 to present) as illustrated in Figure 2 (b). In the first stage, the quantity of publications was relatively low and grew slowly. The annual number of publications remained in single digits to approximately 10. The first two articles were published in 1984 and 1991, representing the initial stage of the PSM studies. During this period, the research field was gradually building and the growth was steady but modest, reflecting the early development phase of the field. From 2008, the number of publications began to gradually increase. By 2017, the number of publications exceeded 50. The years 2006 and 2009 exhibited two small "peaks," with growth rates of 90.91 and 116.67%, respectively. These peaks indicate periods of increased research activity and interest in the field. The growth during this period was more pronounced compared to the initial stage. From 2018 onwards, the number of publications increased significantly. The peak number of publications was reached in 2021 and 2022, at nearly 160. In 2019, the number of publications increased by 58.06% compared to 2018, and in 2021, it increased by 38.46% compared to 2020. This sharp increase in publications reflects the rapid growth of research in the field, driven by advancements in technology, increased funding, and a growing

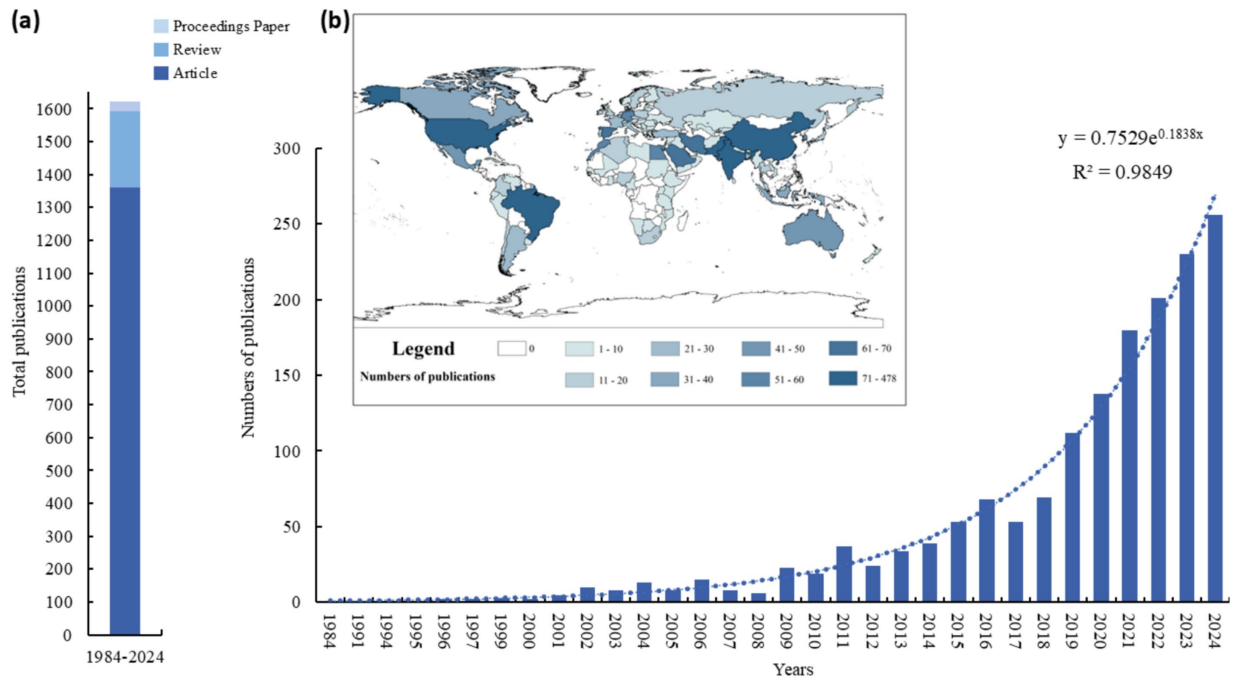


FIGURE 2
Publication output in the field of PSM from 1984 to 2023. **(A)** Stack chart of total publications. **(B)** Annual and cumulative number of publications and geographic visualizations.

recognition of the importance of PSM studies in addressing contemporary challenges.

Based on the cumulative number of publications per year, an equation for fitting the curve can be derived: $y = 0.7529e^{0.1838x}$, $R^2 = 0.9849$, demonstrating excellent fitting properties and conforming to the Price curve. This trend is particularly evident in recent years, with research potentially related to ecological restoration and PSM garnering increasing attention.

3.2 Countries cooperation analysis

From a bibliometric perspective, 101 countries have conducted research on PSM, and 21 countries have published more than 20 articles each. Supplementary Table S1 lists the top 20 countries by number of published papers. Three countries have surpassed the threshold of 100 publications, collectively accounting for 41.47% of the total publications. India leads with 478 publications, closely followed by China with 349. Notably, India initiated research in this field earlier, and the first two articles published in 1984 and 1991 were both from India. Pakistan ranks third with 130 publications.

In the collaboration network shown in Figure 3, India, China, and Pakistan are prominently positioned with larger nodes, signifying their high publication output. India's dominant role is further emphasized by its extensive connections with numerous countries, reflecting strong international collaborations. China also exhibits robust collaborative links, particularly with the USA, Germany, and Australia, highlighting research partnerships. France's high centrality suggests it acts as a key bridge in the network, facilitating collaboration between other major research hubs. Countries like Germany, Iran, and South Korea display moderate centrality, implying active participation but with less influence compared to France and the USA. Clusters of closely connected countries are evident, such as the collaborations between European nations (France, Germany, Spain, and Italy) and between Asian countries (India, China, Pakistan, and Bangladesh). These clusters suggest regional research networks that complement broader international collaborations. Overall, the visualization in Figure 3 demonstrates a well-integrated global research network on PSM, characterized by strong bilateral and multilateral partnerships. This interconnected framework facilitates knowledge sharing and accelerates advancements in sustainable agricultural technologies and ecosystem restoration initiatives.

Co-occurrence network analysis of research topics in the field of PSM and national collaborations reveal seven distinct thematic clusters (Supplementary Figure S1), showing connections between countries and research themes. Cluster #0 (soil fertility), dominated by India and Australia, focuses on improving soil nutrient availability. Cluster #1 (growth-promoting rhizobacteria), linked to Pakistan and Brazil, emphasizes enhancing plant growth. Cluster #2 (oil palm tree), represented by Indonesia, explores PSM applications in tropical agriculture. Cluster #3 (calcareous soil), involving Pakistan and Spain, addresses challenges in alkaline soils. Cluster #4 (phosphorus deficiency), associated with China and the USA, investigates solutions for phosphorus scarcity. Cluster #5 (unlocking agro-ecosystem sustainability), connected to Italy and France, focuses on sustainable farming, while Cluster #7 (environmental remediation), linked to Germany, highlights PSM' role in restoring degraded environments. This network reflects the global collaboration in advancing PSM research for sustainable agriculture.

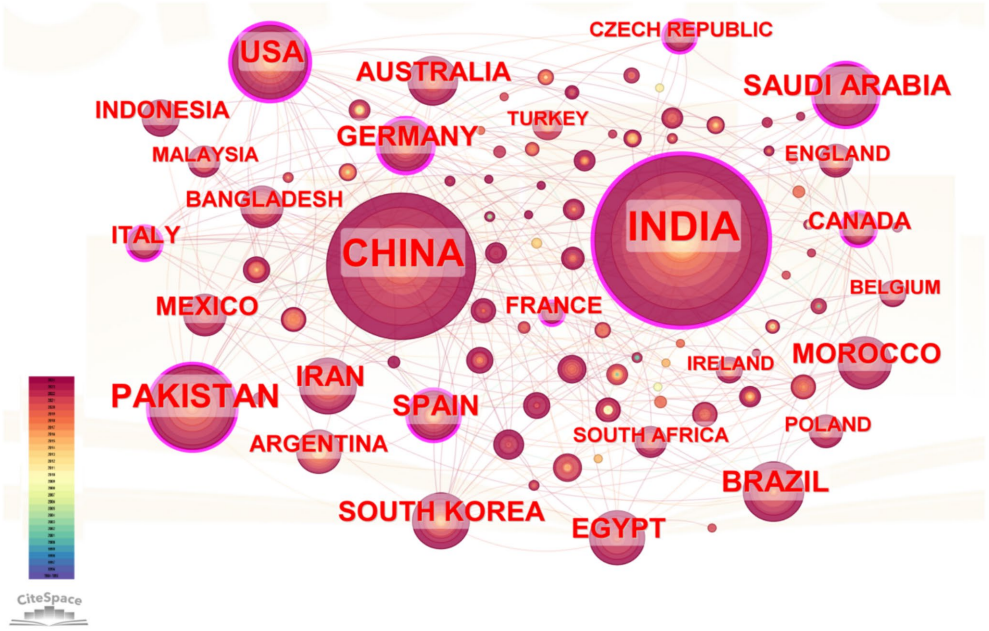


FIGURE 3
Map of country cooperation network analysis of PSM publications. Nodes represent countries involved in the collaboration network, with size indicating their level of participation. Colors reflect collaboration frequency, with darker nodes showing more active partnerships. Lines represent collaborations between countries, with thicker lines indicating stronger or more frequent ties.

3.3 Institution cooperation analysis

The distribution of collaboration and beneficial contribution among diverse institutions in the research field is analyzed using CiteSpace. In the selection criteria, the scale factor k = 5. The analysis revealed that 2097 institutions published research articles in the field of PSM, and the top 20 institutions with the highest number of publications are presented in Supplementary Figure S2. The results indicate that the Indian Council of Agricultural Research (India) ranks first with 111 papers, followed by the Egyptian Knowledge Bank (Egypt) and Chinese Academy of Sciences (China), each with more than 40 publications. Among the top 20 institutions in terms of publication count, India leads with 259, followed by China with 74 and Morocco with 55. The three institutions with the highest centrality are Bahauddin Zakariya University (Pakistan), Huazhong Agricultural University (China), and Al Azhar University (Egypt), suggesting their relatively high comprehensive influence in this field.

The collaborative network of research institutions in the field of PSM highlight the relationship and contributions of key institutions (Figure 4). The full names, abbreviations and countries of these institutions are shown in Table 1. Cluster #0 (Cropping System) is dominated by the Indian Council of Agricultural Research (India) and the Indian Agricultural Research Institute (India), which play central roles in advancing sustainable farming practices. Cluster #1 (Phosphate-Solubilizing Bacteria) features close collaboration between the Chinese Academy of Sciences (China) and the University of Agriculture Faisalabad (Pakistan), emphasizing research on microbial solutions for nutrient availability. In Cluster #2 (*Arbuscular Mycorrhizal* Fungi), institutions like the Spanish National Research Council (Spain) and the Zaidin Experimental Station (Spain) showcase strong cross-regional collaboration on soil–plant interactions. Similarly, Cluster #5 (Nutrient Management) highlights partnerships between King Saud University (Saudi Arabia) and Punjab Agricultural University (India), focusing on optimizing nutrient use efficiency. These thematic clusters illustrate both the diversity of research directions and the interconnected nature of global collaboration in PSM research, with institutions working together to address critical agricultural and environmental challenges.

3.4 Author cooperation analysis

The author cooperation analysis in PSM research highlights the significant contributions and collaborative efforts of key researchers in the field (Supplementary Table S2). Hassan Etesami stands out with the highest number of publications (13) and citations (854), reflecting his central role and influence in advancing PSM studies. Adnane Bargaz (11 publications, 729 citations) and Yadav Ajar Nath (10 publications, 387 citations) are also prominent contributors, indicating active involvement in this research area. Highly cited authors such as Bernard R. Glick (1,484 citations) and Youssef Zeroual (641 citations) underscore the impactful nature of their work, despite slightly fewer publications. The data suggests a network of researchers with diverse expertise, working collaboratively to address critical challenges in the PSM domain. Emerging contributors like Olubukola Oluranti Babalola, Divjot Kour, and Hossein Ali Alikhani also highlight the growing international interest in this field. The distribution of publications and citations demonstrates a balance between well-established and newer authors, emphasizing the importance of both individual impact and collaborative synergy in driving innovation and knowledge dissemination in PSM research.

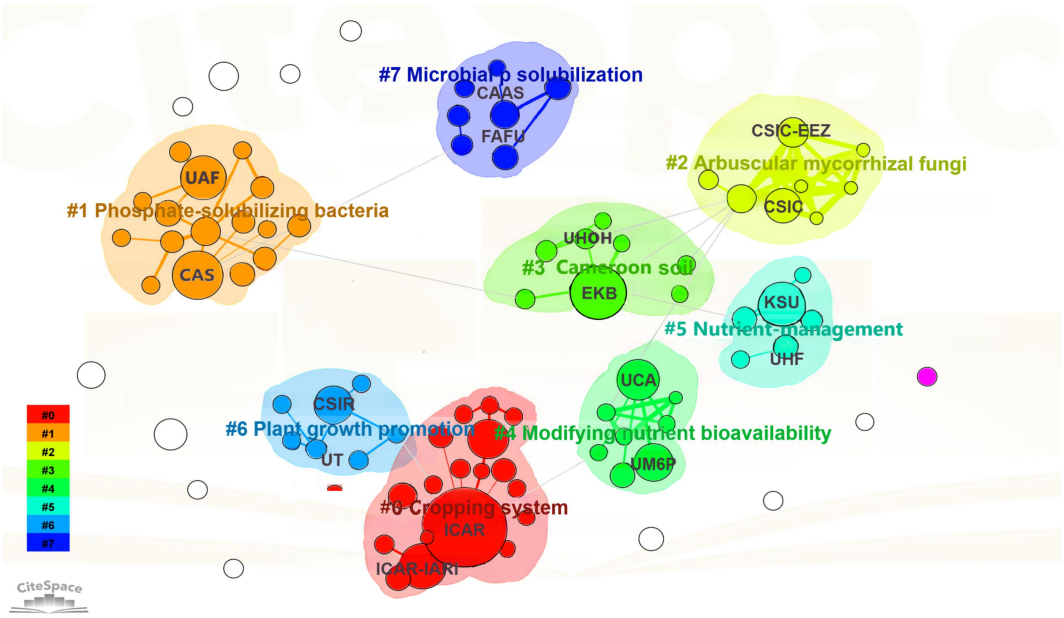


FIGURE 4
Map of collaborative network of global institutions. Colors represent different research clusters. Nodes indicate research institutions, with node size reflecting their influence, and edges represent collaboration links between institutions.

3.5 Keywords analysis

Figure 5 depicts the co-occurrence network of keywords in PSM research, providing a visual representation of the field's intellectual structure. In this network, each node corresponds to a specific keyword, with node size proportional to its frequency of occurrence in the literature (Chen, 2020). The color gradient of the nodes indicates the temporal distribution of studies, where darker red shades represent more recent research (Sabe et al., 2022). The links between nodes signify co-occurrence relationships, with thicker lines denoting stronger associations (Chen, 2020). The network highlights several core research keywords, as evidenced by the larger nodes, including "phosphate solubilizing bacteria," "plant growth," and "rhizosphere. "These keywords represent foundational areas of focus within PSM research. The strong connection between "phosphorus," "rhizosphere," and "microorganisms" suggests a sustained research emphasis on microbial-mediated phosphorus solubilization processes in the soil environment. Additionally, the association between "growth promotion" and "diversity" with mechanisms such as "nitrogen fixation" and "inoculation" reflects the integration of multifunctional microbial strategies that enhancing plant growth and nutrient acquisition. Emerging research fronts are evident in the increasing occurrence of keywords such as "nutrient uptake," "growth promotion," and "sustainable agriculture," indicating a shift towards the practical application of PSM in sustainable crop production systems. The prominence of terms like "biological control" and "plant growth-promoting rhizobacteria" further underscores the expanding interest in leveraging microbial diversity for improving soil fertility and plant health.

Owing to the lengthy interval between the first two papers published in 1984 and 1991, a connection was made with other literature. Consequently, these two documents were not incorporated in the following analysis. Figure 6 offers a comprehensive view of the evolution of research hotspots in agricultural and environmental microbiology from 1990 to 2024, highlighting the development of key topics within the field. Early research themes, such as "organic acids," "plant growth promotion," and "sustainable agriculture," emerge as prominent areas, reflecting a long-standing focus on improving agricultural productivity and sustainability. The figure also reveals the interconnected nature of research, with topics like "phosphate-solubilizing bacteria" and "nitrogen fixation" closely linked, emphasizing the crucial role of microbial processes in nutrient cycling for soil fertility. As the timeline progresses, new clusters, such as "accdeaminase" and "plant growth-promoting bacteria," signal a shift towards mechanistic studies exploring molecular pathways involved in plant stress tolerance and growth promotion. Additionally, keywords like "phytoremediation" and "community structure" indicate a growing interest in the environmental applications of plant growth-promoting microbes, especially for soil health and pollution remediation. The color gradient, transitioning from purple (early years) to yellow (recent years), visually represents the timeline of research development, highlighting when certain topics gained prominence (Chen, 2017; Chen and Song, 2019). Clusters like "bacterial community" and "rock phosphate" further emphasize the critical role of soil microbes and microbial diversity in enhancing soil health and phosphorus availability, which are vital for sustainable agricultural practices. Overall, this visualization provides a detailed overview of how research within soil microbiology and plant growth has evolved, focusing on microbial processes, soil health, and environmental concerns, alongside mechanistic studies aimed at addressing global agricultural challenges like climate change and soil degradation.

Figure 7 illustrates the 25 keywords with the strongest Citation Bursts in the academic literature. Adjacent to each keyword is the year of its citation surge, intensity, start and end times, and the corresponding time period (Hou et al., 2018). Each keyword is accompanied by its surge year, intensity, duration, and corresponding

TABLE 1 Institutions with abbreviations, full names, and corresponding countries.

Abbreviation	Institution	Country
ICAR	Indian Council of Agricultural Research	India
ICAR-IARI	ICAR-Indian Agricultural Research Institute	India
CAS	Chinese Academy of Sciences	China
UAF	University of Agriculture Faisalabad	Pakistan
CSIC	Consejo Superior de Investigaciones Científicas	Spain
CSIC-EZZ	CSIC - Estación Experimental del Zaidín	Spain
UHOH	University of Hohenheim	Germany
EKB	Egyptian Knowledge Bank	Egypt
UCA	Cadi Ayyad University of Marrakech	Morocco
UM6P	Mohammed VI Polytechnic University	Morocco
KSU	King Saud University	Saudi Arabia
UHF	Dr. Yashwant Singh Parmar University of Horticulture & Forestry	India
CSIR	Council of Scientific and Industrial Research, India	India
UT	University of Tehran	Iran
CAAS	Chinese Academy of Agricultural Sciences	China
FAFU	Fujian Agriculture and Forestry University	China

time period. Early bursts like "nutrition" (1997) and "*Aspergillus niger*" (1999) reflect sustained interest in microbial roles in plant nutrient uptake, using traditional microbiological methods. Keywords from the early 2000s, such as "nutrient uptake" and "rhizosphere," indicate growing research on plant-microbe interactions, transitioning towards molecular techniques. Later bursts like "*arbuscular mycorrhizal* fungi" (2006) and "*azospirillum brasilense*" (2010) highlight increased focus on beneficial fungi and nitrogen-fixing bacteria, enhanced by metagenomics and sequencing technologies. Recent bursts, including "promoting rhizobacteria" (2014) and "nitrogen fixation" (2016), signal a shift towards microbial solutions for soil fertility and crop yields, utilizing omics technologies. Keywords like "microbial biomass" (2018) and "solubilization" (2019) emphasize microbial roles in phosphate solubilization, supported by advanced analytical methods. The most recent bursts, such as "gene expression" and "induced systemic resistance" (2020–2024), suggest a focus on molecular mechanisms of plant-microbe interactions, often using gene-editing technologies like CRISPR. Overall, the data highlights a shift in PSM research from basic functions to applied solutions, with increasing attention on enhancing soil health and sustainable farming through microbial interventions. This evolution from conventional to advanced techniques offers insights into modern approaches suitable for current and future studies.

3.6 Reference and journal co-citation analysis

The top 10 co-citation articles in PSM research filed are listed in Table 2. The most influential article, "Phosphate-solubilizing bacteria and their role in plant growth promotion" by Rodríguez and Fraga (1999), with 3,321 co-citation, underscores its foundational role in linking PSM to plant growth enhancement. Sharma et al. (2013) work on sustainable phosphorus management, ranks second with 2,816 citations, highlighting the pivotal role of PSM in addressing phosphorus deficiency in agricultural soils. Other significant studies, such as Nautiyal (1999) and Pikovskaya (1948), focus on an efficient microbiological growth medium and microbial contributions to soil nutrient mobilization, achieving 2,291 and 2,158 citations, respectively. These articles collectively demonstrate high total link strengths, with values exceeding 1900, indicating their substantial connectivity and influence within the research community. Top 20 journals by number of publications from 1,622 articles are listed in Supplementary Table S3. A total of 1,622 articles were published across 472 journals, with 40 journals publishing more than 10 articles, as illustrated in Supplementary Figure S3.

3.7 Subject categories co-occurrence analysis

The co-occurrence analysis of subject categories contributes to the advancement of research frontiers and the identification of interdisciplinary characteristics within specific knowledge fields (Li et al., 2019). Utilizing cluster analysis in CiteSpace software, we identified that research on PSM is both multidisciplinary and interdisciplinary (Figure 8), generating seven distinct clusters. This research encompasses various fields, including medicine, research and experimental, horticulture, physics, engineering environment, water resources, agronomy, biotechnology and applied microbiology, oceanography, chemistry, plant sciences, and ecology.

4 Discussion

Research on PSM from 1984 to 2024 illustrates a remarkable evolution in focus, with growing contributions from diverse institutions and countries, reflecting regional priorities and global

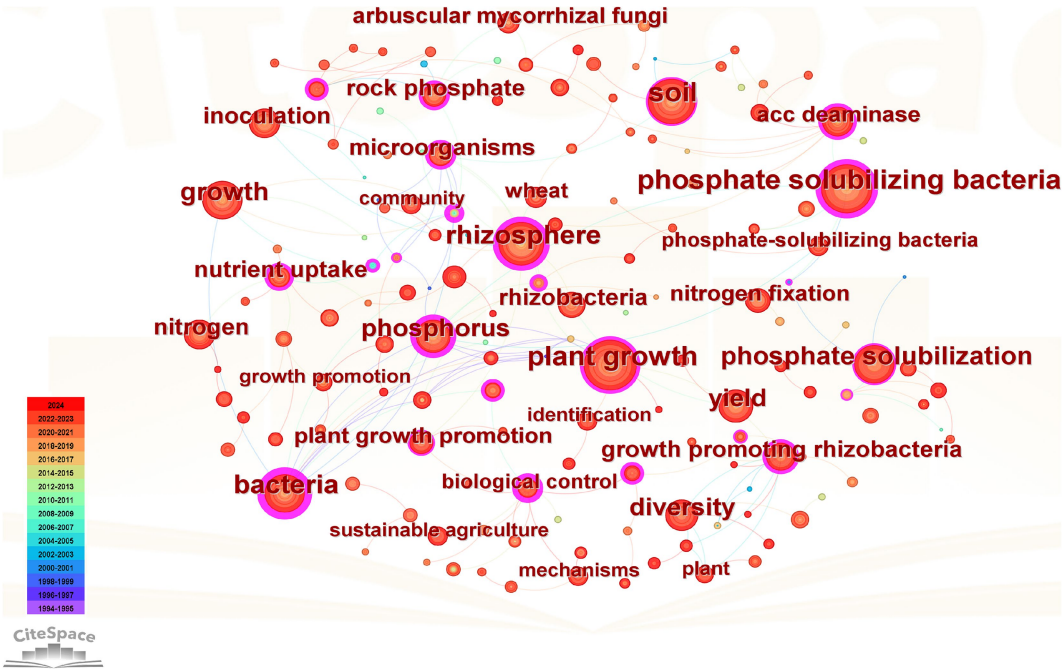


FIGURE 5
Co-occurrence network of core keywords. Each node represents a keyword, with the node size indicating its frequency of occurrence in the literature. The color of the nodes reflects the timeline of publication. Lines represent co-occurrence relationships.

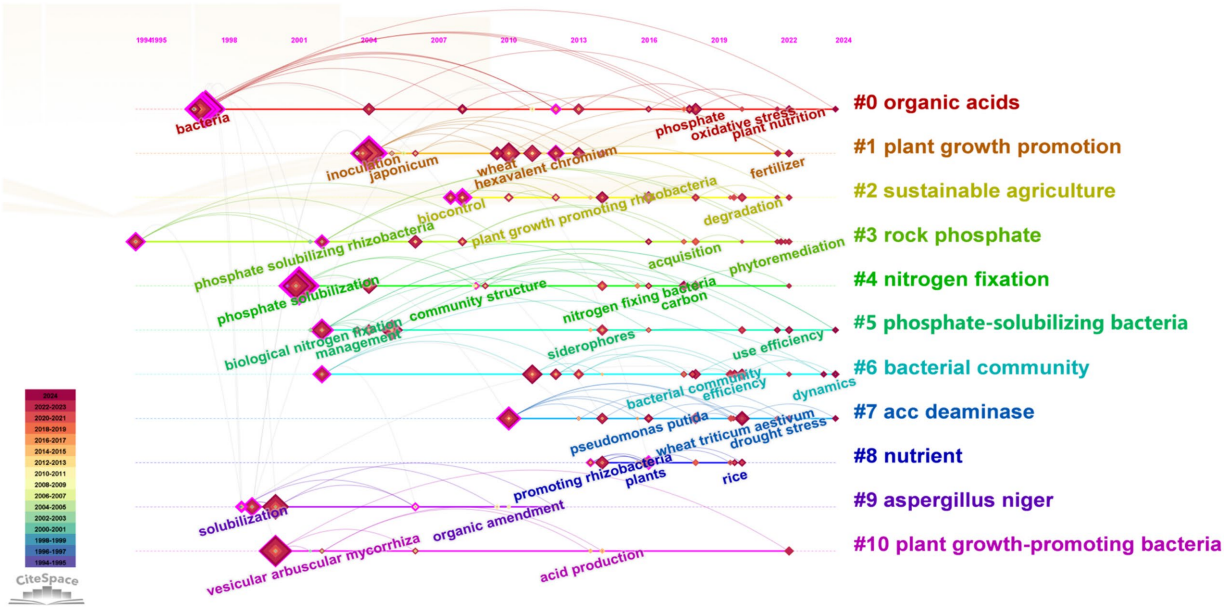


FIGURE 6
Keyword co-occurrence network timeline. The larger nodes represent high-frequency keywords in these fields, indicating the significant influence of these topics over the past few decades. The different colored lines illustrate the connections and interdisciplinary research between various themes.

challenges. Early studies primarily focused on the fundamental mechanisms of phosphate solubilization. Rodríguez and Fraga (1999) emphasized that the secretion of organic acids, such as citric and oxalic acids, is a key mechanism, as these acids lower soil pH and promote phosphate solubilization. Illmer and Schinner (1992) demonstrated that environmental conditions (e.g., pH, temperature, and carbon sources) significantly influence the efficiency of bacterial phosphate solubilization. Kim et al. (1997) identified acid phosphatase and phospholipase as crucial enzymes for the mineralization of organic phosphorus, converting it into plant-available forms. Further molecular studies revealed that certain bacteria (e.g., *Pseudomonas*) possess genes regulating the synthesis

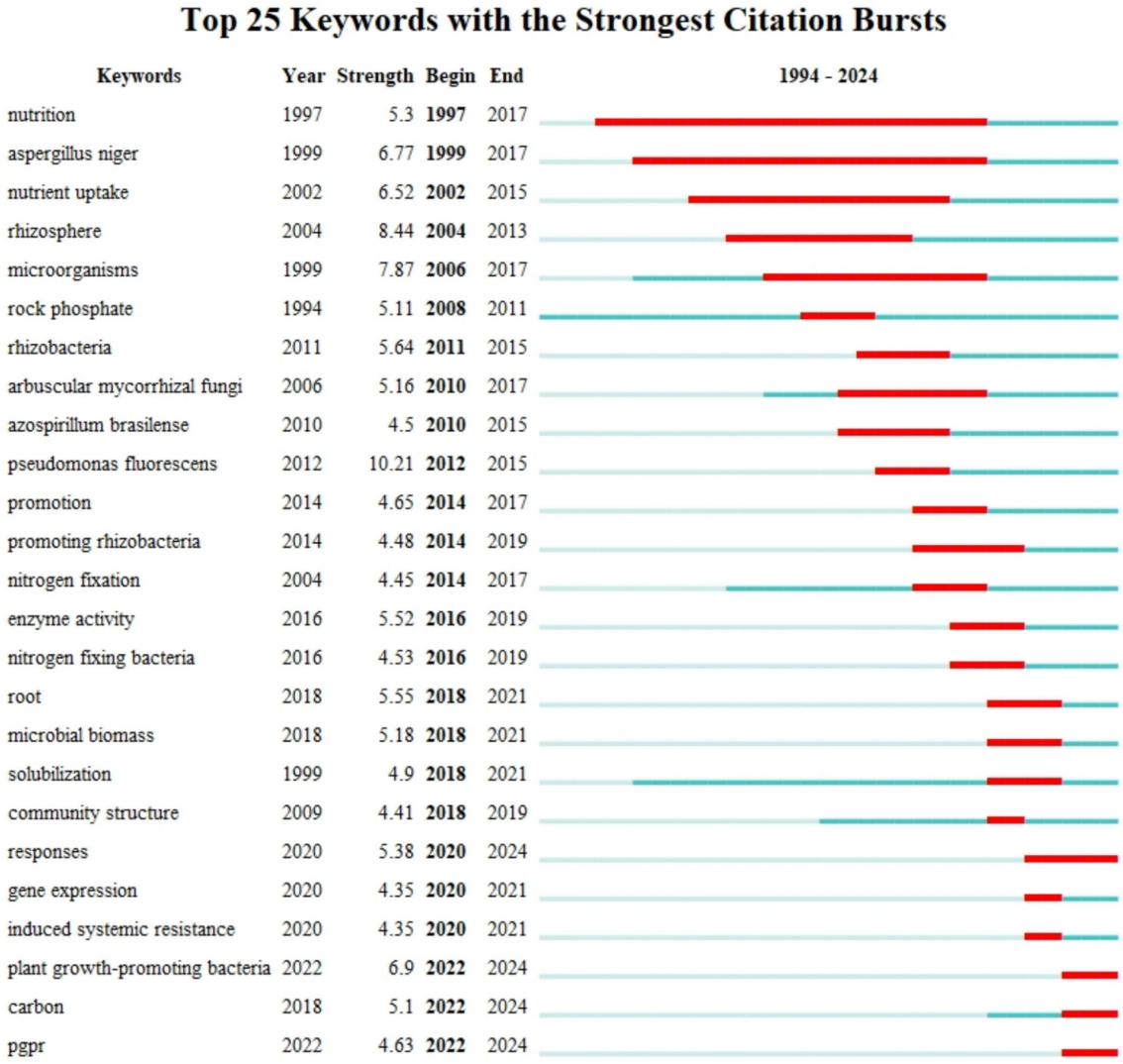


FIGURE 7
Top 25 keywords with the strongest citation bursts from 1994 to 2024.

of organic acids and extracellular enzyme secretion, which are controlled by environmental phosphate levels (Goldstein, 1986). Additionally, microbial interactions, such as the synergistic relationship between phosphate-solubilizing bacteria and AMF, can enhance plant phosphorus uptake (Zheng et al., 2024; García-Berumen et al., 2025). These studies, conducted primarily in Europe and North America, laid a theoretical foundation for developing PSM-based microbial fertilizers. Despite the limited number of publications and modest growth in the field, studies explored the mode of action of PSM (Toro et al., 1997), their diversity (De Souza et al., 2000), and their interactions with plant roots (Sharma, 2003).

In the following decades, PSM research expanded globally, with notable contributions from countries like India, China, and Brazil. Key institutions, including the Indian Council of Agricultural Research and the Chinese Academy of Sciences, advanced PSM applications in agriculture and soil management. Studies emphasized PSM synergies with other microorganisms. Kohler et al. (2007) and Egamberdiyeva and Höflich (2003) highlighted interactions with AMF and plant growth-promoting rhizobacteria, demonstrating enhanced nutrient uptake and plant growth, particularly in tropical and subtropical regions. During this period, policy support in many countries began to emerge, recognizing the role of biofertilizers in sustainable agriculture. For instance, India's National Mission for Sustainable Agriculture and China's Green Development Policy encouraged the use of biofertilizers like PSM to improve soil fertility and reduce chemical fertilizer dependency (Jianbo Shen et al., 2024; Kaur et al., 2024).

Thematic priorities diversified to address nutrient management, ecosystem restoration, and microbial interactions. Research focused on PSM roles in improving soil health, plant growth, and productivity under environmental stress (Rodríguez and Fraga, 1999; Khan et al., 2007). Some studies demonstrated field applications for phosphorus-deficient soils, further supporting the potential of PSM in improving soil fertility and aiding in the bioremediation of heavy metal contamination, particularly lead, cadmium, and chromium (Sharma et al., 2013; Guo et al., 2021a; Guo et al., 2021b; Li et al., 2022). Recent studies have expanded this understanding by examining PSM performance across diverse ecosystems, climates, and soil types. For

TABLE 2 Top ten co-citation articles in PSM research.

Rank	Article title	Authors	Co-citation	Published year	Reference
1	Phosphorous solubilizing bacteria and their role in plant growth promotion	Rodríguez et al.	3321	1999	Rodríguez and Fraga (1999)
2	Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils	Sharma et al.	2816	2013	Sharma et al. (2013)
3	An efficient microbiological growth medium for screening phosphate solubilizing microorganisms	Nautiyal	2291	1999	Nautiyal (1999)
4	Phosphorous solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities	Chen et al.	2158	2006	Chen et al. (2006)
5	Mobilization of phosphorous in soil in connection with the vital activity of some microbial species	Pikovskaya	2123	1948	Pikovskaya (1948)
6	Microbial phosphorus solubilization and its potential for use in sustainable agriculture	Alori et al.	2007	2017	Alori et al. (2017)
7	Universal chemical assay for the detection and determination of siderophores	Schwyn and Neilands	1788	1987	Schwyn and Neilands (1987)
8	Plant growth-promoting bacteria: mechanisms and applications	Glick	1626	2012	Glick (2012)
9	Colorimetric estimation of indoleacetic acid	Gordon and Weber	1103	1951	Gordon and Weber (1951)
10	A modified single solution method for the determination of phosphate in natural waters	Murphy and Riley	1037	1962	Murphy and Riley (1962)

instance, PSM applications in arid and semi-arid regions have shown promise in mitigating drought stress and enhancing soil fertility (Rodríguez and Fraga, 1999). In saline soils, certain halotolerant PSM strains have effectively solubilized phosphorus, contributing to plant resilience and productivity (Kushwaha et al., 2024). Similarly, PSM adapted to acidic soils have improved phosphorus availability and crop yields in tropical regions (Khan et al., 2007). Synergistic interactions with other microbes, such as nitrogen-fixing bacteria (Zaidi et al., 2009) and potassium-solubilizing microorganisms (Yadav, 2022), improved soil nutrient bioavailability and supported ecological restoration on a larger scale. These research advancements aligned with global policy initiatives, such as the European Union's Farm to Fork Strategy, which promotes sustainable agricultural practices by supporting microbial fertilizers through funding and regulatory frameworks (Kour et al., 2020; Dasgupta et al., 2021).

The post-2020 period has seen a global shift in PSM research, driven by pressing challenges such as climate change, environmental stresses, and the need for ecological resilience. This trend is marked by growing contributions from countries like India, China, and the United States, with key institutions such as the Indian Institute of Soil Science, the Chinese Academy of Agricultural Sciences, and the United States Department of Agriculture advancing this field. Some research on PSM has concentrated on their utilization in alleviating environmental stresses induced by climate change, particularly under drought and salt conditions (Kumari et al., 2023); the cooperative interactions between PSM and other microorganisms, enhancing nutrient acquisition and pathogen resistance in crops (Phour and Sindhu, 2023); genomic and metabolomic investigations to elucidate phosphate solubilization mechanisms and identify highly efficient strains (Kumari et al., 2023). In addition, through collaborative efforts between academia and industry, progress has been made in industrial applications that promote psm in sustainable agricultural practices (Phour and Sindhu, 2023). This has a positive impact on soil health and the carbon cycle and can contribute to the sustainable development of agro-ecosystems. Moreover, public-private partnerships have facilitated the commercialization of PSM-based products, bridging scientific research and practical agricultural applications (Verma et al., 2019). However, challenges such as production standardization, quality control, and regulatory disparities across regions must be addressed to maximize PSM's potential in sustainable agriculture (Gopalakrishnan et al., 2015).

Significant progress has been made in the study of PSM from 1984 to 2024, but several challenges remain, particularly concerning their integration into sustainable ecosystem management. Key issues include: (1) limited understanding of molecular and enzymatic mechanisms underlying phosphate solubilization (Rodríguez and Fraga, 1999; Raghothama and Karthikeyan, 2005). (2) The environmental adaptability of PSM remains a critical yet insufficiently explored area, particularly given their variable performance across diverse ecosystems, climatic conditions, and soil types. The underlying mechanisms by which temperature fluctuations, soil pH, moisture levels, and nutrient availability affect PSM survival, colonization, and phosphate-solubilizing efficiency have not been thoroughly elucidated (Alori et al., 2017). (3) underdeveloped multifunctional traits, such as nitrogen fixation and plant growth promotion (Adesemoye et al., 2009). (4) Insufficient research on PSM interactions with soil microbes, particularly in nutrient cycling and ecosystem stability (Richardson and Simpson, 2011; Bashan et al., 2013). (5) Research on the long-term stability and ecological safety of PSM is limited, particularly regarding their impact on native microbial communities and soil functions (Vassilev et al., 2013). (6) Inconsistent policies and regulations across regions hinder the large-scale commercialization and adoption of PSM-based biofertilizers. The absence of unified standards for production quality, application methods, and environmental safety creates barriers to industry adoption and farmer use (Yadav and Yadav, 2024). Addressing these challenges will be vital

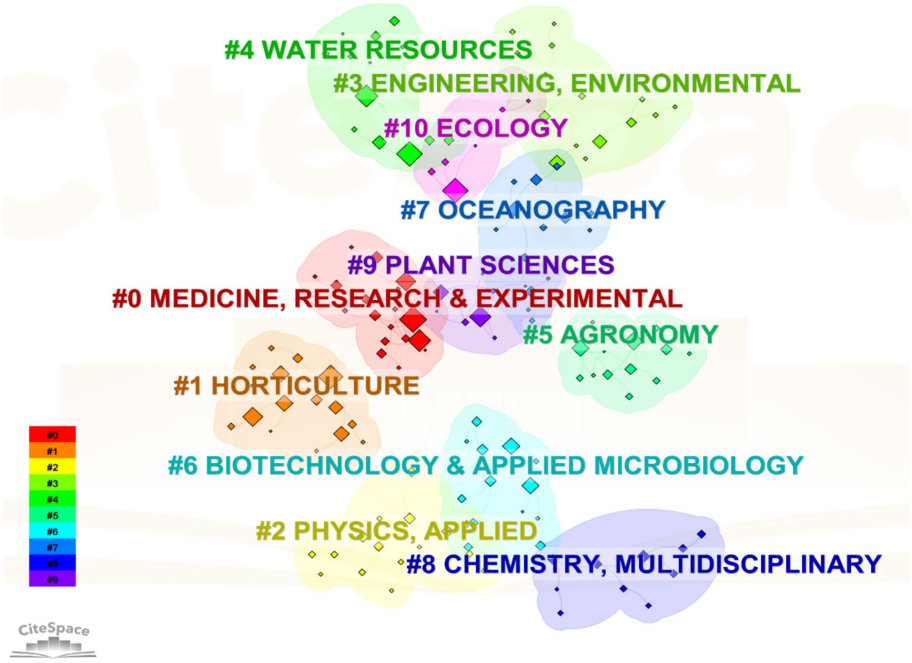


FIGURE 8
Subject co-occurrence analysis in the scientific research field of PSM. Different colors represent distinct clusters research themes. Each color typically indicates a specific area of study.

for advancing PSM-based solutions for sustainable agriculture and ecosystem restoration.

5 Conclusion

This bibliometric study analyzed 1,662 publications on PSM spanning 1984–2024, revealing distinct evolutionary phases in research focus and applications. The initial phase (1984–2008) concentrated on isolating and characterizing PSM, emphasizing their biochemical mechanisms for phosphorus solubilization. The subsequent period (2009–2017), the research expanded to agricultural applications, exploring PSM as biofertilizers within organic farming systems. The recent phase (2018 to present) reflects an increased focus on ecological restoration, soil pollution control, and their role in addressing global challenges such as climate change and nutrient resource recycling.

Despite significant progress, challenges in PSM remain, such as limited understanding of their mechanisms across environments, poor integration of genomic and metabolic analyses, and inconsistent field outcomes due to environmental factors. Future research should focus on: **(1) Unraveling Functional Mechanisms**: Using omics approaches to uncover metabolic pathways and regulatory networks involved in phosphate solubilization. **(2) Environmental Adaptability**: Investigating PSM resilience and adaptability under varying environmental stresses, such as drought, salinity, and extreme temperatures, to ensure consistent field performance. **(3) Multifunctional Microbial Design**: Engineering PSM with additional functionalities such as nitrogen fixation and stress tolerance using advanced biotechnological tools. **(4) Microbial Interactions**: Exploring how PSM influence and are influenced by native soil microbiomes to maintain ecological balance. **(5) Biofertilizer Development**: Focusing on scalable production technologies, formulation stability, and delivery methods to improve the commercial viability of PSM-based biofertilizers. **(6) Policy and Research Integration:** Promoting interdisciplinary research and policy integration to support the sustainable application of PSMs in agriculture and ecological restoration. By expanding research in these directions, PSM can be more effectively harnessed to address global challenges such as soil degradation, food insecurity, and climate change. This comprehensive approach will support resilient agricultural systems and accelerate progress toward global sustainability targets.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://data.mendeley.com/drafts/xrp4hp58gs.

Author contributions

YL: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. YK: Data curation, Visualization, Writing – review & editing. MG: Visualization, Writing – review & editing. HZ: Writing – review & editing. YY: Conceptualization, Writing – review & editing. HH: Funding acquisition, Project administration, Resources, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1546852/full#supplementary-material

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Tripartite microbial augmentation of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54, and *Piriformospora indica* on growth, yield, and nutrient profiling of soybean (*Glycine max* L.)

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Introduction: Enhancing productivity and nutrient content of soybean (*Glycine max* L.) is vital for sustainable agriculture. The utilization of beneficial bacterial and fungal strains has shown promising results in promoting plant growth and improving nutrient uptake. However, the effects of the individual and interactions of such microbes on soybean growth, yield, and nutrient profiling remain inadequately understood. Thus, there is a pressing need to comprehensively investigate the impact of tripartite microbial augmentation on soybean cultivation.

Methods: This field study aims to elucidate the synergistic mechanisms underlying the interactions between *Bacillus* sp. MN54, *Bradyrhizobium diazoefficiens*, and *Piriformospora indica* and their collective influence on soybean growth parameters, yield and nutritional quality.

Results: *In vivo* compatibility tests revealed that consortium applications led to a maximum of 90% soybean germination. The field study demonstrated a significant increase in plant height (17.01%), nodules plant^{-1} (17.35%), pods plant^{-1} (12.11%), and grain yield (20.50%) due to triple inoculation over untreated control. The triple inoculation also significantly increased chlorophyll a, b, and leghemoglobin contents by 19.38, 21.01, and 14.28%, respectively, compared to control. Triple inoculation promoted crude fiber, protein, and oil content by 14.92, 8.78, and 10.52%, respectively, compared to the untreated control. The increase in nitrogen content by 7.33% in grains and 6.26% in stover and phosphorus by 11.31% in grains and 12.72% in stover was observed through triple application over untreated control.

Discussion: Our findings highlight the potential of microbial inoculants as biofertilizers in sustainable soybean production. The triple inoculation with

Bacillus sp. MN54, *Bradyrhizobium diazoefficiens*, and *Piriformospora indica* significantly improved soybean growth, yield, grain quality attributes, and nutrient uptake. This microbial consortium application could help to enhance agricultural productivity by boosting the nodulation in soybean and improving synergism between the microbial strains.

KEYWORDS

triple inoculation, nodulation, *Bradyrhizobium diazoefficiens*, *Piriformospora indica*, *Bacillus* spp., pulses

1 Introduction

Rapid population growth and climate change pose significant challenges to global food production, and cause natural resource scarcity. As the world's population approaches 9 billion by 2050, addressing malnutrition becomes increasingly urgent (Kumar and Dubey, 2020). To maximize the produce from current agricultural land, agricultural pressure generated by population increase has led to extensive usage of chemical fertilizers and pesticides, and among them, about 20–30% of the fertilizers are absorbed by the plants (Saeed et al., 2021). Inefficient nutrient usage in agriculture and soil dynamics results in more than half of fertilizers being lost to the environment (Fageria, 2014). Unfortunately, nitrogen (N) fertilizers given to the soil are easily volatilized, washed away, while Phosphorus (P) fertilizers gets fixed and gradually changed into unavailable forms by natural processes, putting the ecology and biodiversity at greater risk. As a result, total agricultural productivity has decreased, and environmental issues such as habitat loss, carbon emissions, water contamination, and soil pollution have occurred (Zou et al., 2022). Farmers interests are likewise impacted by increasing agricultural input costs and lowering their profits. This situation demands alternative approaches to enhance the efficiency of applied fertilizers and ensure environmental sustainability.

Microbes, particularly those associated with plant roots, collectively termed the plant microbiome, emerge as pivotal allies in reducing the chemical fertilizer demand (Bender et al., 2016). Among these, beneficial microbes known as Plant Growth-Promoting Rhizobacteria (PGPR) and mycorrhizal fungi have been extensively documented for their ability to enhance plant nutrient uptake, promote growth through hormonal interactions, and increase resistance to abiotic stresses such as drought and salinity (Khan et al., 2016; Rajkumar et al., 2017). The mechanisms by which these microbes operate not only underline the potential to overcome nutrient deficiency but also aid crops in adapting to adverse environmental conditions. Furthermore, combining microbial solutions offers a dual benefit: sustaining crop yields and mitigating adverse environmental impacts.

Among leguminous crops, soybean (*Glycine max*) is one of the most crucial crops globally, valued for its high protein content and role in sustainable agriculture through nitrogen fixation. The symbiotic relationship between soybean plants and the nitrogen-fixing bacteria is well-documented, facilitating significant reductions in nitrogen fertilizer requirements (Hungria and Mendes, 2015). Still, the native nitrogen-fixing bacteria usually have nutritional competitiveness and poor survival rates, making them unable to ensure adequate nodulation and sufficient biological nitrogen fixation (Masson-Boivin and Sachs, 2018). One of the methods for increasing biological nitrogen fixation is inoculating seed with biological nitrogen-fixing bacteria. However, further enhancement of soybean productivity may be achieved through co-inoculation with other beneficial soil bacteria, such as *Bacillus* species (Solanki et al., 2023). This integrative approach leverages the combined effects of nitrogen-fixing bacteria, known for its nitrogen-fixing capabilities, and *Bacillus*, recognized for promoting plant growth by various mechanisms, including phytohormone production, phosphate solubilization, and antagonistic activity against plant pathogens (Ahmad et al., 2019; Pankievicz et al., 2021). For example, *Bacillus* strains, which are prolific producers of growth-promoting substances and biocontrol agents, can significantly affect root architecture and thereby improve the efficacy of nitrogen-fixing bacteria in the rhizosphere through a healthier root environment (Xie et al., 2016). This co-inoculation strategy not only boosts the overall health and growth rate of soybean but also contributes to a more sustainable and environmentally friendly farming practice to overcome nutrient deficiency (Compant et al., 2010).

Similarly, it has been found that endophytic growth-promoting microorganisms and nitrogen-fixing bacteria species act synergistically to boost the nitrogen-fixation efficiency of lentils (Saini and Khana, 2012). For the past few decades, *Piriformospora indica* (*P. Indica*) has been a famous and most studied endophytic fungus for vegetative increase and plant resistance to nutrient deficiency (Iqbal et al., 2023). *P. Indica* improves host plants' growth, biomass, and seed yield and confers resistance to various abiotic and biotic stresses (Franken, 2012). The dual and consortium microorganisms have enhanced plant productivity (Varma et al., 2012). It has been found that the effect of single and dual inoculation in legumes was observed in previous studies (Ferreira et al., 2019; Jaiswal and Dakora, 2020; Gupta et al., 2022), however, no study was conducted on multiple inoculation to enhance soybean growth and yield. Therefore, this study aimed to evaluate the synergistic effects of tripartite microbial augmentation of *Bradyrhizobium diazoefficiens* (*B. diazoefficiens*), *Bacillus* sp. MN54 and *P. indica* on soybean growth, yield, and nutrient uptake under field situations. This microbial augmentation was evaluated for synergistic effects to improve nodulation in soybean and nutrient uptake.

2 Materials and methods

2.1 Acquiring endosymbionts and PGPR culture

The microbial cultures of well-characterized plant growth-promoting *Bacillus* sp. MN54 (accession number KT375574) (Naveed et al., 2014; Afzal et al., 2020) was obtained from the Institute of Soil and Environmental Science, University of Agriculture, Faisalabad, Pakistan. *B. diazoefficiens* cultures were obtained from the Japan Collection of Microorganisms,[1] while *P. Indica* culture was obtained from the German Collection of Microorganisms and Cell Cultures, GmbH.[2] The subculture of *B. diazoefficiens* was prepared in Yeast Mannitol extract broth (YMEB) at 28 ± 1°C and 120 rpm for five days. The nutrient broth was used to grow *Bacillus* sp. MN54 at 28 ± 1°C and 120 rpm for 48 h. The *P. indica* was grown in potato dextrose broth (PDB) at 28 ± 1°C and 120 rpm for 1 week. The subcultures were kept at 4°C for further use.

2.2 Evaluation of the compatibility of endophytes and PGPR under axenic conditions

The compatibility of microbial cultures of *B. diazoefficiens*, *P. indica*, and *Bacillus* sp. MN54 was tested through the streak plate method (Raja et al., 2006) and a germination assay. In the streak plate method, *P. indica* biomass was cut with a sterile cutter, placed in the middle of a plate containing nutrient agar medium, and kept for control. *Bacillus* sp. MN54 and *B. diazoefficiens* were streaked on one side, and the *P. indica* culture block was placed on the opposite side with three replications and kept at 28 ± 2°C in an incubator for five days. After incubation, these cultures were observed for compatibility with each other. During the germination assay, healthy soybean seeds were surface sterilized using 3.0% sodium hypochlorite solution for 2 min and then washed thoroughly with sterilized water. The surface sterilized seeds were treated with (1) *B. diazoefficiens* inoculation, (2) *B. diazoefficiens* + *Bacillus* sp. MN54 inoculation, (3) *B. diazoefficiens* + *P. indica* inoculation, (4) *B. diazoefficiens, Bacillus* sp. MN54 and *P. indica* inoculation. The 1 mL log phase culture was used with a ratio of 1:1:1 in triple-inoculation. Treated seeds were kept in Petri dishes comprising 0.7% water agar at 28°C in the growth room for seven days, and each treatment was repeated thrice (Wasike et al., 2009).

2.3 Effect of endosymbionts and PGPR on soybean cultivation under field conditions

A field experiment was conducted in two consecutive summer seasons of 2021 and 2022 at oilseed Research Institute, AARI, Faisalabad, Pakistan. The soil used in this experiment was sandy clay loam having EC 1.4 dS m^{-1}, organic matter 0.67%, pH 7.75, total nitrogen 0.032%, available phosphorus 7.40 mg kg^{-1}, and extractable potassium 116 mg kg^{-1}. The experiment was designed in a randomized complete block design (RCBD) with 24 plots. The soybean variety "Faisal" was used at the rate of 120 kg ha^{-1} with a row-to-row distance of 30 cm in a plot size of 4.0 m × 1.2 m (4.8 m^2). The pooled mean of maximum and minimum temperature during the crop growth period was 39.9 and 27.8°C, respectively. The research location has a subtropical climate, receives 25 mm of rainfall annually, and is between latitude 31.4187°N, longitude 73.0791°E, and 186.0 m above sea level. The treatments in terms of seed coating were (1) Control, (2) *B. diazoefficiens*, (3) *Bacillus* sp. MN54, (4) *P. indica*, (5) *B. diazoefficiens* + *Bacillus* sp. MN54, (6) *P. indica* + *Bacillus* sp. MN54, (7) *B. diazoefficiens* + *P. indica,* and (8) *B. diazoefficiens* + *Bacillus* sp. MN54 + *P. indica* in triplicate.

Soybean seeds were inoculated with respective cultures of *B. diazoefficiens*, *Bacillus* sp. MN54 and *P. indica* alone and in different combinations according to the treatment plan. For this purpose, surface sterilized seeds were inoculated with inoculum-based slurry in 1:1 ratio (50% seed and 50% slurry). This slurry was prepared using 50% inoculum, 30% sterilized peat, 10% clay, and 10% sugar solution. At the time of inoculation, the CFU of *B. diazoefficiens* and *Bacillus* sp. was MN54 1 × 10^8 CFU mL^{-1}, while for *P. indica*, CFU was 1 × 10^6 CFU mL^{-1}. In co-inoculation and triple-inoculation, the *B. diazoefficiens*, *Bacillus* sp. MN54 and *P. indica* were applied to soybean seeds at a 1:1 ratio and 1:1:1 ratio, respectively. The number of seeds was uniform in all the experimental plots. Seeds were thoroughly mixed until a thin, fine layer of inoculum appeared. The seeds were spread in the shade to dry overnight in the laboratory. Field was prepared by giving three ploughings followed by planking. The treated seeds were sown in mid-June with a seed rate of 70 kg ha^{-1}. After germination, the plant-to-plant distance of 5–6 cm was maintained while the row-to-row distance was 45 cm. All the agronomic practices were adopted for cultivating the soybean crop. The recommended doses of chemical fertilizers used in the experiment were 25–25–50 NPK kg ha^{-1} in the form of Urea, single superphosphate (SSP), and sulphate of potash (SOP), respectively. Half of N, all P, and K doses were mixed with soil at the time of sowing, and the remaining N was applied with the first and second irrigations with equal splits. Observations regarding emergence percentage were recorded after 10 days of sowing. Nodular data was recorded at the flowering stage. Plant height, grain yield, number of pods, and dry biomass were recorded at harvesting. Chlorophyll and leghemoglobin contents were recorded after 90 days of sowing (DAS). After harvesting, nutrient contents, seed protein, fiber, moisture, and oil contents were recorded.

2.4 Plant agronomic performance

Ten plants were randomly selected from each replication of a 2-year trial to determine the plant height with the help of a meter rod on a cm scale. Data on emergence count was determined by recording the number of emerged seedlings per meter row length from a central row of each plot after leaving two border rows on each side. Ten randomly selected plants were uprooted from each

1 https://jcm.brc.riken.jp/en/

2 https://www.dsmz.de/

plot and were sun-dried and then oven-dried at 60°C for two days to record the dry weight of the shoot in grams (g).

Symbiotic dependency (SD) was recorded at 90 DAS. To determine the SD of soybean, the formula given by Gerdemann (1975) was used:

$$Symbiotic\ dependency\ = \frac{Dry\ weight\ of\ inoculated\ plants}{Dry\ weight\ of\ uninoculated\ plants} \times 100$$

Nodulation parameters, including the number of nodules and the dry weight of nodules, were recorded by taking an average number of nodules carefully detached from ten randomly uprooted plants. The detached nodules were oven-dried at 60°C for two days, and the dry weight of nodules per plant was recorded in g. The grain yield from each plot (Kg m^{-2}) was recorded, and the final data was presented in kg ha^{-1}.

2.5 Physiological attributes

The chlorophyll content of leaves was estimated using the method of Arnon (1949) at 645 and 663 nm wavelengths through a spectrophotometer (Agilent Technologies, Santa Clara, CA, United States). At noon (between 10:00 and 14:00), after 90 DAS, physiological parameters were recorded from entirely green leaves. For chlorophyll a and b content, plant leaves were crushed in acetone and centrifuged at 10,000 rpm for 10 min. Then, the supernatant was subjected to record absorbance of chlorophyll "a" at 645 nm and that of "b" at 663 nm contents through a spectrophotometer (Agilent Technologies, Santa Clara, CA, United States; Arnon, 1949). Root nodules were taken at the flowering stage to determine the volume of pink bacteroid tissue of nodules and a minor section of nodules (5 μL) was made using a sharp cutter. The amount of pink bacteroid tissue comprising the leghemoglobin in the nodule cortex was determined following the method described by Sadasivam and Manickam (2008). According to this method, 1 g of fresh root nodules was taken and crushed in 5 mL of cold phosphate buffer (0.1 M) with the help of a pestle and mortar in an ice-cold environment. The homogenized mixture was centrifuged at 10,000 rpm for 10 min at 4°C, and a supernatant containing leghemoglobin was collected. Further impurities were removed by adding chilled acetone in 4:1 ratio of nodule extract and incubated for 15 min. The mixture was again centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was discarded, and the precipitate was dissolved in a phosphate buffer. The absorbance of this mixture was read at 540 nm using a spectrophotometer (Agilent Technologies, Santa Clara, CA, United States), and leghemoglobin concentration was calculated using the following formula. The molar extinction coefficient of leghemoglobin was taken around 11 mM cm^{-1}, and the path length (1 cm) refers to the distance that light travels through the sample in the spectrophotometer cuvette.

$$Leghemoglobin\ \left(mg\ g^{-1}\right)\ = \frac{Absorbance\ at\ 540\ nm}{Molar\ extinction\ coeffient\ \times\ Path\ length\ \times\ Sample\ weight}$$

2.7 Assessment of seed quality and nutrient acquisition

Seeds were carefully cleaned and ground to pass through a 0.4 mm screen. AOAC. (1975) method was followed for proximate analysis to determine crude fiber, protein, and moisture content. The protein contents were determined by converting total nitrogen content using a specific factor of 6.25, using the Kjeldahl method. The moisture content in seeds was determined by oven drying 5 g of seeds at 105°C until they reached a constant weight, which was calculated using the following formula.

$$Moisture\ content\ (\%)\ = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100$$

For crude fiber determination, 2 g of dried ground seeds were acid-digested using 1.25% sulfuric acid. The mixture was boiled for 30 min, filtered, and washed with hot distilled water to neutralize the pH. Further alkali digestion was performed through 1.25% sodium hydroxide and boiled for 30 min. The mixture was again washed with distilled water to neutralize the pH. The residue samples were oven-dried at 105°C until constant weight. The crude fiber of residue samples was performed through dry ashing in a muffle furnace at 550°C for 2 h to remove organic matter, and samples were weighed to calculate crude fiber using the following formula.

$$Crude\ fiber\ (\%)\ = \frac{Weight\ of\ dried\ reside - Weight\ of\ dry\ ash}{Weight\ of\ sample} \times 100$$

Seed oil content was determined using Soxhlet's apparatus as described in AOAC. (1984). This method extracted 5 g oven-dried seed samples using hexane solvent in the Soxhlet apparatus. A rotary evaporator was used to recover oil from the solvent-oil mixture. The extracted oil was dried at 105°C for 30 min, and oil contents were calculated using the following formula.

$$Oil\ contents\ (\%)\ = \frac{Weight\ of\ extracted\ oil}{Weight\ of\ sample} \times 100$$

The total nitrogen (N) content of shoot and grain was determined using Kjeldahl's McKenzie and Wallace (1954) technique with slight modification. Estimation of total phosphorous (P) content of shoot in straw and seed was carried out by digestion using a triacid mixture (HNO_3: $HClO_4$: H_2SO_4) (v/v) (Jackson, 1973). Micronutrient contents like iron (Fe), Zinc (Zn), and manganese were determined by following the method of Estefan et al. (2013).

2.8 Statistical analysis

The collected data in triplicate was subjected to analysis of variance (ANOVA) in Randomized Complete Block Design (RCBD) through the software Statistix version 8.1. The Least Significant Difference (LSD) test (Steel et al., 1997) was employed to compare the mean values of each attribute at $p \leq 0.05$. The correlation matrix and principal component analysis were performed using R software version 4.1.2.

3 Results

3.1 *In vitro* and *in vivo* experiments demonstrated the compatibility of triple inoculation

From the streak plate method, it was observed that *B. diazoefficiens and Bacillus* sp. MN54 showed a synergistic effect against *P. indica*. Moreover, during the germination assay, germination was increased by triple inoculations (*B. diazoefficiens*, *Bacillus* sp. MN54, and *P. indica*) than by inoculation *B. diazoefficiens* alone. A maximum increase in germination (90%) was observed by triple inoculations over inoculation with *B. diazoefficiens* alone (Table 1).

3.2 Triple inoculation promoted plant growth and symbiotic traits of soybean under field conditions

Inoculation significantly affected all growth parameters compared to uninoculated control (Figure 1). A maximum increase in plant height (17.01%) was observed in plants that received combined inoculations (*B. diazoefficiens*+ *Bacillus* sp. MN54 + *P. indica*) followed by *Bacillus* sp. MN54 + *P. indica* (13.15%) over uninoculated control (Figure 1A). The highest emergence (16.31%) was recorded when *B. diazoefficiens* was applied in combination with *P. indica* and *Bacillus* sp. MN54 over control (Figure 1B). Moreover, significant increases in the number of leaves and number of branches were observed in all inoculated treatments over uninoculated control, but the maximum increase in number of leaves plant^{-1} (17.83%) and number of branches plant^{-1} (20.40%) were recorded in plants that received triple inoculations over *B. diazoefficiens* application alone (Figures 1C,D).

The results of symbiotic dependency by plants treated with individual symbionts or with triple inoculations are given in Table 2. It was noted the plants inoculated with *B. diazoefficiens* alone and in combination with *Bacillus* sp. MN54, *P. indica* inoculation showed a positive growth response over the uninoculated control. The highest symbiotic efficiency response (161.85%) was observed in plants that received triple inoculation plants (*B. diazoefficiens* + *Bacillus* sp. MN54 + *P. indica*) over control. Similarly, dual inoculation has a more pronounced effect when compared with *B. diazoefficiens* alone, but maximum response (132.95%) was observed in *B. diazoefficiens* + *Bacillus* sp. MN54 + *P. indica* treated plants over the plants solely inoculated with *B. diazoefficiens*. The highest number of nodules plant^{-1} (17.35%) was observed in triple inoculation (*B. diazoefficiens*+ *Bacillus* sp. MN54 + *P. indica*) over uninoculated control (Figure 2A). This treatment response was also higher over the application of *B. diazoefficiens* alone. Data regarding nodular dry weight was also presented in Figure 2B. It has been found *B. diazoefficiens* inoculant increased dry weight (10.52%) over control. This increase was more pronounced when *B. diazoefficiens* was combined with *P. indica* and *Bacillus* sp. MN54. A maximum increase in nodular dry weight (26.31%) was observed with triple inoculation of *B. diazoefficiens*, *P. indica,* and *Bacillus* sp. MN54 over control.

TABLE 1 Compatibility test of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54 and *Piriformospora indica* on seed germination assay under axenic conditions.

Treatments	Germination (%)
Control	60 ± 2.24 e
B. diazoefficiens	70 ± 2.95 d
B. diazoefficiens + *Bacillus* sp. MN54	81 ± 3.64 c
B. diazoefficiens + *P. indica*	83 ± 2.19 b
B. diazoefficiens + *Bacillus* sp. MN54 + *P. indica*	90 ± 3.58 a

Treatment-sharing means with the same alphabetical letters were considered statistically non-significant.

3.3 Triple inoculation response to yield traits of soybean under field conditions

Combined inoculations significantly increased soybean grain yield over the sole application of *B. diazoefficiens* and to uninoculated control (Figure 3A). The maximum increase of 20.50% was observed in triplicate inoculation, i.e., *B. diazoefficiens* + *P. indica* and *Bacillus* sp. MN54 as compared to uninoculated control, as shown in Figure 3A. The co-inoculation of *Bacillus* sp. MN54 + *B. diazoefficiens,* and *P. indica* + *B. diazoefficiens* increased biomass (10.36 and 11.70%, respectively) over *B. diazoefficiens* alone (Figure 3B). However, maximum biomass (18.05%) was recorded in the plants that received a combined application of *B. diazoefficiens*, *Bacillus* sp. MN54 and *P. indica* as compared to uninoculated control. An increase was observed in 100 grain weight and number of pods plant^{-1}. The increase of 11.16% was observed in 100-grain weight in triple inoculation over control (Figure 3C). Similarly, this treatment showed an increase in the number of pods plant^{-1} by 12.50% over the untreated control (Figure 3D).

3.4 Enhancement in physiological and quality traits of soybean under field conditions

A significant improvement was observed in chlorophyll contents by applying single, dual, and triple inoculation over un-inoculated control (Figure 4). It has been observed that the effect of co-inoculation and triple inoculations was more pronounced over the application of *B. diazoefficiens* alone. The highest increased in chlorophyll a and b contents, i.e., 19.38 and 21.01%, respectively, were observed by application of *Bacillus* sp. MN54 and *P. indica*, along with *B. diazoefficiens*, compared to control (Figures 4A,B). Similarly, significant increases in leghemoglobin contents were observed in treated plants against untreated control. A 7.10 and 10.71% increase was observed by co-inoculation of *P. indica* along with *B. diazoefficiens*, and *Bacillus* sp. MN54 mixes *B. diazoefficiens*, respectively, over control. But maximum leghemoglobin contents (14.28%) were recorded in the combination of all three inoculants (*B. diazoefficiens* + *Bacillus* sp. MN54, and *P. indica*) over

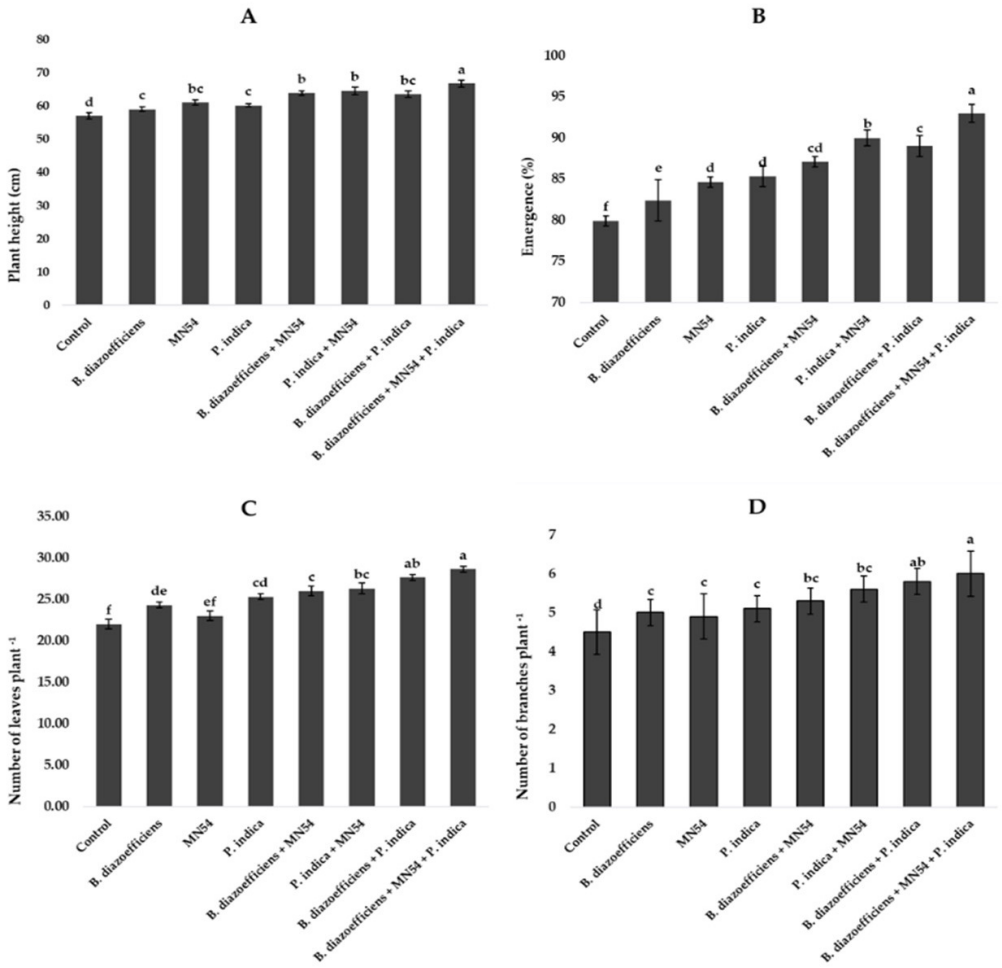


FIGURE 1
Effect of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54 and *Piriformospora indica* on **(A)** plant height, **(B)** emergence (%), **(C)** number of leaves plant^{-1}, **(D)** number of branches plant^{-1} in soybean under field conditions. The error bars represent the least significant difference among treatments at $P \leq 0.05$.

un-inoculated control as shown in Figure 4C. On the pooled mean basis, it has been found that a significant increase in all quality parameters has been observed in treated plants as compared to untreated (Figure 5). The co-inoculation increased quality parameters followed by single inoculation over control. The maximum increase in crude fiber, crude protein, moisture percentage, and oil contents was recorded in triplicate inoculation of *B. diazoefficiens, Bacillus* sp. MN54, and *P. indica* (14.92, 8.78, 19.28, and 10.52%, respectively) over uninoculated control (Figures 5A–D).

TABLE 2 Effect of *Bradyrhizobium diazoefficiens, Bacillus* sp. MN54 and *Piriformospora indica* inoculation on symbiotic dependency (%) of soybean under field conditions.

Treatments	Symbiotic dependency (%)	
	Over control	Over *B. diazoefficiens*
B. diazoefficiens	121.73 ± 12.2 d	–
B. diazoefficiens + *Bacillus* sp. MN54	125.71 ± 11.4 c	103.07 ± 14.5 c
B. diazoefficiens + *P. indica*	150.08 ± 9.8 b	123.29 ± 17.7 b
B. diazoefficiens + *Bacillus* sp. MN54 + *P. indica*	161.85 ± 14.2 a	132.95 ± 25.9 a

Treatment-sharing means with the same alphabetical letters were considered statistically non-significant.

3.5 Microbial inoculations promoted the nutrient profile of soybean under field conditions

Results showed that dual and triple inoculation had a significant effect on nutrient contents in soybean over uninoculated control as shown in Figures 6, 7. Soybean plants that received triple inoculations had high grain and stover N and P contents, followed by co-inoculation as compared to untreated control and single inoculation. Maximum increases in N contents (7.33 in grain and 6.26% in stover) were observed in treatment where *B. diazoefficiens* was applied along *Bacillus* sp. MN54 and *P. indica* over uninoculated control (Figures 6A,B). Similarly, an increase in P contents was observed in the co-inoculation of

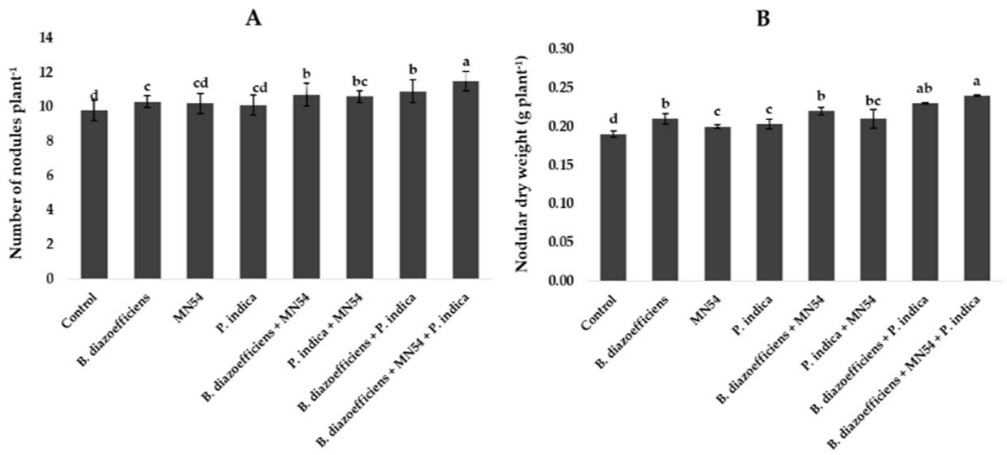


FIGURE 2
Effect of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54 and *Piriformospora indica* on **(A)** number of nodules plant^{-1}, **(B)** nodular dry weight in soybean under field conditions. The error bars represent the least significant difference among treatments at $P \leq 0.05$.

B. diazoefficiens and *P. indica* over *B. diazoefficiens* alone. But maximum P in grain (11.31%) and stover (12.72%) was found in a combination of *B. diazoefficiens, Bacillus* sp. MN54 and *P. indica* over untreated control, as shown in Figures 6C,D. Results also showed that single, co-inoculation, and triple-inoculation had significantly improved micronutrient contents in soybean grains over uninoculated control. The highest Fe (15.60%), Zn (11.11%), and Mn (13.25%) contents were recorded in triple inoculations treatment (*B. diazoefficiens*, *Bacillus* sp. MN54, and *P. indica*) over control (Figures 7A–C).

3.6 Results from Pearson correlation and principal component analysis

A strong positive correlation was observed in all studied growth, yield attributes, and mineral contents of soybean crops under field conditions (Figure 8). Principal component analysis (PCA) was performed to observe interrelationships among various parameters as shown in Figure 9. The first biplot showed that among all the components, the first two components viz. PC1 (Dim1) and PC2 (Dim2) exhibited maximum contribution, accounting for 87.8% of the total dataset. Principal components 1 (Dim1) and 2 (Dim2) explained 73.9 and 13.9% of the variability among the variables studied. The distribution of all treatments showed that all inoculated treatments positively affected plant growth, yield, and nutrient contents over control. All treatments with single or co-inoculation were displaced from control. Still, the treatment where triple-inoculation was used had a more significant displacement from control, indicating a more pronounced effect.

4 Discussion

In this study, we found considerable improvement in the growth of soybean under the application of *B. diazoefficiens*, *Bacillus* sp. MN54, and *P. indica* either alone or in combination (Figure 1). A significant positive response was observed in plant height where treatments were applied compared to uninoculated control. This increase may be directly associated with the acceleration of plant growth by producing phytohormones (Kaur and Sharma, 2013; Aziz et al., 2020; Rafique et al., 2021). A significant increase in emergence, number of leaves per plant, and number of branches per plant were also observed with the alone application of microbes, but the combined application effect was more pronounced. According to Orrell and Bennett (2013), *Rhizobia* are critical in encouraging plants' hostile behavior toward diseases and herbivores. This encourages and improves several growth metrics, including seed germination, emergence, seedling vigor, plant stand, root and shoot growth, and total biomass of the plants, including seed weight (Ravikumar, 2012). Studies revealed that plant growth-promoting bacteria (PGPB) exhibit a variety of traits that encourage plant growth, including the synthesis of exopolysaccharides and the solubilization of phosphate, zinc, production of indole acetic acid (IAA), and HCN. When exposed to various environmental and soil conditions, different PGPB frequently exhibit one or more features that promote plant growth (Olanrewaju et al., 2017). According to Kalam et al. (2020), numerous plant growth-promoting activities, including IAA and GA production, and P solubilization have been linked to different *Bacillus* species (Miljakovic et al., 2020; Magotra et al., 2021). Seed germination is a crucial factor and essential to generating total biomass and yield. It has been found that inoculation with *Bacillus* sp. showed increased in seedlings germination of Arachis hypogea (Shifa et al., 2015). Because they produce antibiotics, phytohormones, and the ability to solubilize phosphate, *Bacillus* sp. like *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus pumilus*, and *Bacillus polymyxa* are well known for their capacity to promote plant growth and development (Majumdar, 2017). Moreover, Tsimilli-Michael and Strasser (2013) reported that *Piriformospora indica* has also been identified as a new candidate symbiont capable of delivering significant growth-promoting activity to various plants, including agricultural and medicinal crops.

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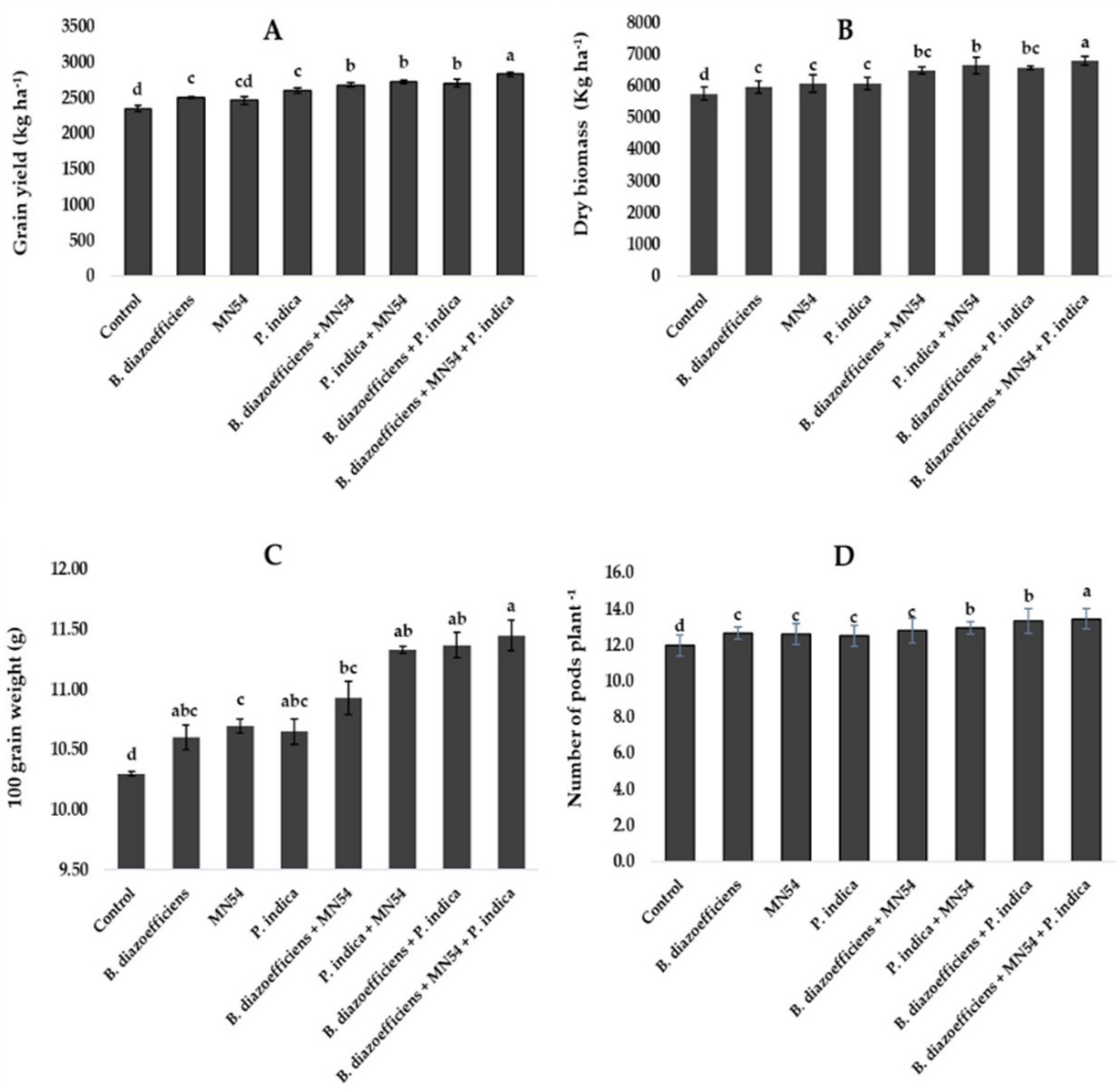


FIGURE 3
Effect of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54 and *Piriformospora indica* on **(A)** grain yield, **(B)** dry biomass, **(C)** 100 grain weight, **(D)** number of pods in soybean under field conditions. The error bars represent the least significant difference among treatments at $P \leq 0.05$.

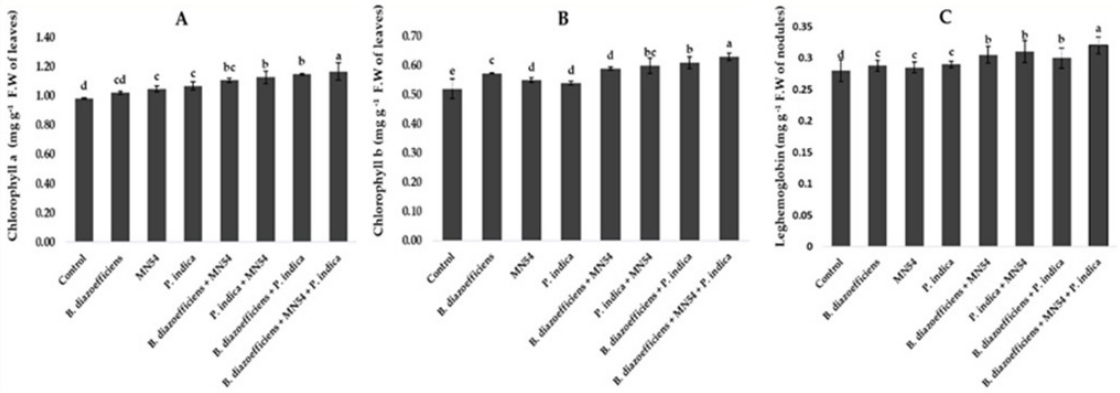


FIGURE 4
Effect of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54 and *Piriformospora indica* on **(A)** chlorophyll a contents, **(B)** chlorophyll b contents, **(C)** leghemoglobin contents in soybean under field conditions. The error bars represent the least significant difference among treatments at $P \leq 0.05$.

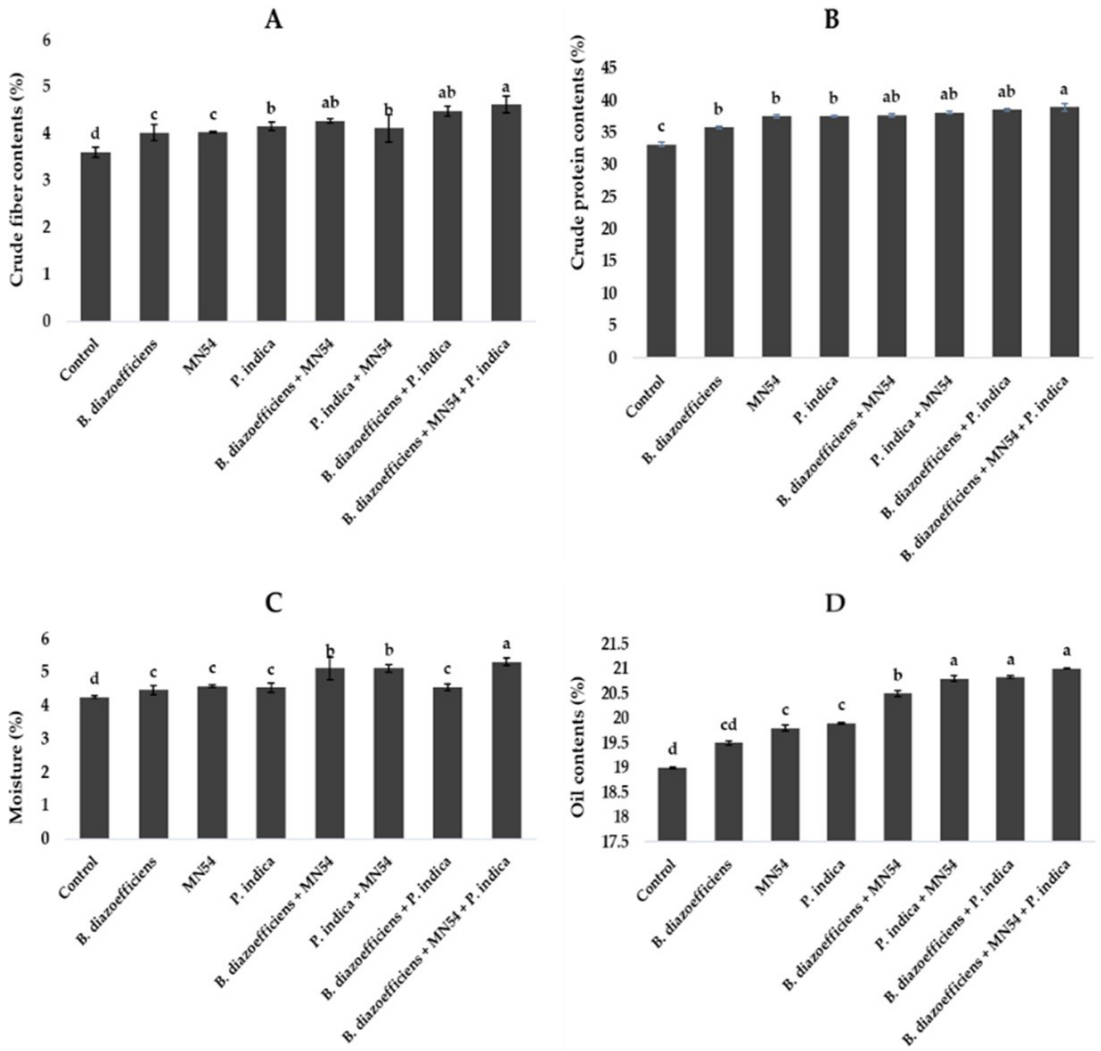


FIGURE 5
Effect of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54 and *Piriformospora indica* on **(A)** crude fiber contents, **(B)** crude protein contents, **(C)** moisture contents, **(D)** oil contents in soybean under field conditions. The error bars represent the least significant difference among treatments at $P \leq 0.05$.

In our study, all the treated plants showed increased nodule count and nodule dry weight (Figure 2). The current results are in line with the findings of Hungria et al. (2015) and Janagard and Ebadi-Segherloo (2016), who discovered that plants grown from infected seeds had more nodule growth and dry weight than plants grown from uninoculated seeds. As a result of the symbiotic interaction between *Rhizobia* and soybean plants, root nodules often start to form and expand, increasing the amount of nitrogen fixation in the plant (Tirichine et al., 2006). According to Vasileva and Ilieva (2012), rhizobia are of considerable scientific and commercial significance due to their capacity to fix atmospheric nitrogen in leguminous plants. Through synthesizing unique signal molecules known as Nod factors, rhizobia promote the development of root nodules in leguminous plants (Boundless com, 2021). *Rhizobia* produces ammonia inside the nodules, which host plants utilize as a source of absorbed nitrogen. This use significantly reduces the need for chemical fertilizers (Lin et al., 2019). According to research, the *Bacillus* sp. was able to assist the plant growth and solubilize the insoluble nutrients. According to Bhutani et al. (2018), *Bacillus* is the most prevalent non-rhizobial endophytic species in summer crops and can promote plant root development. Auxin-responsive genes are regulated by PGPR, which alters root architectural characteristics by controlling endogenous IAA levels in rice roots (Ambreetha et al., 2018). The boost in nodulation in the current study could also be attributed to higher metabolism in *P. indica*-infested plants, which have enabled them to deliver more significant amounts of carbohydrates to the *Rhizobia*. Our results are supported by earlier research (Rokhzadi and Toashih, 2011; Verma et al., 2012), which shows that PGPR can increase native rhizobia's capacity to produce more chickpea nodules. Chickpea nodule formation may have benefited from native AMF and inoculated *P. indica* mobilizing phosphates from the soil. Our findings are consistent with those of Nautiyal et al. (2010), who showed that *P. indica* and PGPR consortia improved the ability of native rhizobia to nodulate chickpeas. According to Tavasolee et al. (2011), the opposite is true; PGPR bacterial

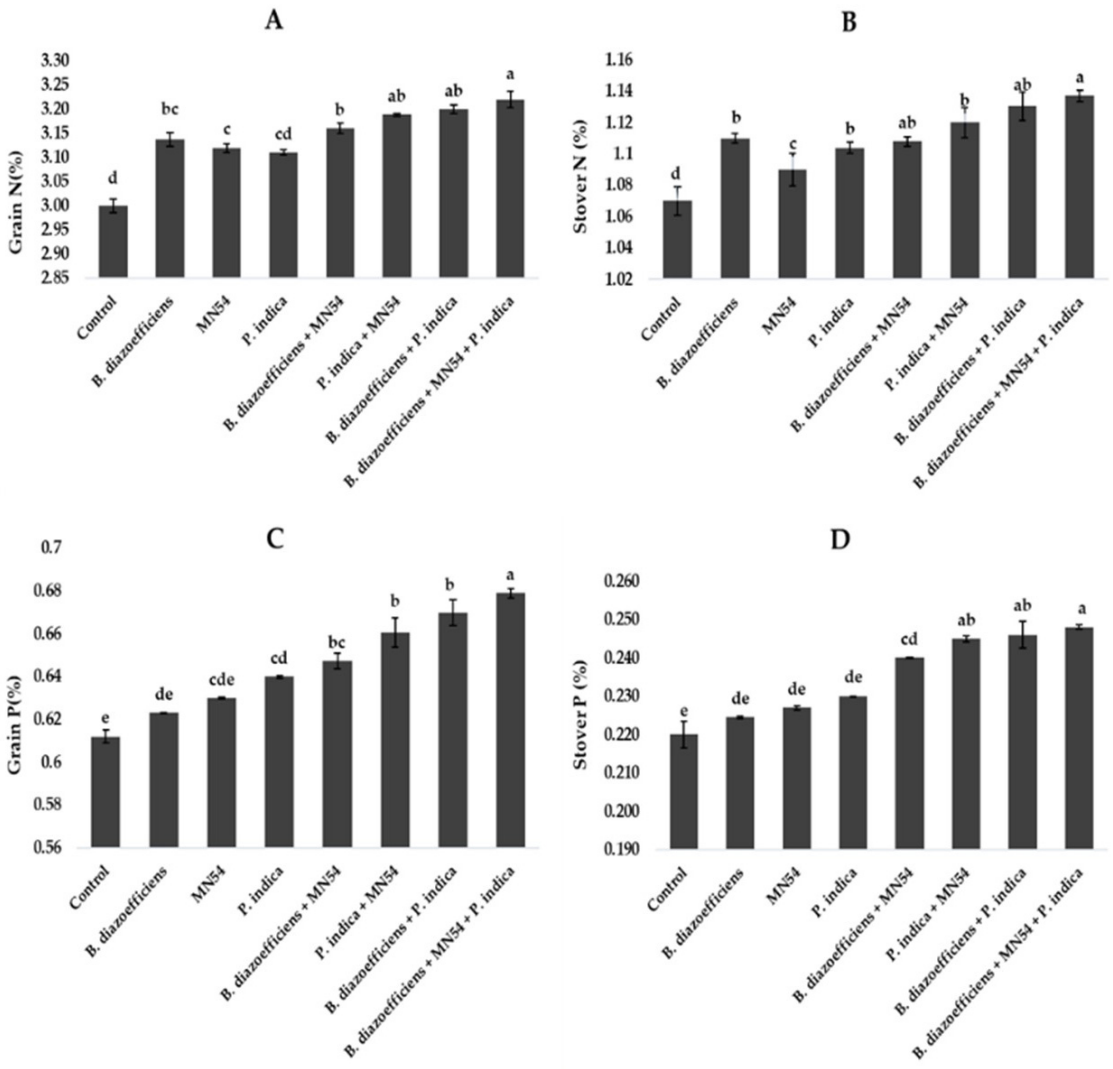


FIGURE 6
Effect of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54 and *Piriformospora indica* on **(A)** grain N, **(B)** stover N, **(C)** grain P, **(D)** stover P in soybean under field conditions. The error bars represent the least significant difference among treatments at $P \leq 0.05$.

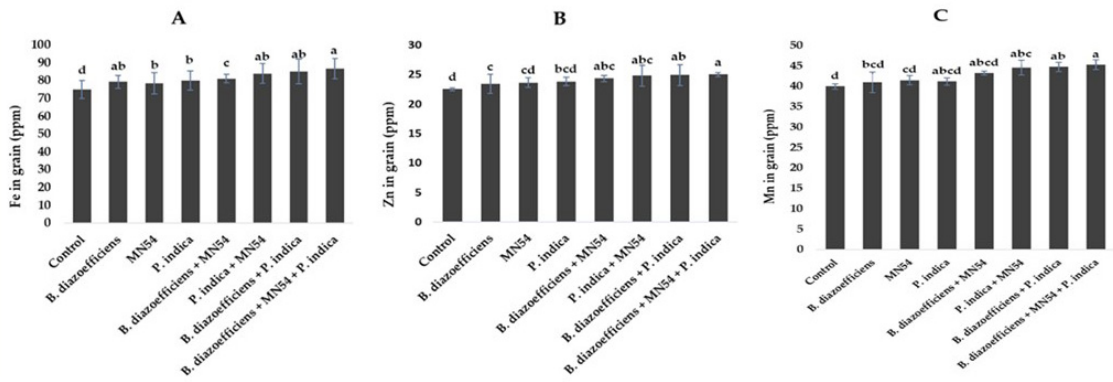


FIGURE 7
Effect of *Bradyrhizobium diazoefficiens*, *Bacillus* sp. MN54 and *Piriformospora indica* on **(A)** Fe in grain, **(B)** Zn in grain, **(C)** Mn in grain in soybean under field conditions. The error bars represent the least significant difference among treatments at $P \leq 0.05$.

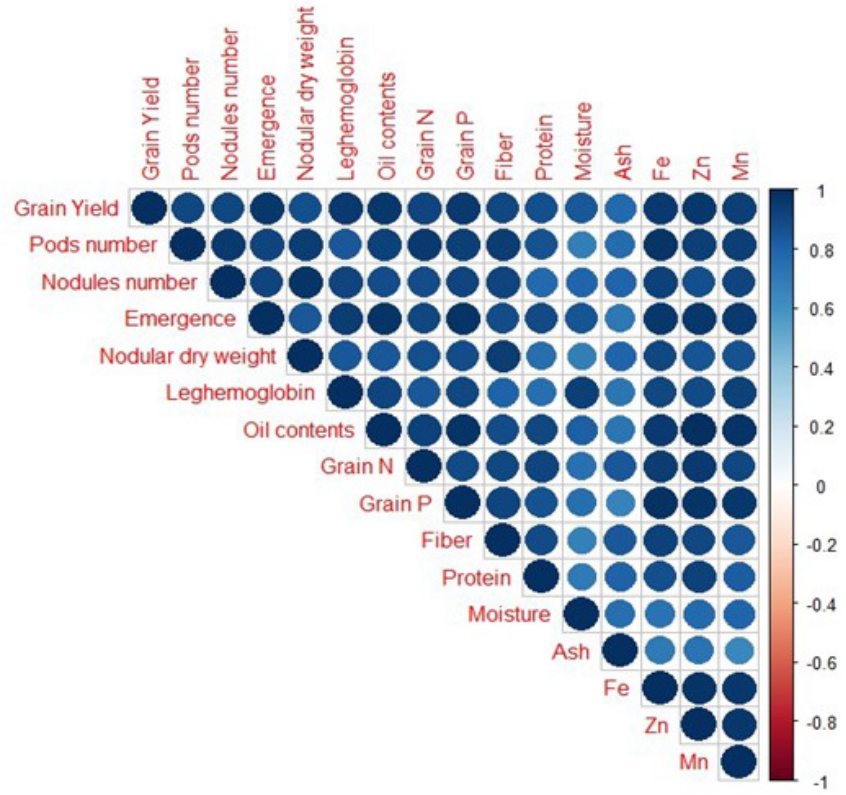


FIGURE 8
Correlation among measured parameters of soybean grown under field conditions. Positive correlations are displayed in blue color. The color intensity and the size of the circle are proportional to the correlation coefficients.

strains decreased the fresh mass of nodules in chickpeas due to competition for photosynthetic materials.

The findings of our investigation showed that seed inoculation with *Rhizobia* boosted grain yield, biomass, and pod number; however, this reaction was further strengthened by adding *Bacillus* and *P. Indica* (Figure 3). Alam et al. (2015) revealed that soybean plants inoculated with *Rhizobia* produced more pods than uninoculated plants. It may be due to increased nitrogen availability that directly enhanced photosynthesis and biomass production. More biomass generally includes increased leaf area, which is crucial for photosynthesis and energy production, thus supporting more extensive growth and enabling the plant to support more reproductive structures, including pods. Moreover, using *Bacillus* sp. to boost *V. radiata* growth and yield proved successful. According to Kumari et al. (2018), *Bacillus megaterium*, *Bacillus pumilus*, and *Bacillus subtilis* all lengthened shoots by 55.55, 46.46, and 46.20% over control, respectively. Good plant development was seen in *Arachis hypogea* exposed to *Bacillus licheniformis* (Shifa et al., 2015). A promising method for increasing plant development and decreasing the use of toxic chemical fertilizers is to inoculate soil or crops with PGPB (Alori et al., 2012). Additionally, Meena et al. (2010) found that *P. indica*-inoculated plants had higher P contents, and Achatz et al. (2010) found that colonized plants had higher photosynthetic rates and improved production. Similarly, Ray and Valsalakumar (2010) reported that co-inoculation with *Rhizobia* and AM fungi enhanced plant vigor and nutrient uptake and significantly boosted the yield of green gram.

In our investigation, treated plants showed a substantial increase in photosynthetic pigments (Figure 4) that could be a result of the synergistic action of applied microbes, which increased the supply of N to the plant, which is a crucial structural component of chlorophyll (Zohaib et al., 2019). This study found that inoculations of *Bradyrhizobium*, *Bacillus* MN54, and *P. indica* significantly improved leghemoglobin content compared to *Bradyrhizobium* alone treatment. This might result from higher nodulation and N_2 fixation increasing the occupancy of effective nodules, which might have increased the leghaemoglobin content. These findings fit well with the results of Kunal, and Sharma (2012), who reported a positive correlation between leghaemoglobin content and nitrogen fixation in chickpeas due to *Rhizobia* inoculation. Furthermore, one of the causes of enhanced nodulation in such plants is higher P nutrition in legumes connected to AMF. AMF are known to increase the amount of N available to legumes by promoting the growth of nodules with high levels of leghemoglobin. This might result from higher nodulation and N_2 fixation increasing the occupancy of effective nodules, which might have increased the leghaemoglobin content.

According to the findings of our study, soybean with single, dual, and triple inoculations had higher fiber, protein, oil, and nutrient contents (Figures 5–7). The inoculation of legume crops with beneficial microbes such as *Rhizobia*, *Bacillus* spp., and *P. indica* can significantly enhance the nutrient content of the seeds, particularly protein, fat, and fiber. Each microorganism contributes uniquely through various biological mechanisms, improving plant

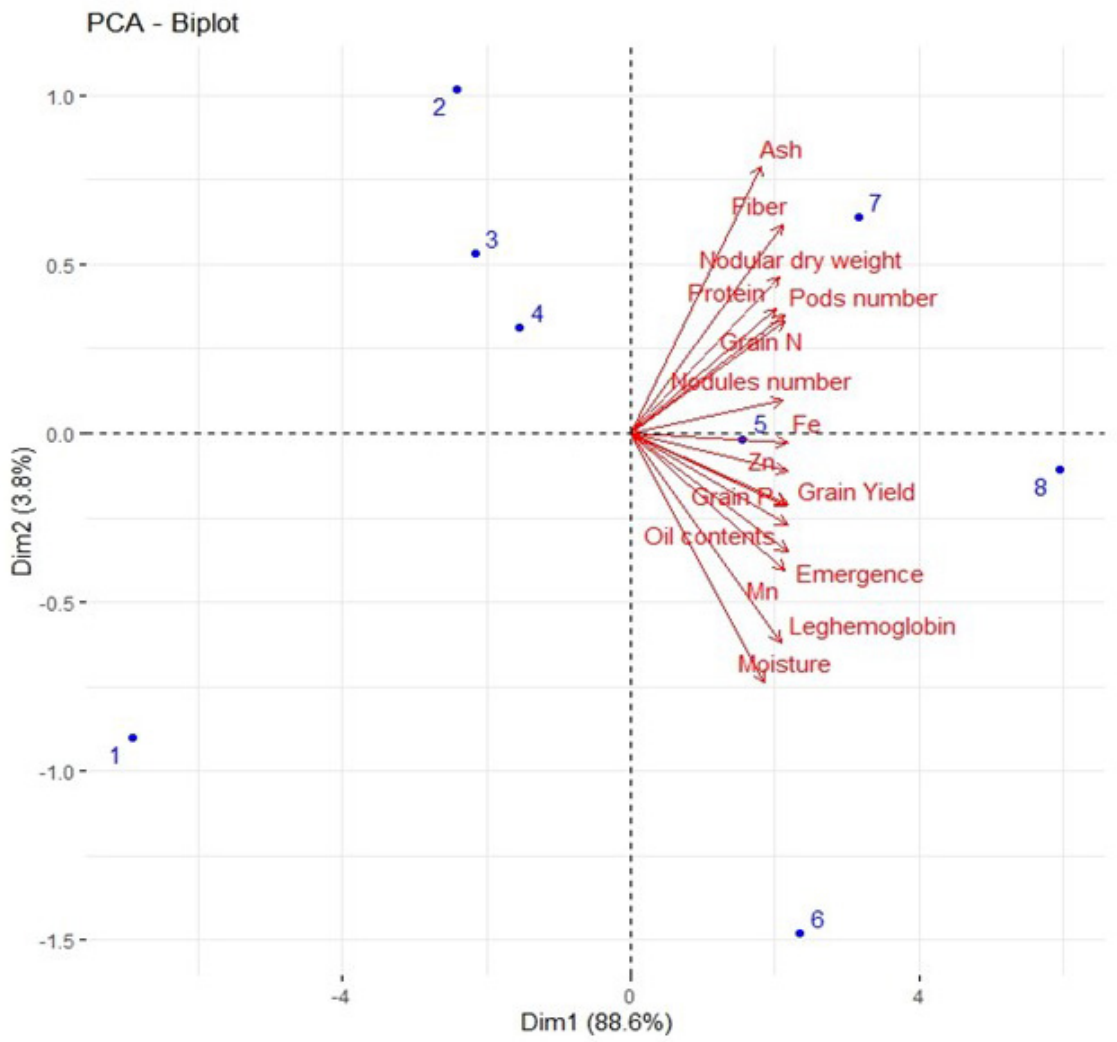


FIGURE 9
Represents the PCA biplot among measured parameters of triple inoculation in soybean under field conditions. Treatments are as T_1. Control, T_2. *B. diazoefficiens* inoculation, T_3. *Bacillus* sp. MN54 inoculation, T_4. *P. indica* inoculation, T_5. *B. diazoefficiens* + *Bacillus* sp. MN54, T_6. *P. indica* + *Bacillus* sp. MN54, T_7. *B. diazoefficiens* + *P. indica*, T_8. *B. diazoefficiens* + *Bacillus* sp. MN54 +*P. indica*.

growth, nutrient uptake, and metabolic efficiency. *Rhizobia* are best known for their role in nitrogen fixation. They form nodules on the roots of leguminous plants, where they convert atmospheric nitrogen into ammonia, making it available to the host plant. This directly enhances protein synthesis in the plant because nitrogen is a critical component of amino acids, the building blocks of proteins (Sulieman et al., 2015). Meanwhile, *Bacillus* sp. MN54 and *P. indica* have a significant role in enhancing phosphorus solubilization and nutrient uptake. These processes are crucial for efficient metabolic functioning and can increase seed oil (fat) and possibly fiber content by promoting biosynthetic pathways (Bhattacharyya and Jha, 2012; Gill et al., 2016).

In soy bean the combined effect of *R. japonicum* and *P. striata* on seed N, protein, and oil contents was significantly influenced by post-harvest plant sample analysis (Waghmare et al., 2012; Jaga and Sharma, 2015). Compared to uninoculated treatments, seed N and protein content rise due to *Rhizobia* and *Bacillus* inoculation because *Bacillus* promotes N availability and improves seed accumulation (Fatima et al., 2007). Our study's improvement in total N content aligns with previous findings in chickpeas (Nautiyal et al., 2010) and soybean (Lima et al., 2011). This may be due to PGPB promoting root growth and root structure by increasing the number of root hairs and surface area, which thus increases plant nutrient intake and acquisition (De Sousa et al., 2021; Calvo et al., 2017; Adesemoye et al., 2008).

According to Iqbal et al. (2023), phosphate-solubilizing bacteria boost the production of auxin and cytokinin and make inaccessible P available to plants. These microorganisms improve plant P uptake and make it available in inorganic form (Qadir et al., 2024). Earlier research by Malla et al. (2004) found that *P. indica* contained significant amounts of an acid phosphatase with the potential to solubilize phosphate in the soil and deliver it to the host plant, improving P content in *P. indica* treatments. These findings are in line with our study. Moreover, the investigation has been found coherent with the results of Nautiyal et al. (2010), who reported that consortia of *P. indica* and PGPR along with native

chickpea rhizobia increased the P content. PGPR might contribute to soil phosphate pool available for extraradical hyphae of AMF like fungi, to pass on to the plant, especially in soils with low P bioavailability. Furthermore, due to the ability of *P. indica* to colonize host plant roots and create a robust root system increases the soil's ability to absorb nutrients (Hosseini et al., 2019). In addition, PGPR may contribute to the amount of soil phosphate pool available for extraradical hyphae of AMF transmitted to the plant, particularly in soils with low P bioavailability. Additionally, because *P. indica* may colonize host plant roots and develop a robust root system, the soil's capacity to absorb nutrients increases (Hosseini et al., 2019).

5 Conclusion

The tripartite microbial augmentation of Bradyrhizobium diazoefficiens, *Bacillus* sp. MN54 and P. indica significantly influenced soybean growth, yield, and nutrient contents. Our findings demonstrate the substantial enhancements in crucial growth parameters, indicating the positive impact of microbial symbiosis on soybean growth. The application of the microbial consortium resulted in notable improvements in soybean yield. Importantly, our analysis of nutrient profiling revealed significant enhancements in essential nutrients such as nitrogen, phosphorus, zinc, iron, and manganese, highlighting the potential of tripartite microbial augmentation to enhance the nutritional quality of soybean crops. These findings emphasize the importance of harnessing compatible consortium inoculation for sustainable agriculture practices to improve crop productivity and nutritional value. Further research into the mechanisms underlying microbial interactions and their effects on plant-microbe-soil interactions will be valuable for optimizing microbial-based strategies in soybean cultivation and advancing agricultural sustainability.

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

MR: Writing – original draft. MN: Writing – original draft. MM: Writing – review & editing. AN: Writing – original draft. SA: Funding acquisition, Writing – review & editing. SR: Writing – original draft. MS: Writing – review & editing. AM: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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PGPR and nutrient consortia promoted cotton growth, antioxidant enzymes, and mineral uptake by suppressing sooty mold in arid climate

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Introduction: Cotton (*Gossypium hirsutum* L.) plays a vital role in Pakistan's economy, providing significant employment opportunities and supporting the country's textile industry. However, cotton productivity is severely impacted by pests and diseases, such as black spots caused by sooty mold, posing critical challenges to sustainable agriculture. This study investigates a novel integration of plant growth-promoting rhizobacteria (PGPR) with recommended NPK fertilizers and micronutrients to enhance cotton growth, yield, disease resistance, and post-harvest soil properties.

Methodology: A consortium of *Bacillus megaterium* (ZR19), *Paenibacillus polymyxa* (IA7), and *Bacillus* sp. (IA16) were evaluated under six treatments: control (T1), PGPR (T2), recommended NPK (T3), recommended NPK + PGPR (T4), recommended NPK + micronutrients (T5), and recommended NPK + micronutrients + PGPR (T6).

Results: The results depicted a significant increase in antioxidant activities of 19% in superoxide dismutase (SOD), 29% peroxidase (POX), 28% peroxidase dismutase (POD), and 14% catalase (CAT) activity under T6 as compared to control. Similarly, growth parameters substantially improved root length (39%), shoot length (19%), and root and shoot biomass by up to 31 and 20%, respectively, under T6. Moreover, the yield attributes like single boll weight and lint percentage were also enhanced by 32 and 13%, respectively, under the integration. In contrast, the PGPR consortium demonstrated considerable biocontrol potential against sooty mold, as disease incidence was reduced by 68% in cotton, the disease index was 75%, and control efficacy reached 75%. The PGPR consortium also substantially improved post-harvest soil biological and chemical properties, including bacterial populations, microbial biomass nitrogen, organic matter, and essential nutrient availability.

Discussion: So, these findings witnessed the dual behavior of the *Bacillus* and *Paenibacillus* strains with balanced nutrition and can lead us to the development of an effective biopesticide cum biofertilizer for the sustainable production of cotton in arid conditions by combating sooty mold effectively.

KEYWORDS
sooty mold, balanced nutrition, PGPR, nutrient uptake, sustainable agriculture

1 Introduction

Cotton is the backbone of Pakistan's economy, playing a vital role in providing employment opportunities and supplying raw materials for the textile industry (Rana et al., 2020). Globally, Pakistan is the 4th largest cotton producer and ranks as the 3rd largest consumer of cotton. The textile sector is the country's largest industrial domain, employing approximately 40% of the workforce (Economic Survey of Pakistan, 2022; Mehmood et al., 2021; GOP, 2019). Beyond its significance as a fiber crop, cotton is also a key oilseed crop in Pakistan, alongside other major oilseeds like sunflower, canola, and rapeseed (PBS, 2022).

Cotton is grown mainly in arid and semi-arid regions due to its lower water requirements than other cash crops (Chen et al., 2019). However, high temperatures and drought stress in these areas exacerbate sooty mold severity, as plants under stress are more susceptible to pest and fungal attacks. These regions are less fertile and have poor nutrient availability, especially having low diffusion coefficients (Zhang et al., 2017). Agricultural productivity in these regions mainly depends upon the agrochemicals (fertilizers, pesticides) as an integral part of farming systems in these regions. Applying fertilizers and enhancing crop production are responsible for enhancing input costs and environmental deterioration (Ali et al., 2023). For example, despite the abundance of phosphorus in soils, it often remains unavailable and insoluble for plants, making it a major limiting factor in crop production and may accumulate in surface water by soil erosion and runoff from the fertilized field (Mahmood et al., 2024).

Another alarming threat in the arid regions is the attack of sooty mold, which has become a significant threat to cotton production worldwide in the last decade (Belachew and Jenber, 2024). Sooty mold (black fungus) thrives on honeydew secretions from insect pests, *viz.* aphids and whiteflies, forming a black, soot-like coating on cotton leaves and bolls, impairing photosynthesis and reducing crop yield by 40% under higher infestation (Mondal et al., 2020). *Bemisia tabaci* is one of the most destructive pests among 160 insect pests of cotton throughout its growth (Kouser et al., 2019; Naeem-Ullah et al., 2020). *B. tabaci* not only causes direct damage but also facilitates the growth of black fungus through its gummy secretions, impairs photosynthetic activity, and can lead to plant death (Shah et al., 2020). Along with yield reduction, sooty mold deteriorates the fiber strength and lint quality, leading to price penalties for affected cotton (Wrather et al., 2008). Chemical fungicides are used to control the black fungus infection in cotton, but none has been found effective and registered against sooty mold control (Hameed et al., 2023). Moreover, the increased dosage and repeated use may cause fungicide resistance in the fungus and deteriorate the environmental quality (Ziółkowska et al., 2021; Tooker and Pearsons, 2021). Approximately 0.3 billion US$ has been spent annually on pest control, of which 80% is used on cotton crops alone (Khan et al., 2015; Shuban et al., 2024).

Growing concerns about human and environmental health have prompted researchers to shift their focus from synthetic products to safer alternatives for enhancing nutrient use efficiencies and pathogen control (Lahlali et al., 2022). Numerous studies have urged the use of sustainable options for safe crop production and controlling crop pests (Abdullah and Zahoor, 2023; Ayilara et al., 2023; Hezakiel et al., 2024; Dar et al., 2024a, 2024b). One such strategy is to use plant growth-promoting rhizobacteria (PGPR), which reside in the rhizosphere and compete with other microorganisms for food and survival (Kloepper and Okon, 1994). The PGPRs increase crop production following various direct and indirect mechanisms. The direct mechanisms involved nitrogen fixation, phytohormones production [gibberellins, auxins (IAA) and cytokinins], nutrient solubilization (phosphorus, potassium, iron, and zinc), siderophores and exopolysaccharides production (Islam et al., 2013: Maheshwari et al., 2015; Murad et al., 2024). Whereas PGPRs indirectly boost crop growth, *viz.* antibiotics production, lytic enzymes, ACC-deaminase, hydrogen cyanide (HCN), competition, induction of systemic resistance, and secondary metabolites production to cope with crop pests (pathogens and weeds), and abiotic stress tolerance (Beneduzi et al., 2012; Ramadan et al., 2016; Ajinde et al., 2024; Dar et al., 2024a). These bacteria increase nutrient concentration by nutrient solubilization in soil, improving nutrient availability and plant uptake (Hussain et al., 2019). PGPRs alleviate stress and enable crop production under abiotic stress, *viz.* drought (Arzanesh et al., 2011), salinity (Arora et al., 2012), and flooding stress (Tewari and Arora, 2016). In addition, these are also the major contributors to the bioremediation of metal-polluted sites (Zheng et al., 2024; Guo et al., 2021; Kong and Glick, 2017; Mishra et al., 2017).

With the advancement of research and development, the indirect mechanisms of PGPR are being used to suppress pests (weeds and pathogens) in field crops (Hassan et al., 2024). Recently, bacteria, one of the safe alternatives to pesticides, has shown promising results in alleviating sooty mold damage (McLaughlin et al., 2023). Certain strains of *Bacillus subtilis* and *Pseudomonas fluorescens* have demonstrated their antifungal properties by producing secondary metabolites against sooty mold and reducing disease severity by up to 60% in field trials (Kumar et al., 2017). Singh et al. (2016) reported the induction of systemic resistance to boost defense mechanisms and antifungal activities in cotton crops.

The knowledge gap lies in integrating PGPR and balanced nutrition to control cotton sooty mold. Therefore, the present study was designed to test the dual action of PGPR and balanced nutrition for plant growth promotion and fungal disease suppression for sustainable cotton production. This study aimed to investigate the synergistic impact of a specific PGPR consortium (*Bacillus megaterium* ZR19, *Paenibacillus polymyxa* IA7, and *Bacillus* sp. IA16) in combination with balanced nutrition (NPK and micronutrients) application on the growth of two native cotton varieties of Pakistan (IUB13 and IUB4). We also aimed to integrate the PGPR consortium and balanced nutrition to promote nutrient uptake and control sooty mold attacks on cotton. Thus, the novelty of this study lies in using bacteria with dual functions of growth promotion and sooty mold suppression with balanced nutrition, which provides valuable insights for sustainable cotton production tailored to the challenging arid climate. Previously, we explored the PGPR and balanced fertilizers individually for cotton growth (Unpublished). However, there is a gap in integrating PGPR and balanced nutrition for sooty mold control, which we have explored in the present investigation.

2 Materials and methods

2.1 Collection of rhizobacterial strains and cotton seeds

Three rhizobacterial strains were obtained from the culture bank of Soil Microbiology and Biotechnology Laboratory, Department of Soil Science, The Islamia University of Bahawalpur. These bacterial strains were identified as *Bacillus megaterium* ZR19 (MN007186) by Iqbal et al. (2020) and *Paenibacillus polymyxa* IA7 (NM005923) and *Bacillus* sp. IA16 (NM005924) by Ahmad et al. (2021). These strains could solubilize insoluble minerals and demonstrated the production of siderophores, exopolysaccharides, and ammonia, and were positive for cellulase and protease activities (Iqbal et al., 2020; Ahmad et al., 2021). Moreover, these bacterial strains were compatible with growing simultaneously. Seeds of two cotton varieties, i.e., IUB13 and IUB4, were collected from the National Cotton Breeding Institute (NCBI), The Islamia University of Bahawalpur. These cotton varieties were selected for this study due to their local farmer preference and possess high yield potential in the study area, making them relevant for regional agricultural practices.

2.2 Preparation of inoculum and seed inoculation

The collected strains were grown in DF (Dworkin and Foster) salt minimal media (Mumtaz et al., 2022) for 48 h at 100 rpm shaking and 28 ± 2°C to inoculate the cotton seeds. Before coating, seeds were surface sterilized by using ethanol (95%) and $HgCl_2$ (0.2%) and then washed gently with sterilized water (Abd-Alla et al., 2012). The bacterial consortium was developed in a sterilized media storage bottle by taking equal volumes of three bacterial strains in a 1:1:1 ratio of the bacterial cultures and homogenized through vortexing. The cotton seeds of both varieties were coated with the bacterial culture by a slurry-based carrier coating prepared by mixing the inoculum with sterile peat and sugar solution in a 4:5:1 ratio, as reported in our previous work (Mumtaz et al., 2022).

2.3 Pot trial

The effectiveness of bacterial strains was tested along with chemical fertilizers to boost cotton growth and yield under natural conditions. The treatments *viz.* treatments: control (T1), PGPR (T2), recommended NPK (T3), recommended NPK + PGPR (T4), recommended NPK + micronutrients (T5), and recommended NPK + micronutrients + PGPR (T6) were laid out on a completely randomized design (CRD) under factorial settings. Six cotton seeds were sown in each earthen pot with dimensions of 18″ × 12″ (height × diameter) filled with 10 kg sieved (using 2 mm mesh) and dried soil. The soil was obtained from a farmer's field and characterized for the physicochemical attributes (Table 1) using the methods detailed in Handbook 60 (Regional Salinity Laboratory Staff (US), 1954). Recommended fertilizer [urea was used as the source of nitrogen (N), diammonium phosphate for nitrogen (N) and phosphorus (P), and muriate of potash for potassium (K) doses (NPK at 310:170:110 kg ha^{-1}, respectively)] were applied in the pots as basal doses. Nitrogen fertilizer was applied in 3 equal split intervals, including basal, early flowering, and early bol formation stages. These fertilizer sources were chosen based on their availability, effectiveness, and cost-efficiency for the cotton crop, ensuring optimal nutrient supply. The trial was conducted in the wirehouse of the Department of Soil Science, The Islamia University of Bahawalpur. The pots were regularly irrigated with good-quality water to fulfill the irrigation requirements. Antioxidant enzymatic status was estimated at the flowering stage and other growth and yield parameters were determined during harvesting. The biocontrol potential of the strains was evaluated by spraying the PGPR consortium (2 liters per hectare in 1:1:1 ratio for each strain) in T2, T4, and T6 treatments at 55, 85, and 115 days after germination. While T3 and T5 were sprayed with pyriproxyfen (Axxiprox, Swat Agro Chemicals) at 55 days, acephate (FMC) at 85 days, and acetamiprid (Mospilan, Arysta Life Sciences) at 115 days after cotton germination.

TABLE 1 Soil pre-sowing analysis for physicochemical attributes.

Characteristics	Value
pH_s	7.8
EC_e	1.64 dS m^{-1}
Sand	45%
Silt	42%
Clay	13%
Textural class	Loam
Saturation percentage	36%
Total nitrogen	0.022%
Available phosphorous	5.2 mg kg^{-1}
Extractable potassium	80 mg kg^{-1}
Organic matter	0.29%
Iron	3.8 mg kg^{-1}
Zinc	0.66 mg kg^{-1}

2.4 Growth and yield properties

The growth parameters, such as root and shoot lengths, were measured using a meter rod, and the root and shoot fresh using a portable balance immediately after harvesting. The cotton bolls were counted manually, and the seed cotton from the open boll was picked manually and weighed through a portable weight balance. Lint yield was determined by separating the cotton and seeds.

2.5 Biocontrol efficacy of *Bacillus megaterium* (ZR19), *Paenibacillus polymyxa* (IA7) and *Bacillus* sp. (IA16)

The efficacy of the *Bacillus megaterium* (ZR19), *Paenibacillus polymyxa* (IA7), and *Bacillus* sp. (IA16) consortium as a biocontrol agent against sooty mold was evaluated through a foliar application (spray) on cotton plants, and the effect was compared to the impact of synthetic chemicals spray. Disease severity was assessed using a standard rating scale from 0 to 4, quantifying the percentage of plant tissue exhibiting symptoms such as chlorosis, leaf necrosis, or defoliation (0 = healthy plant, 1 = 1–33% affected, 2 = 34–66%

affected, 3 = 67–99% affected, 4 = dead plant). Disease incidence (percentage of infected plants), disease index (average disease severity), and control efficacy (relative reduction in disease incidence) were calculated from the Equations 1–3 developed by Zhu et al. (2013) given below:

$$Disease\ incidence\,(\%) = \left[\left(n_1 + n_2 + n_3 + n_4\right)/n\right] \times 100 \quad (1)$$

$$Disease\ index = \left[\left(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4\right)/4n\right] \times 100 \quad (2)$$

$$Control\ efficacy\,(\%) = \left[\frac{\left(Disease\ index_{control} - Disease\ index_{treatment}\right)}{Disease\ index_{control}}\right] \times 100 \quad (3)$$

Where n_0-n_4 represents the number of plants assigned to each corresponding disease rating, and n denotes the total number of plants assessed.

2.6 Determination of antioxidant enzymatic status

The fresh leaf sample of 0.25 g was thoroughly mixed with 4 mL of pre-cooled phosphate buffer solution (pH 7.8; 0.0663 g of $NaH_2PO_4.2H_2O$ + 16.385 g of $Na_2HPO.12H_2O$ dissolved into 1,000 mL distilled water) in a pre-cooled mortar placed on ice. The homogenized mixture was centrifuged for 20 min at 4°C and 10,000 rpm to collect enzyme extract. After that, the supernatant was taken in Eppendorf tubes and analyzed for antioxidant enzyme activities. For ascorbate peroxide, 2.5 mL of phosphate buffer solution was used, and reading was noted on a spectrophotometer at 290 nm wavelength by following the method of Prochazkova et al. (2001). Catalase and peroxidase activity was measured by following the protocol of Chance and Maehly (1955) at 240 and 470 nm wavelengths, respectively. The assay mixture was prepared by mixing 2.6 mL of 1 mM KH_2PO_4 buffer, 400 µL of H_2O_2, and 40 µL of enzyme extract. The process explained by Giannopolitis and Ries (1977) was employed to assess the superoxide dismutase activity at 560 nm. Antioxidant activities were expressed in terms of units per mg fresh leaf weight (U mg^{-1} FW).

2.7 Macro and micronutrients determination in roots and shoots

Wet digestion of plant samples was done by following the protocol described by Wolf (1982). Five mL of the digested sample was added to the Kjeldahl flask and attached to the Kjeldahl distillation unit by adding 10 mL NaOH (40%). A conical flask containing 5 mL of boric acid (4%) was attached at the receiving point. After collecting about 30–40 mL distillate, the flask was removed from the distillation unit and 5–10 drops of mixed indicator were added. The flask contents were titrated against 0.01 N standard sulfuric acid solution until the pink endpoint. Phosphorus was determined in digested plant samples by adding Barton reagent (Ashraf et al., 1992). For this purpose, 5 mL of aliquot was taken in a 50 mL volumetric flask and 10 mL of Barton reagent. After incubating for 30 min the readings were measured on a UV–visible spectrophotometer at 420 nm (Model G6860A, Agilent Technologies, Australia) and compared with the standard curve of known concentration potassium dihydrogen phosphate standards. Potassium concentration was determined from the digested samples by using a flame photometer (Model: BWB-XP, BWP Technologies, United Kingdom). The concentration of K was calculated by comparing instrument readings with the KCl calibration curve. The Fe and Zn concentrations in samples were determined using an Atomic Absorption Spectrophotometer (Model 240FS AA, Agilent Technologies Australia).

2.8 Post-harvest soil sample collection and analysis

After crop harvest, post-harvest rhizosphere soil samples were collected, air-dried, and sieved through a 2-mm sieve and stored at 4°C. The prepared soil samples were analyzed within 5 days for biological properties, including bacterial population, microbial biomass N, and organic matter. The bacterial population was enumerated in terms of colony-forming units (cfu) using standard serial dilution and pour plate technique (Alexander, 1982). Microbial biomass nitrogen was calculated by subtracting the biomass nitrogen in chloroform-fumigated soil from the non-fumigated soil sample using the method developed by Okalebo et al. (1993). For the analysis of organic matter, the method of Nelson and Sommers (1982) was used. To ensure data reliability and precision, all measurements were conducted in triplicates. The soil's chemical properties, including ammonium N, nitrate N, available phosphorus, and extractable potassium, were also analyzed using standard protocols. Ammoniacal N and nitrate N were determined using the methods of Kamphake et al. (1967) and Sims and Jackson (1971), respectively. The available phosphorus was determined according to Watanabe and Olsen's (1965) method, and the extractable potassium was noted using a flame photometer (Model: BWB-XP, BWP Technologies, United Kingdom).

2.9 Statistical analysis

The obtained data was statistically analyzed by performing a two-way ANOVA interaction at Statistix 8.1 Analytical Software, Tallahassee, Florida (Steel et al., 1997). Treatment means were computed using an honestly significant difference test (HSD) at 5% probability. The graphs were prepared using R studio. The principal component analysis (PCA) and Pearson's correlation among growth, biochemical, antioxidants, yield attributes of cotton, and biocontrol potential of treatments was performed through Origin 2025 software (Origin Lab, Massachusetts, United States).

3 Results

3.1 Antifungal activities

Consortium spray as a biocontrol agent demonstrated substantial potential in reducing disease incidence and disease index, as illustrated in Figure 1. The effectiveness of the consortium spray was consistent across both varieties tested. The highest disease control (Figure 1a) was observed in the IUB13 and IUB4 varieties, with a maximum reduction

of 68 and 65%, respectively, when the consortium spray was used with the recommended NPK + micronutrients + PGPR. Similarly, a reduction of 61 and 60% in disease incidence was noted in the IUB13 and IUB4 varieties, respectively, when the consortium spray was combined with the recommended NPK + PGPR treatment. The disease index also showed a significant reduction under these treatment conditions compared to the application of synthetic chemical sprays (Figure 1b). Specifically, both varieties reduced the disease index by 75 and 74% when the consortium spray was used with the recommended NPK + micronutrients + PGPR treatment. In contrast, the impact of the synthetic chemical sprays on the disease index was not as pronounced as the control in both varieties. Control efficiency was also significantly improved in treatment where the PGPR consortium was sprayed compared to other treatments (Figure 1c). However, the impact was highest in T6, where PGPR was sprayed and recommended NPK + micronutrients + PGPR was applied.

3.2 Cotton growth and yield

3.2.1 Root parameters

The PGPR significantly affected root growth parameters of cotton varieties IUB13 and IUB4 when recommended NPK and micronutrients were also applied over control (Figures 2a–c). It was evident from the experiment that NPK + micronutrients + PGPR (T6) increased the root length and fresh and dry biomasses. The highest

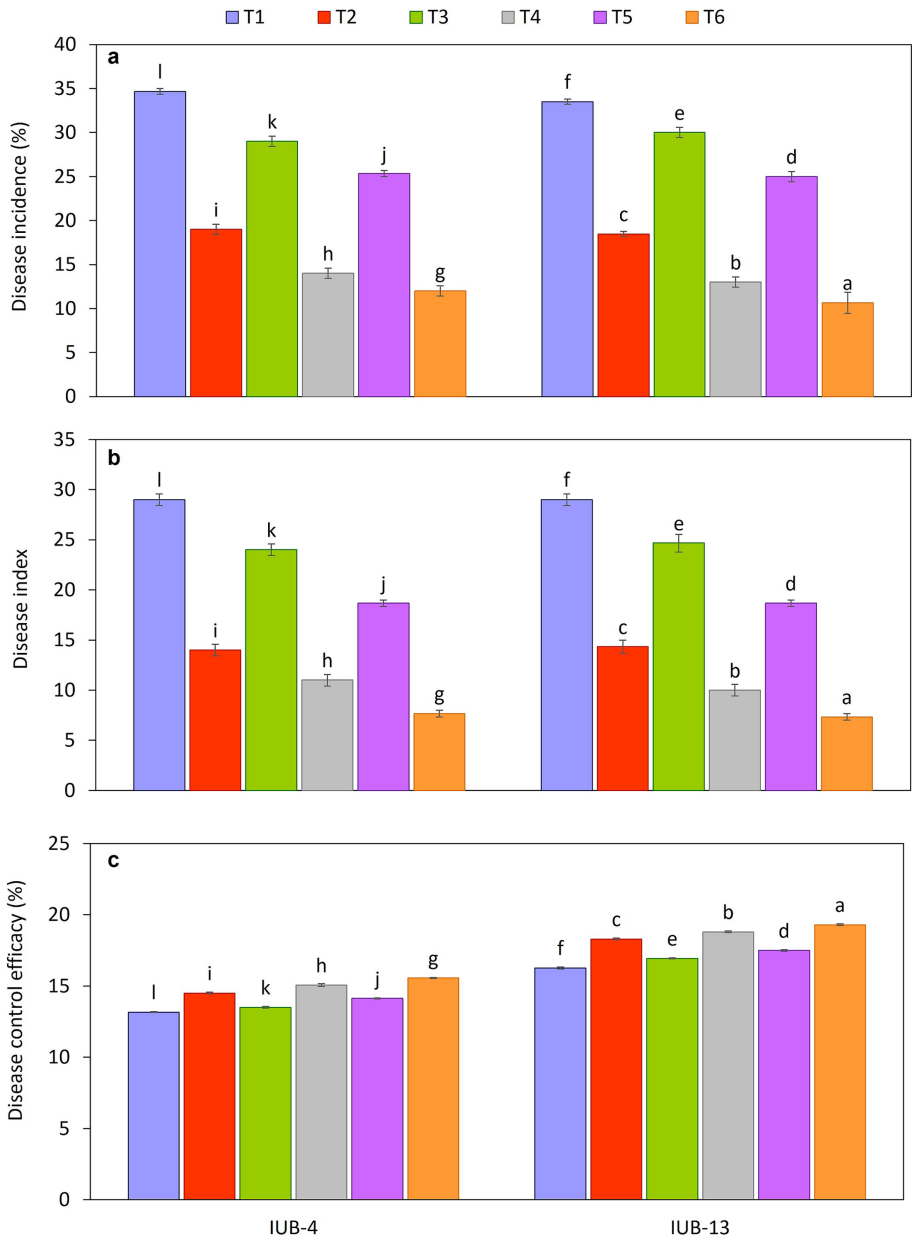


FIGURE 1
Impact of fertilizer and PGPR-based consortium on antifungal activities of disease incidence **(a)**, disease index **(b)** and disease control efficiency **(c)** of cotton. Bars sharing the same letter (s) are not significantly different from each other at $p \leq 0.05$.

increase in root length of IUB13 and IUB4 was 39 and 30%, followed by 40 and 24% under recommended NPK + micronutrients + PGPR and recommended NPK + PGPR application, respectively, compared to respective controls. Root fresh and dry biomasses were also significantly increased due to the application of recommended NPK + micronutrients + PGPR, the increase was 30 and 29% in fresh biomass and 31 and 30% in dry biomass under IUB13 and IUB4, respectively. All the treatments were significant compared to the control but non-significant compared to each other. The application of recommended NPK was the treatment that showed the minimum increase in root growth parameters for both cotton varieties.

3.2.2 Shoot parameters

Inoculating PGPR in cotton varieties IUB13 and IUB4 and applying combinations of the recommended NPK and micronutrients improved the shoot length and shoot fresh and dry biomass. Data showed (Figures 2d–f) that the highest increase in shoot length of cotton variety IUB13 was in treatment where recommended NPK + micronutrients + PGPR were used. The increase was observed by 19%, followed by the treatment of recommended NPK + PGPR (15%) compared to the control. Application of recommended NPK + micronutrients + PGPR also performed well for improving the shoot fresh and dry biomass of both cotton varieties, where the increase was 20 and 19% in fresh biomass and 19 and 18% in dry biomass as compared to the control in IUB13 and IUB4, respectively. The statistical data showed that all the treatments had caused significant improvements in the shoot parameters compared to the control treatment. However, these were non-significant as compared to each other.

3.2.3 Yield attributes

Data regarding single boll weight and lint percentage of cotton varieties IUB13 and IUB4 are depicted in Table 2. It was clear from the statistical data that all the treatments performed better in increasing the single boll weight and lint percentage of the cotton crop. Treatment with recommended NPK + micronutrients + PGPR (T6) caused 32 and 29% increases in single boll weight over the controls of IUB13 and IUB4, respectively, followed by recommended NPK + PGPR, which showed 28 and 23% increases in single boll weight, respectively. The recommended NPK (T3) treatment was the non-significant treatment, which showed a minimum increase in single boll weight that was 17 and 5% over the respective controls in the case of IUB13 and IUB4, respectively. The lint percentage was also improved by the treatment of recommended NPK + micronutrients + PGPR, which was 13 and 12% in IUB13 and IUB4, respectively, over the respective controls.

3.3 Antioxidant enzyme activities

The antioxidative activity of SOD, POX, POD, and CAT enzymes in cotton varieties IUB13 and IUB4 leaves is presented in Figures 3a–d. A significant increase in enzymatic status was observed with the combined application of recommended NPK + micronutrients + PGPR. Most of the treatments were statistically significant compared to the control except for the single use of recommended NPK. Maximum increase in SOD (19%), POX (29%), POD (28%), and CAT (14%) was observed due to the application of recommended NPK + micronutrients + PGPR, followed by 16, 22, 21, and 13% increase in SOD, POX, POD, and CAT over the control because of the use of recommended NPK + PGPR, respectively. While the maximum increase in the case of IUB4 was SOD (18%), POX (26%), POD (25%), and CAT (13%) over the control due to the treatment of recommended NPK + micronutrients + PGPR. Recommended doses of NPK were the treatment that showed a minimum increment in antioxidative

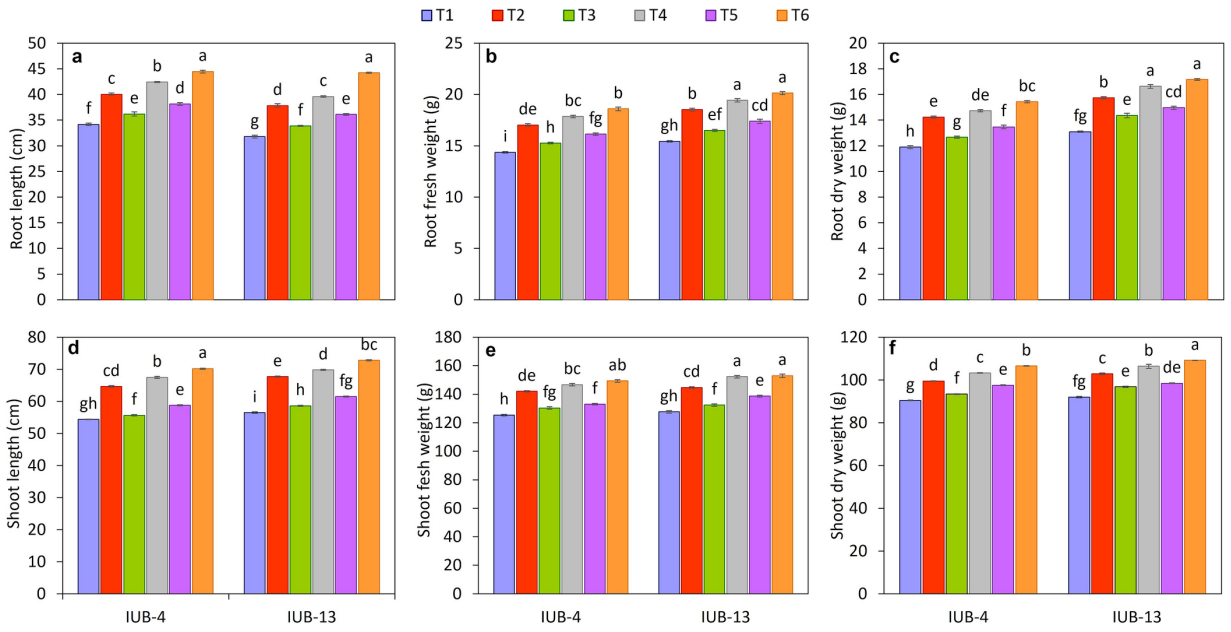


FIGURE 2
Impact of fertilizer and PGPR-based consortium on cotton growth as root length **(a)**, root fresh weight **(b)**, root dry weight **(c)**, shoot length **(d)**, shoot fresh weight **(e)** and shoot dry weight **(f)**. Bars sharing the same letter (s) are not significantly different from each other at $p \leq 0.05$.

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TABLE 2 Impact of fertilizer and PGPR-based consortium on yield of cotton and soil organic matter of rhizosphere soil.

Treatment	Single boll weight (g)		Lint percentage (%)		Organic matter (%)	
	IUB13	IUB4	IUB13	IUB4	IUB13	IUB4
Control	2.17 ± 0.03^{fg}	2.07 ± 0.03^{g}	30.87 ± 0.19^{h}	29.7 ± 0.06^{i}	0.58 ± 0.12^{fg}	0.57 ± 0.13^{g}
PGPR	2.7 ± 0.03^{abc}	2.37 ± 0.03^{de}	33.7 ± 0.06^{c}	32.3 ± 0.06f	0.63 ± 0.37^{c}	0.64 ± 0.27^{d}
Recommended NPK	2.53 ± 0.03^{cd}	2.17 ± 0.03^{fg}	32.6 ± 0.06^{ef}	30.5 ± 0.06 h	0.59 ± 0.25^{e}	0.59 ± 0.21^{f}
Recommended NPK + PGPR	2.77 ± 0.03^{ab}	2.53 ± 0.03^{cd}	34.23 ± 0.07^{b}	32.77 ± 0.03e	0.64 ± 0.51^{b}	0.64 ± 0.16^{c}
Recommended NPK + Micronutrient	2.63 ± 0.03^{bc}	2.27 ± 0.03^{ef}	33.2 ± 0.06^{d}	31.27 ± 0.03 g	0.60 ± 0.14^{de}	0.60 ± 0.11^{e}
Recommended NPK + Micronutrient + PGPR	2.87 ± 0.03^{a}	2.67 ± 0.03^{bc}	34.9 ± 0.06^{a}	33.4 ± 0.06 cd	0.66 ± 0.18^{a}	0.65 ± 0.22^{b}
HSD ($p \leq 0.05$)	0.1708		0.3856		2.5891	

Note: the treatments sharing similar letters didn't differ significantly at $p = 0.05$ as per tuckey's test.

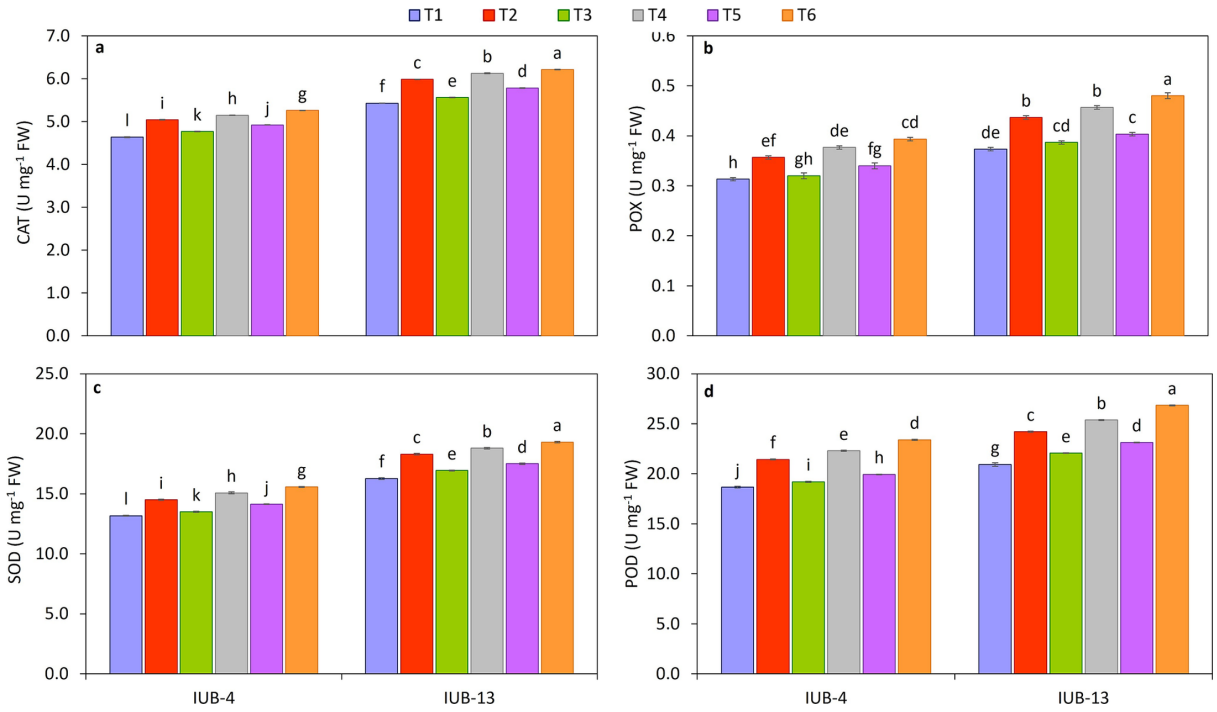


FIGURE 3
Impact of fertilizer and PGPR-based consortium on CAT **(a)**, POX **(b)**, SOD **(c)**, and POD **(d)** of cotton plants. Bars sharing the same letter (s) are not significantly different from each other at $p \leq 0.05$.

activity in the case of both cotton cultivars IUB13 and IUB4 as compared to the control.

3.4 Nutrients uptake

3.4.1 Nutrient concentration in root

The impact of different fertilizer combinations with the PGPR-based consortium considerably improved nutrients in the roots of cotton varieties IUB13 and IUB4. Macronutrient concentration is presented in Figures 4a,c,e, and the micronutrients are shown in Figures 5a,b. The statistical data showed that treatment with recommended NPK + micronutrients + PGPR significantly improved the crop roots' NPK Fe and Zn concentrations. Maximum increases in NPK contents were 12, 27, and 15% over the respective controls in IUB13. All the treatments were significant except the sole application of recommended NPK. The Fe and Zn concentration increase was 23 and 30% in IUB13 and 21 and 27% in IUB4 due to the treatment of recommended NPK + micronutrients + PGPR. However, applying the recommended NPK showed a minimum increase in nutrient concentration in the roots of both cotton varieties.

3.4.2 Nutrient concentration in shoot

The effectiveness of fertilizer application and PGPR inoculation for improving the macro and micronutrient status in shoots of cotton varieties IUB13 and IUB4 are presented in Figures 4b,d,f, 5c,d, respectively. The minimum increase in nutrient concentration in shoots was noted due to the impact of recommended NPK in both cotton varieties. While the maximum increase was because of the treatment of recommended NPK + micronutrients + PGPR, which increased NPK 17.0, 31.0 and 22.0% in case of IUB13 and 13.0, 27.0 and 18.0% in case of IUB4, respectively, while the increase in Fe and

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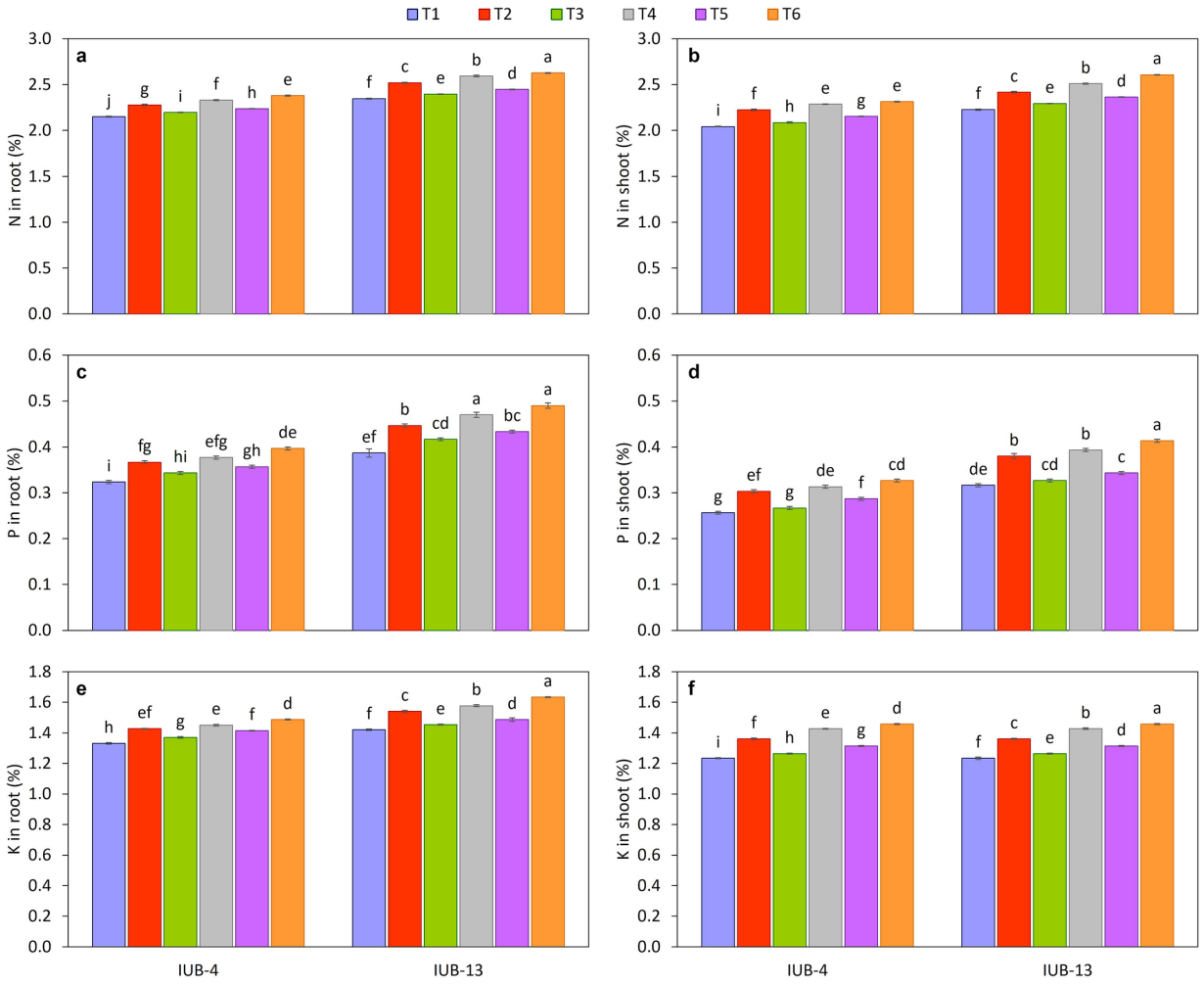


FIGURE 4
Impact of fertilizer and PGPR-based consortium on Macronutrients concentration in root and shoot, N in root **(a)**, N in shoot **(b)**, P in root **(c)**, P in shoot **(d)**, K in root **(e)**, K in shoot **(f)**. Bars sharing the same letter (s) are not significantly different from each other at $p \leq 0.05$.

Zn was 20 and 28% in IUB13 and 18 and 27% in IUB4, respectively over their respective controls as showed in Figures 5c,d. Data showed an increase in nutrient concentration in shoots by the recommended NPK treatment, which was non-significant and lowest among all the treatments.

3.5 Soil health indices

The results demonstrate a significant improvement in the post-harvest biological properties of the rhizosphere soil in cotton under the treatment of recommended NPK + micronutrients + PGPR (T6) compared to the other treatments (Figure 6). The bacterial population was highest under T6, with values of 41×10^4 cfu in both IUB13 and IUB4 varieties, marking a significant increase over the control, which recorded the lowest bacterial population at 32×10^4 cfu (Figure 6a). Similarly, microbial biomass nitrogen (MBN) showed a considerable increase under T6, with values of 10.73 mg kg^{-1} in IUB13 and 10.3 mg kg^{-1} in IUB4, compared to the control, which exhibited the lowest values of 6.63 and 6.53 mg kg^{-1}, respectively (Figure 6b). In terms of organic matter, T6 also showed the highest percentage in rhizosphere soil of both varieties, with 0.66% in IUB13 and 0.65% in IUB4 rhizosphere, which was significantly more significant than the control, where organic matter content was recorded at 0.58 and 0.57%, respectively, (Table 2). Other treatments, such as recommended NPK + PGPR (T4), also demonstrated notable improvements but were less effective than T6. Overall, the integration of PGPR with recommended NPK fertilizers and micronutrients (T6) consistently outperformed all other treatments, significantly enhancing bacterial population, microbial biomass nitrogen, and organic matter in the rhizosphere soil, thereby indicating its potential to improve soil health and fertility post-harvest.

Integrating the PGPR-based consortium with fertilizers significantly improved the post-harvest chemical properties of the soil in both IUB13 and IUB4 cotton varieties. For ammoniacal nitrogen (Figure 6c), the highest mean values were observed by the impact of recommended NPK + micronutrients + PGPR (T6) treatment, recording 12.8 mg kg^{-1} for IUB13 and 12.5 mg kg^{-1} for IUB4, significantly higher than the control, which had the lowest values (9.1 mg kg^{-1} for IUB13 and 8.8 mg kg^{-1} for IUB4). Similarly, nitrate nitrogen levels peaked under the same treatment with 13 and 12.8 mg kg^{-1} for IUB13 and IUB4, respectively (Figure 6d). The

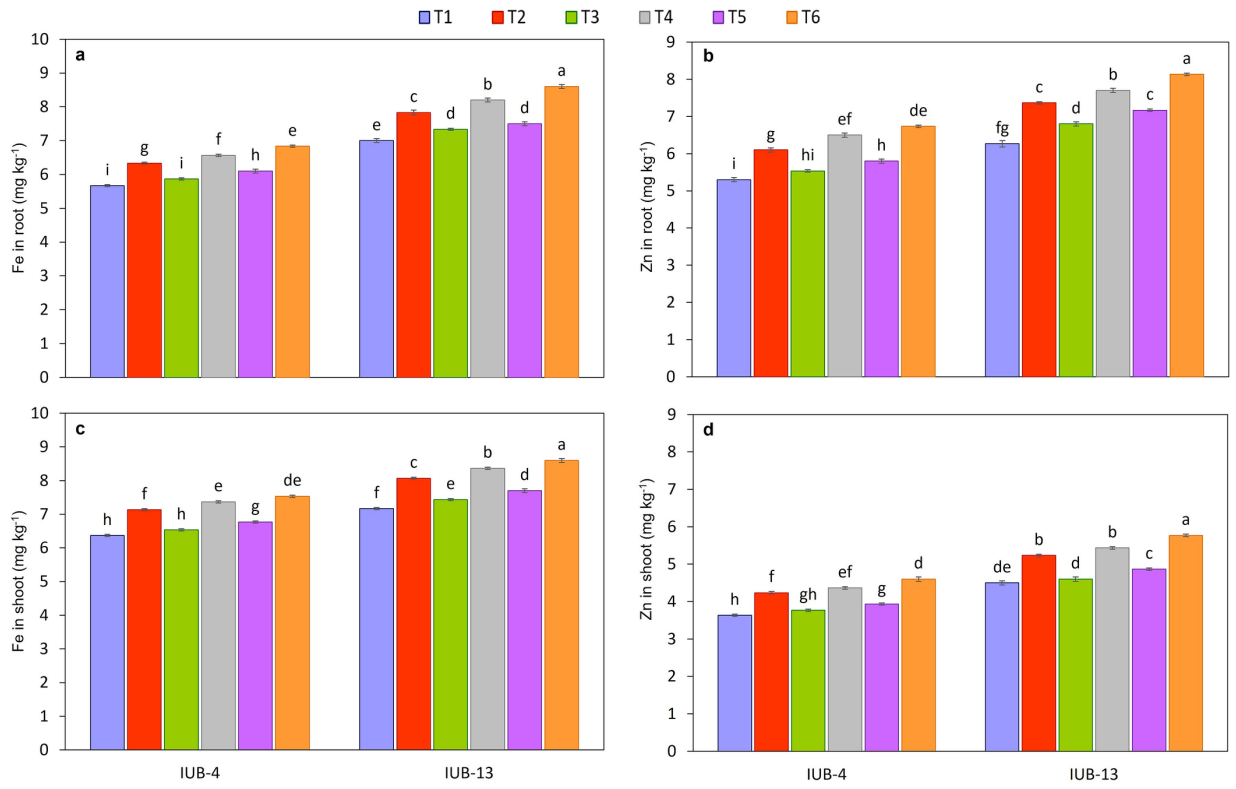


FIGURE 5
Impact of fertilizer and PGPR-based consortium on Fe **(a)** and Zn **(b)** concentration in roots and Fe **(c)** and Zn **(d)** concentration in roots of cotton plants. Bars sharing the same letter (s) are not significantly different from each other at $p \leq 0.05$.

available phosphorus content (Figure 6e) also followed this trend, with the recommended NPK + micronutrients + PGPR treatment yielding the highest values (11.47 mg kg^{-1} for IUB13 and 10.98 mg kg^{-1} for IUB4) compared to the lowest values in the control. For extractable potassium (Figure 6f), the same treatment resulted in the maximum extractable potassium levels, which showed a 29% increase in extractable potassium content in both cotton varieties compared to their respective controls. Intermediate increases were observed in other treatments, but they did not match the effectiveness of the fully integrated treatment. These findings highlight the synergistic effect of PGPR and balanced fertilizers in enhancing soil fertility, particularly under nutrient-deficient conditions, demonstrating their potential as a sustainable soil management strategy.

3.6 Relationship between observed attributes in response to applied treatments

The relationship between the observed attributes of cotton variety IUB-4 and IUB-13 is depicted in Figure 7 in the form of Pearson correlation and PCA biplot. The biplot of PCA depicted that the first and second components of the IUB-13 cotton cultivar showed 98.2 and 1.2% variations in cotton growth, antioxidants status, chlorophyll contents, and yield attributes. On the other hand, the cotton cultivar IUB-4 showed 98.6 and 0.6% variability in growth, antioxidant status, chlorophyll contents, and yield attributes of cotton. Moreover, the negative values of the disease incidence % and disease index for both cotton cultivars depicted suppressing the sooty mold attack by applying the recommended NPK + micronutrient + PGPRs. Pearson's correlation also described that the growth, yield, and physiological parameters of both cultivars (IUB-13 and IUB-4) were positively correlated by the integration of recommended NPK + micronutrient + PGPRs; however, the disease incidence% and disease index were found to be negatively correlated. These analyses justified our concept of dual action by applying PGPR as a growth promoter of cotton and sooty mold suppressor on the cotton leaf by spraying bacterial consortium at different intervals.

4 Discussion

The present study demonstrated the significant impact of PGPR and recommended NPK and micronutrients on cotton growth, yield, nutrient uptake, and disease resistance in the challenging arid climate. Moreover, this investigation highlights the comparison of bacteria and other pesticides for controlling sooty mold. The calcareous sandy soils, characterized by pH (>8) and low organic matter (<0.5%), present substantial hurdles for optimal crop yield. In this context, seed inoculation with PGPR proved to be an effective strategy for enhancing crop productivity by improving nutritional balance and nutrient uptake in crop plants (Fatima et al., 2024; Murad et al., 2024). The consortium of *Bacillus megaterium* (ZR19), *Paenibacillus polymyxa* (IA7), and *Bacillus* sp. (IA16), when applied with recommended NPK and micronutrients, significantly improved cotton growth, yield attributes, nutrient concentrations, antioxidant enzyme activities

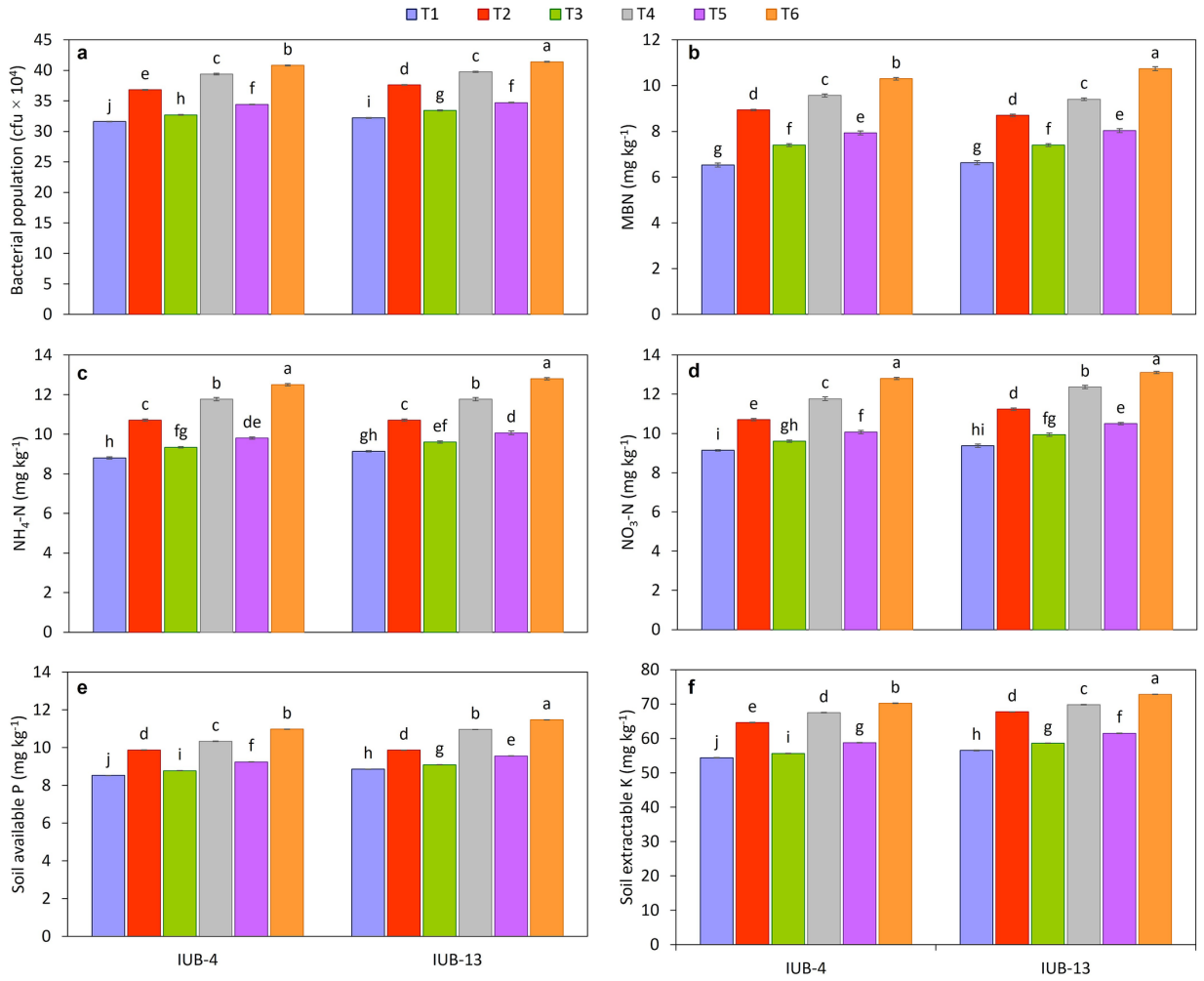


FIGURE 6
Impact of fertilizer and PGPR-based consortium on biological and chemical properties of rhizospheric soil, bacterial population **(a)**, microbial biomass nitrogen [MBN] **(b)**, NH_4-N **(c)**, NO_3-N **(d)**, soil available P **(e)**, soil extractable K **(f)**, of cotton plants, values sharing the same letter (s) are not significantly different from each other at $p \leq 0.05$.

compared to the control. These strains have previously been reported to enhance crop growth and yield (Ahmad et al., 2021; Iqbal et al., 2022), and our results further confirm their efficacy in cotton cultivation. However, their role in biological control has not been studied earlier. The present investigation filled that gap by determining the incidence of sooty mold attack and resistance caused by the foliar spray of the studied strains compared with chemical pesticides.

The consortium of *Bacillus megaterium* (ZR19), *Paenibacillus polymyxa* (IA7), and *Bacillus* sp. (IA16), when applied with recommended NPK and micronutrients, significantly improved cotton root and shoot growth parameters compared to the control. Another aspect of the study was comparing biological and chemical sooty mold control. The biocontrol potential demonstrated by the PGPR consortium is the key finding of this study. Although sooty mold can significantly reduce cotton yield and quality by interfering with photosynthesis (Belachew and Jenber, 2024), but the result of the present investigation demonstrated a significant reduction in disease incidence and disease index, coupled with high sooty mold control efficacy compared to synthetic pesticides. This reduction of sooty mold under treatments of foliar spray of PGPR may follow the following mechanism, i.e., (i) induction of systemic resistance in cotton against sooty mold (Eski et al., 2019), (ii) reducing honeydew secretions on cotton leaves by killing whitefly and aphids, for example, *Bacillus subtilis* strains is best known biological control agent for cotton whitefly (Çelik et al., 2023; Rumyantsev et al., 2023), (iii) secretions of secondary metabolites with antifungal properties for example, volatile organic compounds (VOCs) inhibiting sooty mold mycelium on leaves (Calcagnile et al., 2022). The strains under study have already proved their potential for VOCs and antibiotics production (Unpublished). Moreover, the improved plant nutrition resulting from PGPR inoculation may enhance disease resistance by inducing systemic resistance. The superior performance of the PGPR consortium compared to synthetic pesticides suggests that these bacteria may offer a more holistic approach to pest management, simultaneously addressing both plant health and pest control. This aligns with the findings by Eski et al. (2019), who reported the remarkable impact of *Bacillus* strains as biocontrol agents for cotton pests. Various *Bacillus thuringiensis* strains have been previously reported as effective biopesticides against different insect orders (Zhang et al., 2009; Eski et al., 2017; Kovendan et al., 2011). The

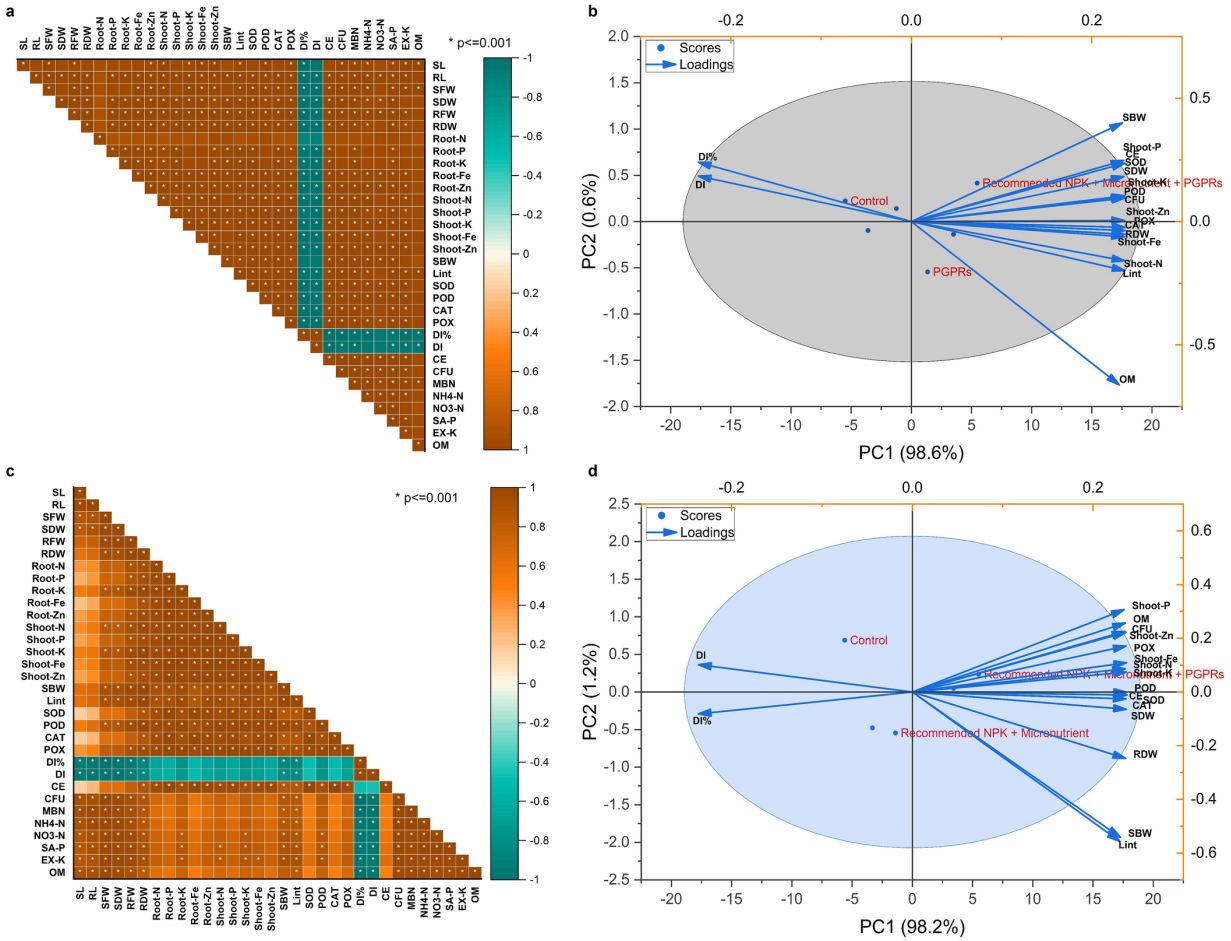


FIGURE 7
The correlation between the observed attributes in terms of Pearson correlation of IUB4 **(a)** and IUB13 **(c)** and PCA biplot of IUB4 **(b)** and IUB13 **(d)**.

potential of PGPR as an environmentally friendly alternative to chemical pesticides is particularly significant in sustainable agriculture, offering a way to reduce the environmental impact of cotton cultivation while maintaining or improving yields.

The growth enhancement can be attributed to multiple mechanisms adopted by PGPRs, i.e., production of phytohormones like auxins, cytokinins, and gibberellins, which directly stimulate root and shoot growth (Ahmad et al., 2021; Iqbal et al., 2022). Additionally, these bacteria can solubilize phosphates and mobilize other nutrients, making them more available to plants. The production of phytohormones and the nutrient solubilization ability of the PGPR strains might be the possible reason for the observed increases in growth parameters like root length, biomass, and shoot parameters. The synergistic effect of PGPR with NPK and micronutrients suggests that the bacteria may enhance the nutrient use efficiencies of NPK fertilizers and reduce their fixation in the soils of arid climates. These findings align with the findings of Ejaz et al. (2020), who reported that microbially secreted hormones and microbially enhanced nutrient efficiencies are possible reasons for higher cotton production. Chen et al. (2023) also found similar positive effects on tillering, spike length, and grain production in wheat. Moreover, the higher phosphorus solubilization by the applied *Bacillus* strains might be another reason for growth enhancement, as described by Bahadir et al. (2018) and Anwar et al. (2024). Grossi et al. (2024) described another reason for growth enhancement in crops: crop growth enhancement is linked with microbially produced auxin in the rhizosphere, which modifies the root architecture for higher nutrient use efficiencies and water intake. The better development of roots and photosynthesis is responsible for the higher yield of the plants, and our results are in line with the findings of Saleem et al. (2021), who demonstrated the role of IAA-producing rhizobacteria in improving the vegetative growth and yield of cotton.

The improvement in antioxidative enzyme activities in cotton plants treated with the PGPR consortium-recommended NPK and micronutrients is a noteworthy finding that sheds light on the stress tolerance mechanisms of PGPR. The observed increases in SOD, POX, POD, and CAT activities suggest an improved capacity to manage oxidative stress caused by abiotic factors (high temperature of arid climate) and sooty mold attack. This improvement can be attributed to the modulation of gene expression by PGPR under stress. PGPRs potentially upregulate genes involved in antioxidant production in biotic or abiotic stress (Noreen et al., 2024; Ahmad et al., 2023). Our findings are consistent with studies by Li and Jiang (2017), who found that *Bacillus aquimaris* DY-3 significantly enhanced the activities of catalase, superoxide dismutase, peroxidase, and ascorbate peroxidase in maize under salt stress. Similarly, Khan et al. (2020) observed increased

SOD activity by 3.1 folds in soybean plants inoculated with PGPR strains SA1 under heat stress. The enhanced CAT activity suggests improved capacity to neutralize hydrogen peroxide, a common reactive oxygen species produced under stress (Fatima et al., 2024; Pandey et al., 2017). These results align with Santos et al. (2018), who reported increased CAT and SOD activities in cowpea nodules co-inoculated with bacteria. This improved antioxidative status likely contributes to the overall enhanced growth and yield observed in the treated plants by mitigating the negative impacts of high temperatures in arid areas.

The results of the present study highlight significant improvements in post-harvest soil biological and chemical properties. The bacterial population in the rhizosphere was notably higher under T6 compared to other treatments, demonstrating the ability of PGPR to enhance microbial activity and diversity. These findings are consistent with the work of Gouda et al. (2018), who reported that PGPR enhances microbial population in the crop rhizosphere due to the secretion of carbohydrates in root exudates and improved soil nutrient availability. Similarly, the increase in microbial biomass nitrogen (MBN) and organic matter observed under T6 aligns with studies by Hussain et al. (2019), which documented that PGPR enhances microbial-mediated nutrient cycling, contributing to higher MBN and organic matter percentage. The enhancement of ammoniacal and nitrate nitrogen and available phosphorus under T6 is consistent with studies by Murad et al. (2024) and Noreen et al. (2024), who demonstrated that PGPR improves mineralization and availability of essential nutrients. This improvement is particularly valuable in calcareous soils (the soil under study), where phosphorus availability is often restricted due to fixation. The observed differences in the response of the two cotton varieties to the treatments may be attributed to their genetic makeup, which influences their nutrient uptake efficiency, antioxidant enzyme activity, and inherent stress tolerance. Additionally, variations in root architecture, nutrient assimilation capacity, and interaction with PGPR strains could contribute to the differential responses. Our findings provide strong evidence for the role of PGPRs in integrated nutrient management and pest management to enhance soil health, crop productivity, and environmental degradation by fertilizers and pesticide application. Future studies should further explore the long-term impacts of these practices on soil fertility and their scalability for broader agricultural applications.

5 Conclusion

This study demonstrated the potent efficacy of PGPR combined with recommended NPK fertilizers and micronutrients in enhancing cotton growth, yield, and soil properties. The consortium of *Bacillus megaterium* (ZR19), *Paenibacillus polymyxa* (IA7), and *Bacillus* sp. (IA16) significantly improved antioxidant enzyme activities, growth parameters, yield, and nutrient concentrations in cotton varieties IUB13 and IUB4. Additionally, PGPR integration significantly enhanced post-harvest soil properties, showcasing the synergistic effect of biological and chemical approaches. Integrating PGPR with balanced fertilization is particularly significant for improving cotton productivity and mitigating the impact of sooty mold in arid regions, where nutrient availability and disease pressure are major challenges. These findings underline the role of PGPR in improving nutrient uptake and soil fertility, which is crucial for sustainable cotton production. This integrated approach not only boosts cotton productivity but also reduces dependence on chemical inputs. The PGPR consortium also proved effective in controlling pests, offering an eco-friendly alternative to synthetic pesticides. This dual benefit of growth promotion and pest control is vital for sustainable cotton cultivation. Future adoption of PGPR-based strategies by farmers could enhance yields and soil health, while policymakers can support this transition through field trials and awareness campaigns. Further research on the long-term effects of PGPR on soil health and cotton production is needed to explore its full potential.

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

ML: Writing – original draft. MA: Writing – original draft. AD: Writing – original draft. AH: Writing – original draft. UZ: Writing – original draft. MM: Writing – original draft, Writing – review & editing. AM: Writing – review & editing. AM: Writing – review & editing. ME: Writing – review & editing.

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Conflict of interest

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

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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Mechanistic insights into phosphorus transformation mediated by *Arthrobacter* and *Sordariomycetes* under long-term high-volume swine manure application in a wheat-rice rotation system

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Introduction: Understanding the impacts of sustained high-input swine manure on soil phosphorus (P), along with identifying and functionally characterizing P-associated microorganisms, can provide a scientific foundation for effective management of soil P in relation to swine manure application. This study provides novel insights into the functional roles of P-associated microorganisms in mediating phosphorus dynamics under long-term excessive swine manure application.

Methods: The study investigated the prolonged impact of high-volume swine manure application on soil P fractions over an 8-year continuous, randomized field trial involving rotating wheat (wet conditions) and rice (flooded conditions) crops. And the soil treated with the prolonged high- volume swine manure application was selected to isolate and identify specific microorganisms, which were subsequently inoculated into soil previously treated with long-term NPK fertilizer (F) and swine manure application (M) for indoor cultivation and functional characterization verification.

Results: The sustained high input of swine manure markedly enhanced soil P activity and microbial P content ($P < 0.05$), specifically extracting P-associated microorganisms, namely *Arthrobacter* sp. M4 bacteria and *Sordariomycetes 2 MS-M4* fungi. Upon separate inoculation of these microorganisms into high-Carbon (C) and high-P soils (M soil, Olsen P > 70 mg kg^{-1}, ROC > 150 mg kg^{-1}), it was observed that both microorganisms effectively converted available P sources (Ca_2-P, Ca_8-P) into organic P reserves through biological immobilization. Conversely, under conditions of low C and low P (F soil, Olsen P < 10 mg kg^{-1}, ROC < 75 mg kg^{-1}), there was an enhancement in the decomposition and utilization of soil organic C which resulted in increased

effective P content via the breakdown of organic phosphates—demonstrating a robust capacity for P transformation. Furthermore, when these phosphate-related microorganisms were introduced to long-term fertilized soils enriched with NPK fertilizer (F), they exhibited a significantly greater enhancement in soil P availability compared to those inoculated into soils subjected to prolonged high inputs of swine manure.

Discussion: The P-related microorganisms *Arthrobacter* sp. M4 and *Sordariomycetes 2 MS-M4* extracted from soils with high P availability were confirmed to have the key functions of enhancing the fixation of inorganic P into organic P (high-C and high-P condition) or promoting the activation of organic P into rapidly available P (low C and low P level). Which may plays an important role in the management of agricultural P nutrients.

KEYWORDS

functional validation, P related microorganisms, swine manure, soil P fractions, wheat-rice rotation

Highlights:

- The prolonged application of swine manure enhances the bioavailability of phosphorus in soil.
- Isolation of soil microorganisms, specifically Arthrobacter sp. M4 bacteria and *Sordariomycetes 2 MS-M4* fungi, was performed.
- The isolated microorganisms facilitate the biological fixation of inorganic phosphorus in nutrient-rich environments.
- The isolated microorganisms effectively degrade organophosphorus in nutrient-poor environments.

1 Introduction

The rapid advancement of livestock farming has led to a significant increase in the volume of livestock waste (Peng et al., 2015; Guo et al., 2018). However, the effective utilization rate remains approximately 60% (Nobile et al., 2020; Bai et al., 2016), with unused pig manure frequently contributing to environmental issues. The decomposition and incorporation of livestock manure into field represent a crucial method for its resourceful utilization. Swine manure is abundant in P, predominantly in inorganic form (approximately 70%). Many studies have shown that when both short—and long-term swine manure applied to soil as organic compost, it can directly enhance the P content and may induce alterations in soil P fractions, thereby improving P availability (Chen et al., 2022; Zhang C. L. et al., 2024), however, the study of phosphorus components in soil with high amount of swine manure application is still neglected. Concurrently, swine manure contains a lot of organic matter and other mineral nutrients, the application of swine manure induces modifications in the structure of soil microbial communities, which may influence the P cycling processes within the soil.

Soil microorganisms can significantly mitigate the content of soil available P (Olsen P) fixation by converting Olsen P into microbial P (MBP) (Dai et al., 2017). Under certain conditions, they enhance the utilization of unavailable P fractions by solubilizing inorganic P from calcium phosphate and hydroxyapatite (Chen et al., 2019; Qin et al., 2024), as well as various fractions of organic P in the soil through enzymatic activity and other metabolic products (Huang et al., 2019). Long-term application of organic fertilizers can form a more interactive community and enhance the function of microorganisms, thereby increasing the multi-functional potential of soil ecosystems, and may cause microbial functional redundancy (Lin et al., 2019; Sun et al., 2015). The application of swine manure facilitates the transformation of unstable P compounds into bioavailable organic P via soil microorganisms (Chen et al., 2015; Tian et al., 2016) and concurrently reducing soluble inorganic P levels in the soil (González et al., 2019; Lemming et al., 2019). However, an accumulation of recalcitrant organic matter may increase demand for stable organic matter (such as humus), prompting mineralization processes that release active organic P (Tiecher et al., 2017). Thus, introducing swine manure stimulates the proliferation of certain functional soil microorganisms; however, current research predominantly emphasizes short-term studies and low-input scenarios regarding swine manure's impact on these microorganisms. This may overlooks their feedback mechanisms under excessive input conditions. While a judicious and substantial application of swine manure is advantageous for enhancing resource utilization efficiency.

Consequently, investigating the interaction between functional microorganisms associated with soil P and the availability of soil P from specific P-functional microorganisms holds significant implications for practical applications in agricultural production. Given the dominance of bacterial and fungal taxa in organic P mineralization and inorganic P solubilization (Tian et al., 2016), we focused on isolating and validating these functional groups to elucidate their roles under contrasting nutrient conditions. To clearly suggest the differentiated application of microorganisms in high and low phosphorus soils and enhance the practical guiding significance of the research. In our study, based on an 8-year field experiment involving continuous high-rate application of swine manure, assessed soil P content and selected the prolonged high-rate swine manure treatments to isolate, screen, and functionally characterize bacteria and fungi that constitute a

substantial portion of the soil microbial community. The cultured bacterial and fungal solutions were subsequently introduced into the soil and incubated in a laboratory setting for functional verification, concentrating on three key areas: (1) elucidating the characteristics of soil P fractions under sustained high-dose swine manure input; (2) selecting P-functional microorganisms and identifying them via the NCBI platform; (3) clarifying the impacts of these selected bacteria and fungi on both soil P availability and transformations within P fractions. We hypothesized that long-term high-volume swine manure application would: (1) shift soil P fractions toward organic forms via microbial immobilization; (2) select for specific P-transforming microorganisms capable of dual-functionality (P fixation vs. activation) depending on soil nutrient status.

2 Materials and methods

2.1 Long-term field trial

The general situation of the experimental site is described in our previous research by Zhang C. L. et al. (2024). The field experiment commenced in 2012, incorporating two treatment modalities: a single application of NPK fertilizer (F) (The fertilizer application rate was according to the local farmer practices) and swine manure at a rate of 30,900 kg ha^{-1} (M, representing 150% FN and 600% FP), as detailed in Table 1. The field trial site was laid out as a completely randomized block, with each treatment being applied to three random plots. The experimental area employed a wheat-rice rotation planting pattern, with each plot measuring 20 m^2. To mitigate water and nutrient runoff, the plots were delineated by plastic film barriers. All fertilizers were applied simultaneously prior to transplanting or sowing during the rice season (*Oryza sativa* L., F-498, June-October) and the wheat season (*Triticum aestivum* L., Wheat 863, November-May).

In 2020, soil samples (generating 24 samples) were collected by earth auger from the surface layer (0-20 cm, this depth represents the primary root zone for wheat and rice cultivation and is most responsive to manure-induced changes in P dynamics) of the paddy field during the rice harvest season and the wheat harvest season following 8 years of treatments. The sampling was conducted using a soil auger at five designated points according to the five-point sampling method, and the samples were subsequently mixed utilizing a four-point technique, retaining 1.00 kg of soil for analysis. The collected soil was dried and sieved through 1.00 and 0.149 mm nylon mesh before being sealed and stored.

The concentration of total P (TP) in soil is quantified using the NaOH fusion-molybdenum-antimony complexometric titration method (Lu, 2000). Soil available P (Olsen P) was determined by extraction with 0.5 mol L^{-1} $NaHCO_3$ (pH 8.5) and the molybdenum-antimony resistance coloration method (Olsen et al., 1954). Microbial biomass C (MBC) and microbial biomass P (MBP) was determined using chloroform fumigation extraction (Brookes et al., 1984). Alkaline phosphatase (ALPase) activity was determined using the benzene disodium phosphate colorimetric method (Schneider et al., 2000). The primer utilized for sequencing the *phoD-* gene is ALPS-F730/ALPS-R1101 (CAGTGGGACGACCACGAGGT/GAGGCCGATCGGCATGTCG) (Huang et al., 2019). Dissolved organic C (DOC) using an elemental analyzer, and readily oxidized organic C (ROC) using a potassium permanganate oxidation method (Lu, 2000).

Soil inorganic P fractions were extracted in sequence using the methods of Jiang and Gu (1990), which classify the inorganic P present in soil into six distinct categories: dicalcium phosphate (Ca_2-P), octacalcium phosphate (Ca_8-P), aluminum phosphate (Al-P), iron phosphate (Fe-P), occluded phosphate (O-P), and decacalcium phosphate (Ca_{10}-P). Soil organic P fractions were extracted in sequence using the method of Bowman and Cole (1978), which classify the organic P present in soil into four distinct categories: Labile organic P (LPo), moderately labile organic P (MLPo), moderately resistant organic P (MRPo) and highly resistant organic P (HRPo). Details of P fractions were described in our previous research by Zhang C. L. et al. (2024).

2.2 Isolation and functional validation of key P-utilizing bacteria

2.2.1 Isolation and characterization of specific bacterial strains

Prior to microbial isolation, fresh soil samples were homogenized and sieved (<2 mm), with subsamples stored at 4°C for immediate processing. The culture medium employed for the isolation of soil bacteria (and fungi) via the dilution spreading method is a streptomycin-bengal red medium (Martin et al., 2022; Devkota et al., 2024). The specific procedures are outlined as follows:

In a sterile condition, use a sterile spoon to weigh about 5 g of fresh soil from the M treatment and transfer it into a 150 mL triangular flask. Previously, add 45 mL of deionized water and 10 glass beads to the flask, which were sterilized and prepared for utilization. Then seal it with a sealing film, shake it at 200 rpm for 30 min, and use it as the mother liquid to dilute it 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} times for plate coating. The coating volume is 200 μL, and it is evenly coated with a sterile glass rod. After sealing it with a sealing film, keep it right side up for 30 min and then place it in a 25°C incubator upside down for 2-3 days for bacterial culture and 4-5 days for fungal culture. During the culture, pay attention to observation and select 10 or so well-separated colonies on the plate. Perform bacterial line-pure culture on the beef extract peptone agar plate using a spatula, and perform fungal line-pure culture on the potato dextrose agar plate using a spatula. The specific method of transmission is to dig out a single small colony with a spatula and transfer it to the center of a new beef extract peptone agar (or PDA) plate culture medium, and then cover it immediately. Note that to prevent cross-contamination, the alcohol lamp flame should be used to sterilize the spatula before and after picking up each colony. Cultivate at 5°C for 5-7 days and perform the next transmission. In this experiment, a total of 23 bacterial strains and 7 fungal strains were isolated.

2.2.2 Microbiological inoculation and cultivation assessment

After isolating pure strains to a specified number, the resulting strains were cultured in the medium. Four colonies with a diameter

TABLE 1 Experimental fertilization application (kg.ha^{-1}).

Treatment	Manure	Urea	Superphosphate	KCl	P application (P_2O_5)	Nitrogen application (N)	Potassium application (K_2O)
F	0	776	1250	250	150	360	150
M	30900	0	0	0	900	540	370

The amount of fertilizer application in rice-wheat rotation.

of 1 cm (including the medium) were inoculated into 500 mL triangular flasks containing 200 mL of appropriate bacterial beef extract peptone liquid culture medium or fungal PDA liquid culture medium. The cultures were incubated at 25°C with shaking at 200 rpm in constant darkness for a duration of 7 days. Filter the culture solution under sterile conditions, discard the filtrate, and rinse thoroughly with sterile deionized water to ensure that all media components are removed. The colonies were then resuspended in 200 mL of sterile deionized water to obtain a uniform suspension. Inoculation involved adding 30 mL of microbial suspension (10^8 CFU ml^{-1}) to 3,300 g of air-dried soil (F or M treatment) in triplicate. Control groups received sterile deionized water. Detailed operational steps for bacterial and fungal inoculation and cultivation tests are illustrated in Figure 1.

Deionized water was utilized in the experiment to minimize experimental errors. All treatments were incubated in a light-controlled environment for a duration of 6 weeks. The cultivation conditions comprised 14 h of illumination and 10 h of darkness. The temperature during the light phase was maintained at 28°C, while the dark phase was kept at 20°C, with relative humidity consistently held at 70%.

Following the cultivation process, the Olsen P content of each soil sample was assessed, and variations in soil Olsen P content were utilized to further identify and select P-related microbial strains, leading to the isolation of one bacterial strain and one fungal strain. The methodology for determining the Olsen P content is detailed in section 2.1.

2.2.3 Identification of high-quality bacterial strains associated with Olsen P

Classify and identify the strains derived from the soil inoculation experiment. Genomic DNA was extracted using the FastDNA SPIN Kit (MP Biomedicals, United States), following the manufacturer's protocol. Amplify the extracted DNA using it as a template. Bacterial amplification will be conducted with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGYTACCTTGTTACGACTT-3′) (Lane, 1991), while fungal amplification will utilize ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGGTAAAAGTCAAGG-3′) (Furan et al., 2022). Compare the resulting sequences against those in the NCBI database to ascertain the classification of the corresponding strains.

2.2.4 Validation of functional characteristics in specific strains

Assess relevant indicators for soil inoculated with selected bacteria and fungi, based on changes in soil Olsen P content. β-glucosidase (β-glu) is chosen as an enzyme activity indicator for the soil C cycle, while alkaline phosphatase (ALPase) serves as an enzyme activity indicator for the soil P cycle (Schneider et al., 2000). Enzyme activities were quantified using a SpectraMax® M5 microplate reader (Molecular Devices, United States) via colorimetric assays (Tiecher et al., 2017). Concurrently, measurements of soil C and P fractions indicators are conducted as outlined in section 2.1.

2.3 Statistical analyses

Data normality was verified using Shapiro-Wilk tests and non-normal data were analyzed via Kruskal-Wallis test. Tukey's *post-hoc* test ($P < 0.05$) was carried out using SPSS 23 (IBM) to test the significance of differences in P related index and fractions across the treatments. Pearson correlation coefficient analysis ($P < 0.05$) was applied to the P related index and fractions. Utilizing DNA sequencing technology and the NCBI data platform for comparative analysis, specific microorganisms were identified, and a phylogenetic tree of these microorganisms was constructed using MEGA X. Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA X with 1000 bootstrap replicates.

3 Results

3.1 P related index and fractions

Long-term application of swine manure enhanced the P content and bioavailability in the soil. In comparison to conventional NPK fertilizer application (F), long-term swine manure application (M) resulted in a marked increase in both TP and Olsen P levels, with increases of 35.82 and 683.77%, respectively ($P < 0.05$, Figures 2a,b). These results indicate that sustained high-input swine manure profoundly altered soil P availability. No significant differences were observed in soil phosphorus content between rice and wheat growing seasons under identical treatments. While the application of swine manure notably elevated MBC and MBP levels, the trend for MBP mirrored that of swine manure addition. Furthermore, this practice significantly boosted ALPase activity as well as the abundance of *phoD*- gene copies within the soil, with increases in *phoD*- gene copy numbers surpassing those seen for ALPase activity. The disproportionate increase in *phoD* gene copies (220% higher in M soil; $P < 0.055$) compared to ALPase activity (58% increase; $P < 0.05$) (Figures 2d,e) suggests functional redundancy within the P-cycling microbiome. Thus, it can be concluded that swine manure application substantially augmented populations of P-related microorganisms in the soil; however, their overall metabolic activity remained relatively lower (Figures 2c-f). Additionally, easily available organic C forms such as DOC

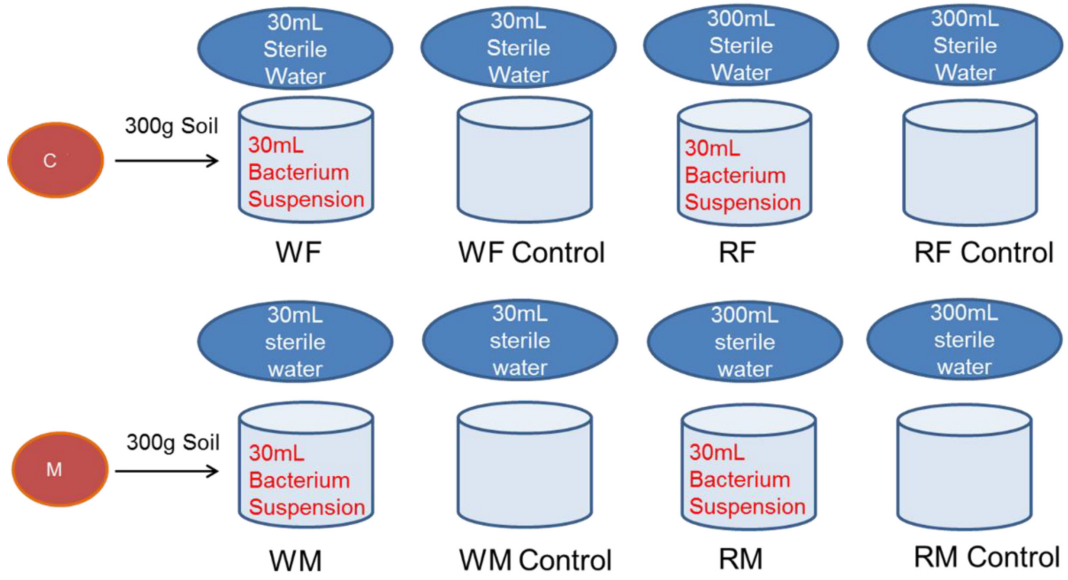


FIGURE 1
Flow chart of microbial inoculation culture test. The capital letters R and W represent the flooded conditions (rice season) and the wet conditions (wheat season), respectively, the same below. The capital letters F and M on the horizontal axis represent the fertilizer NPK application and the swine manure application, respectively. Bacteria and fungi were cultured separately in beef extract peptone and PDA media, respectively.

and readily ROC exhibited significant increases following swine manure applications, with ROC being primarily responsible for this enhancement (Figures 2g,h).

The long-term application of swine manure enhanced the content of various P fractions in the soil and altered their relative proportions (Figure 3). Among the increased P fractions, Ca_8-P, Fe-P, and MLPo emerged as predominant fractions, notably; Ca_2-P and LPo exhibited relatively higher increases of 375.15 and 360.71% (WM), respectively. Concurrently, with respect to the distribution of each P fraction, long-term swine manure application markedly elevated the proportions of Ca_2-P, Ca_8-P, Fe-P, O-P, LPo, MRPo, and HRPO within the soil matrix. In contrast, stable P fractions (Ca10-P) remained unchanged, confirming that microbial activity preferentially mobilized bioavailable P pools (Supplementary Figure 1). This indicates a significant enhancement in both active and medium-active P fractions in the soil while improving P bioavailability. The TPi/TPo ratio decreased from 3.86 (RF) to 3.20 (RM), suggesting an increase in organic P proportion within the soil environment due to long-term swine manure application. The observed rise in P bioavailability alongside an increased proportion of organic P may contribute to greater diversity among P-related microbial communities.

3.2 Isolation and characterization of microorganisms associated with P

Select long-term high-rate swine manure input soil for treatment (M), isolate using the dilution plate method, and obtain bacterial and fungal 16S rRNA and ITS sequences through high-throughput sequencing. The bacterial 16S rRNA sequence was assembled to yield a complete length of 1429 bp. Based on this sequence information, the identified species corresponds to a bacterium classified within the *Bacteria*, *Actinomycetota*, *Actinomycetes*, *Micrococcales*, *Micrococcaceae*, *Arthrobacter*, *Arthrobacter* sp. M4. The microbial data is presented in Table 2 and Figure 4.

Upon assembling the fungal IFS sequence, a complete sequence of 588 bp was acquired. Utilizing this sequence information, a comparative analysis with the NCBI database revealed that the extracted sequence was *Eukaryota*, *Fungi*, *Dikarya*, *Ascomycota*, *Saccharomycet*a, *Pezizomycotina*, *Leotiomyceta*, *Sordariomyceta*, *Sordariomycetes*, unclassified *Sordariomycetes*, *Sordariomycetes 2 MS-M4*. The microbial data is presented in Table 3 and Figure 5.

3.3 Inoculation and cultivation of P-associated microorganisms for functional validation

The bacterium *Arthrobacter* sp. M4 and the fungus *Sordariomycetes 2 MS-M4*, previously isolated and identified, were individually inoculated into long-term NPK fertilizer-treated soil (F, characterized by low C and low P) and soil enriched with excessive swine manure (M, characterized by high C and high P) for *in vitro* cultivation and functional characteristic assessment, with distilled water serving as the control treatment (Figures 6, 7). Results from the microbial inoculation cultivation experiment indicated that inoculation with *Arthrobacter* sp. M4 significantly enhanced the Olsen P content of the NPK-treated soil, achieving a maximum increase of 8.34 mg kg^{-1}(RF), while concurrently reducing the Olsen P content in the M-treated soil by a maximum of 26.24 mg kg^{-1} (WM). Additionally, it markedly increased MBC, MBP, ALPase, and β-glu activity in both NPK- and M-treated soils while decreasing DOC and ROC levels (Figure 6). Overall, the influence of *Arthrobacter* sp. M4 inoculation on NPK-treated

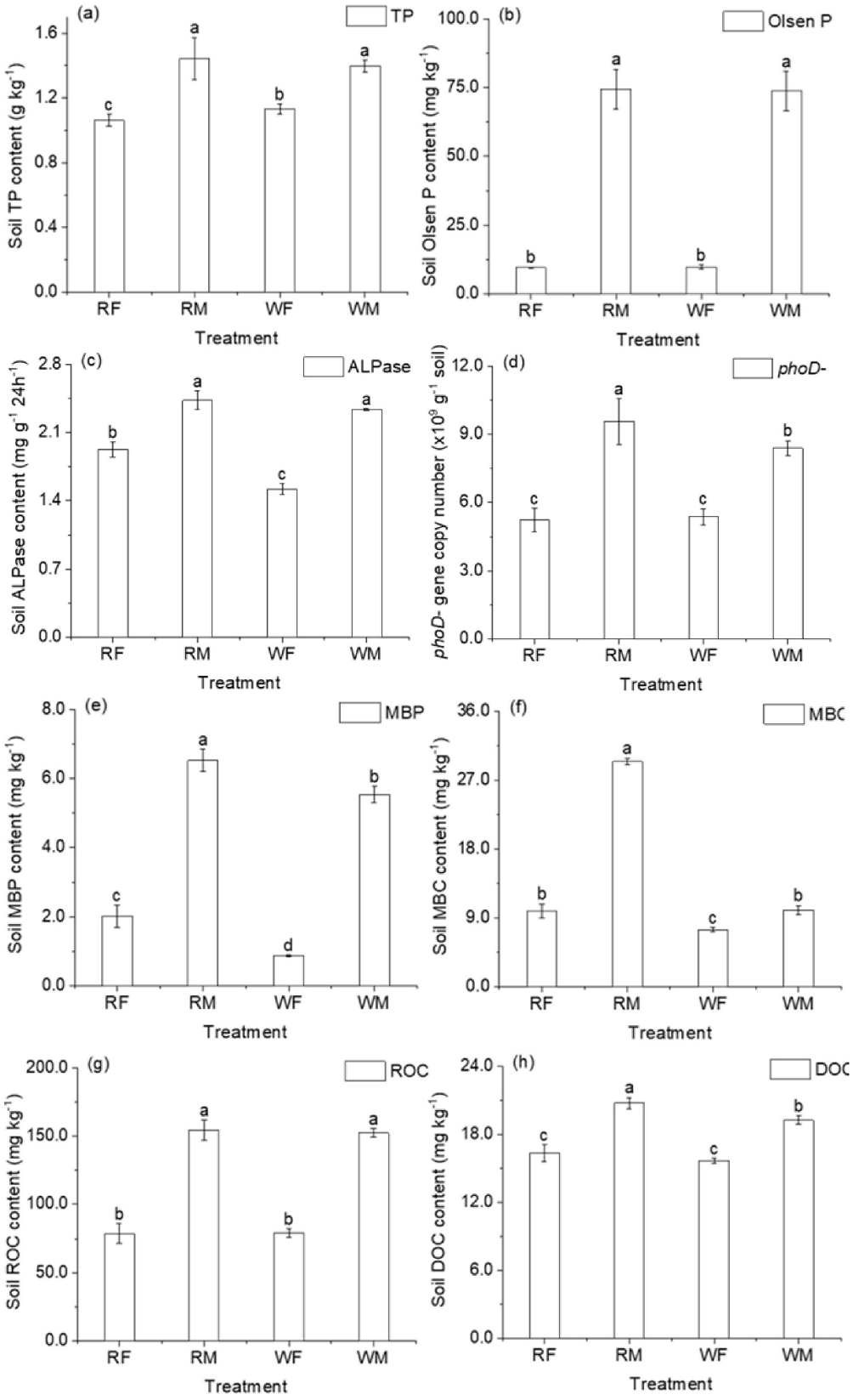


FIGURE 2
Effects of swine manure returning on chemical properties in the Ultisol. This figure shows the change characteristics of different indexes of soil applied with long-term fertilizer and swine manure, and **(a–h)** were refer to the TP, Olsen P, ALPase, *phoD*-, MBP, MBC, ROC, DOC, respectively. The capital letters R and W on the horizontal axis represent the rice season and the wheat season, respectively, the same below. The capital letters F and M on the horizontal axis represent the fertilizer NPK application and the swine manure application, respectively, the same below. Different letters indicate significant difference among the treatments ($P < 0.05$).

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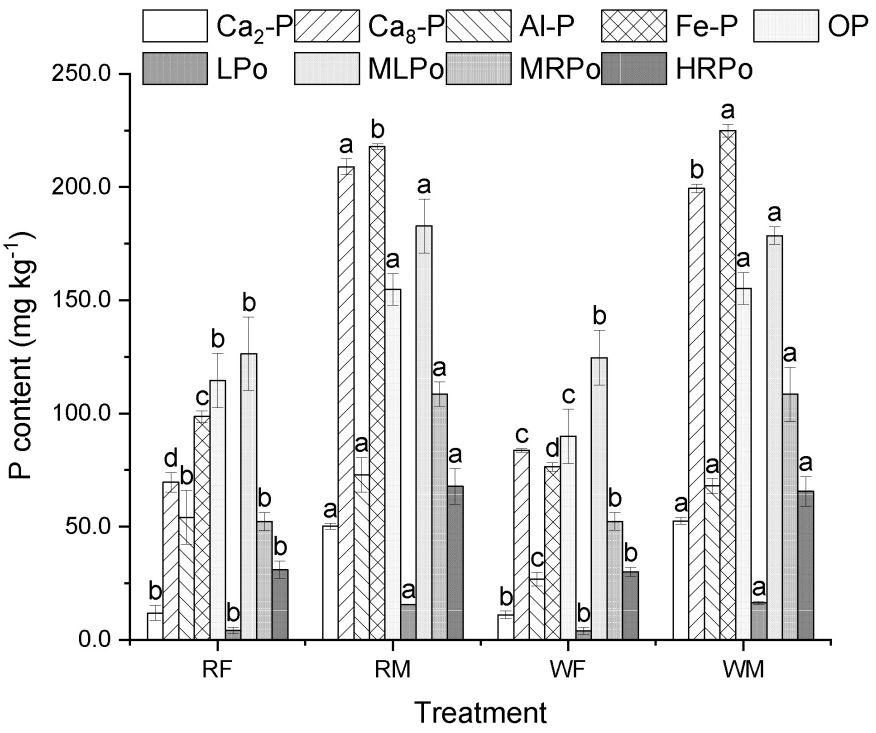


FIGURE 3
Effect of high-volume swine manure application on soil P fractions. The Ca_{10}-P content remain relatively stable, making them the highest among all phosphorus fractions, to highlight the changing characteristics of other phosphorus fractions more effectively, the Ca_{10}-P content on the Supplementary Figure 1. Different letters indicate significant difference among the treatments of the same P fractions ($P < 0.05$).

TABLE 2 Information table for identification of P-related bacteria.

Description	Query cover	*E*-value	Per. Ident	Accession
Arthrobacter sp. M4 16S ribosomal RNA gene, partial sequence	97%	0.0	99.78%	OR742067

soil was more pronounced than on M-treated soil; furthermore, changes in soil indicators under wet conditions were greater than those observed under flooding conditions (Figure 6).

Consistent with the findings from bacterial addition, inoculation with *Sordariomycetes 2 MS-M4* markedly enhanced the Olsen P content in NPK-treated soil, with an increase from 9.69 mg kg−1 to 18.03 mg kg−1 (Figure 7). Control groups (uninoculated soils) showed negligible changes in Olsen P ($\Delta < 2$ mg kg−1) and enzyme activities ($\Delta < 5\%$ for ALPase and β-glu), confirming that observed effects were driven by microbial inoculation rather than experimental artifacts. For instance, in NPK soil inoculated with *Sordariomycetes 2 MS-M4*, ALPase activity increased by 68% ($P < 0.01$) compared to the control, while β-glu activity rose by 55% ($P < 0.05$), indicating enhanced C-P co-metabolism (Figure 7). In contrast, no significant effect on Olsen P content was observed in M-treated soil. Following the inoculation of *Sordariomycetes 2 MS-M4*, there were substantial increases in soil MBC, MBP, DOC levels, as well ALPase and β-glu activities, conversely, a significant reduction in ROC content was noted. Overall, the impact of *Sordariomycetes 2 MS-M4* inoculation on NPK-treated soil was more pronounced than that on M-treated soil, and variations in soil indicators under wet and flooded conditions remained relatively minor. Furthermore, *Sordariomycetes 2 MS-M4* inoculation demonstrated superior efficacy in enhancing available phosphorus levels within NPK-treated soils compared to *Arthrobacter* sp. M4 inoculation (Figure 7).

The microbial inoculation markedly altered the soil P fractions. With the most significant changes observed in the inorganic P fraction Ca_2-P and the organic P fractions LPo, MLPo, and MRPo. Notably, the alterations in Ca_2-P and LPo were pronounced both in magnitude and extent (Figure 7). Both microbial inoculations led to a substantial increase in Ca_2-P content within NPK-treated soil, specifically, *Sordariomycetes 2 MS-M4* exhibited greater efficacy than *Arthrobacter* sp. M4, achieving a maximum enhancement of 97.71% (WF). Conversely, the M treatment resulted in a significant reduction of Ca_2-P content by up to 31.52% (bacteria, RM), decreasing it from 50.56 to 34.63 mg kg^{-1}. Furthermore, both microbial inoculations significantly elevated LPo levels in the soil, aligning with changes observed in MBP. For LPo specifically, microbial inoculation proved more advantageous for enhancing its concentration in NPK-treated soils; *Sordariomycetes 2 MS-M4* demonstrated a significantly higher increase compared to *Arthrobacter* sp. M4 with an impressive rise of 236.28%, elevating LPo from 4.29 to 14.43 mg kg^{-1}. The MRPo fraction exhibited changes solely when microbial inoculation was applied to soils treated with M, both *Arthrobacter* sp. M4 and *Sordariomycetes 2 MS-M4* notably decreased MRPo levels by as much as 18.43% (WF).

The Ca_8-P fraction exhibited changes solely upon the inoculation of microorganisms into M-treated soil, with these

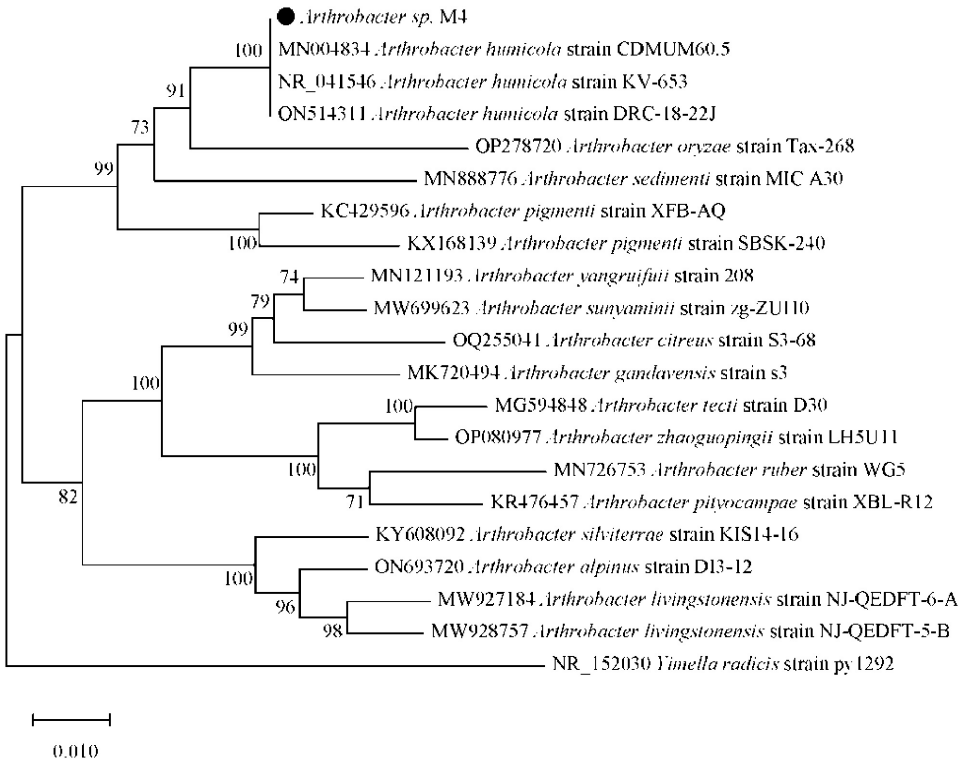


FIGURE 4
Isolated and screened bacteria 16S rRNA phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method, the same as below. The optimal tree with the sum of branch length = 0.47953567 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches, the same as below. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree, the same as below. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site, the same as below. This analysis involved 21 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option), the same as below. There were a total of 1,548 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

TABLE 3 Information table for identification of P-related fungi.

Description	Query cover	*E*-value	Per. Ident	Accession
Sordariomycetes 2 MS-M4 genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, isolate 2266c	78%	0.0	94.64%	OR742068

alterations being relatively minor. Inoculation with *Arthrobacter* sp. M4 led to a significant reduction in the Ca_8-P content within the soil, achieving a maximum decrease of 5.75% in the WM treatment group. Conversely, *Sordariomycetes 2 MS-M4* inoculation resulted in a notable increase in the inorganic P fraction of Ca_8-P only within the WM treatment; other treatments did not demonstrate any significant impact on soil Ca_8-P levels. Overall, *Sordariomycetes 2 MS-M4* inoculation had a more pronounced effect on soil P fractions compared to that of *Arthrobacter* sp. M4, particularly evident in NPK treatment soils.

4 Discussion

4.1 The response of soil phosphorus fractions to prolonged input of swine manure

In this study, the long-term application of swine manure significantly enhanced both the content and proportion of soil active P fractions. This phenomenon can be attributed to the relatively high P content in swine manure (ranging from 0.93 to 5.20%) (Guo et al., 2018) and its predominant presence as orthophosphate post-fermentation (approximately 70%) (Zhang et al., 2021). Consequently, this elucidates the substantial increases observed in TP and Olsen P levels in soil following prolonged swine manure application within this research context. In our study, the average annual total phosphorus content of swine manure in 8 years was 29.23 g kg^{-1}, of which inorganic phosphorus accounted for 72.02% and organophosphorus accounted for 17.98%. However, an excessive accumulation of Olsen P may expedite P leaching from the soil, thereby exacerbating the P load on adjacent aquatic environments. Although swine manure increased the proportion of organic P (TPi/TPo decreased from 3.86 to 3.20), this does not imply reduced plant availability. Microbial-mediated conversion of labile inorganic P (Ca2-P) to microbial biomass P (MBP) and

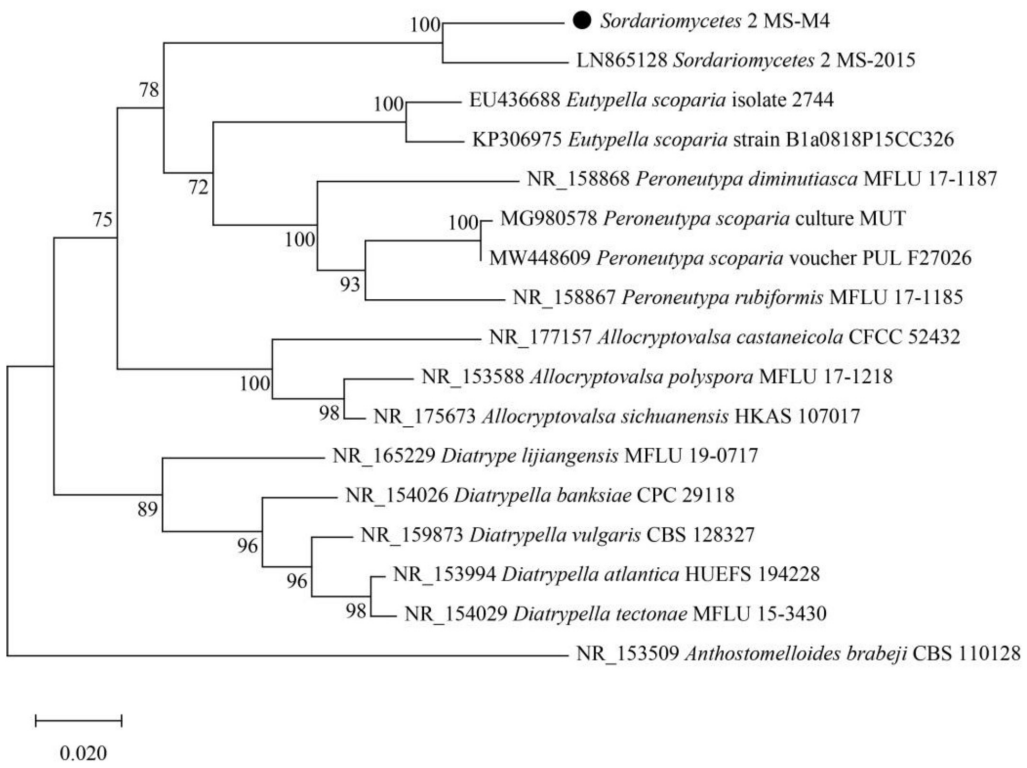


FIGURE 5
Isolated and screened fungal IFS phylogenetic tree. The optimal tree with the sum of branch length = 0.78737578 is shown. This analysis involved 17 nucleotide sequences. There were a total of 1,451 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

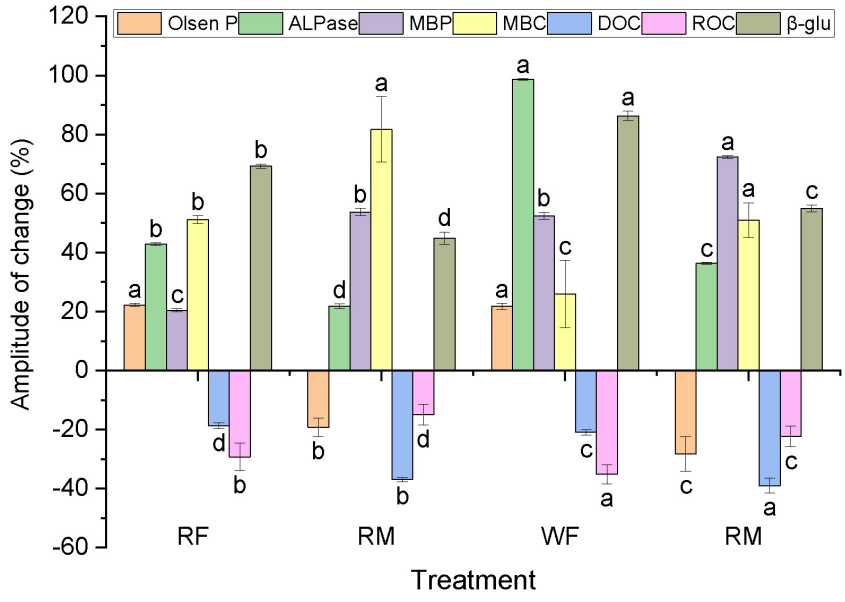


FIGURE 6
Compared with the control, the change amplitude of the functional characteristics of the soil inoculated by bacteria. In the horizontal axis, R represents flooding treatment in simulated rice season and W represents wetting treatment in simulated wheat season. NPK is the field experiment (2.3.1) NPK fertilizer input (F) treatment, M4 is the field experiment (2.3.1) 900 kg P hm^{-2} swine manure input, the same below. Different lowercase letters represent the same index with significant difference between different treatments ($P < 0.05$), the same as Figure 7.

labile organic P (LPo) ensures a slow-release P pool, which reduces leaching risks while maintaining plant uptake through gradual mineralization (Tiecher et al., 2017). This is corroborated by the significant rise in ALPase activity and phoD gene abundance, indicating enhanced organic P turnover capacity. While this study focused on a high swine manure dose (150% N and 600%

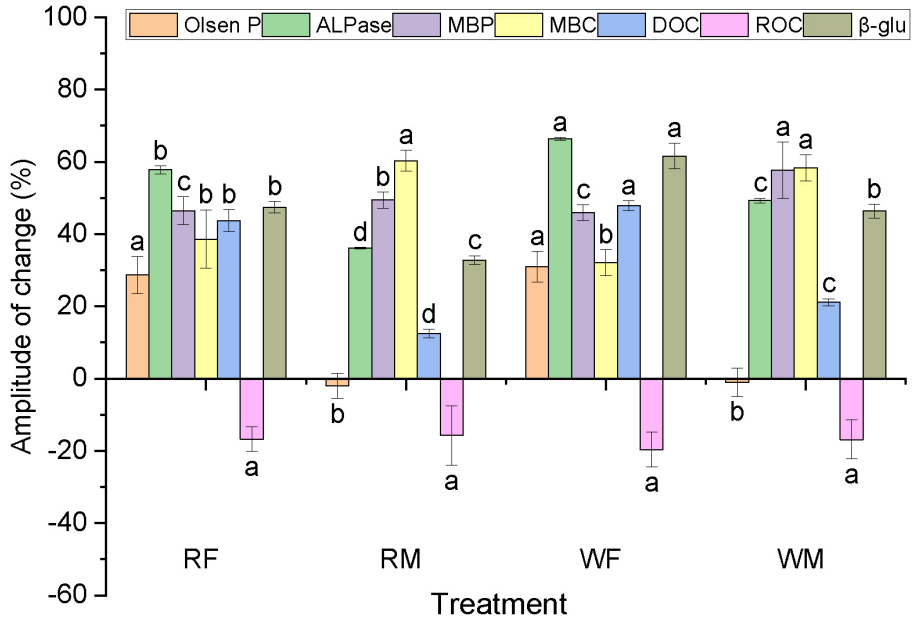


FIGURE 7
Compared with the control, the change amplitude of the functional characteristics of the soil inoculated by fungi.

P of conventional fertilization), the absence of low-to-medium dose treatments limits our understanding of dose-dependent P dynamics. Our prior studies suggest that moderate manure inputs (e.g., 50–100% P requirement) optimize P availability without saturation risks (Zhang C. L. et al., 2024). Therefore, it is imperative to monitor soil P leaching during agricultural practices reliant on long-term swine manure applications and implement strategies aimed at reducing soil P availability—such as converting active inorganic P into organic fractions for stabilization.

Prolonged swine manure application substantially enhanced soil P bioavailability. This improvement may augment the efficiency of soil microorganisms in P utilization, thereby increasing both the diversity and functionality of the soil P microbial community (Lonardo et al., 2019). In this study, the observed increase in organic P proportion within soils subjected to long-term swine manure input can be attributed to microbial activity facilitating the conversion of active inorganic P into biological forms. The notable increases in soil MBP content, ALPase activity, and *phoD*- gene copy number further corroborate this assertion. Notably, when compared to conventional fertilizer applications, the significant rise in *phoD*- gene copy number exceeded that of ALPase activity, indicating functional redundancy among soil microorganisms associated with phosphorus cycling. Despite the benefits of enhanced P availability, prolonged swine manure application may elevate ecological risks. Studies report soil acidification (pH decline by 0.5-1.0 units) and heavy metal accumulation (e.g., Cu, Zn) under high manure inputs (Guo et al., 2018). Additionally, antibiotic resistance genes in manure could propagate in soil microbial communities (Peng et al., 2015). Future research must integrate multi-parameter assessments to balance agronomic benefits with environmental sustainability.

4.2 Functional attributes of phosphorus-associated microorganisms in soil

In this study, the selected bacterium *Arthrobacter* sp. M4 is a member of the genus *Arthrobacter* within the phylum *Actinobacteria*, which has been extensively documented in research concerning antibacterial properties (Ait et al., 2023; Baranova et al., 2022; Ebrahimi et al., 2022). In agricultural contexts, it has been reported to synergistically interact with plants to mitigate abiotic stress (Narsing et al., 2022), remediate pesticide residues in agricultural soils (Saez et al., 2022; Giaccio et al., 2023; Emelyanova et al., 2023), fix N, solubilize P and K (Boubekri et al., 2022; Mao et al., 2024), and enhance the solubility of phosphate fertilizers from 2,000 to 2,020, accumulating a total of 949 reports. Consequently, it is frequently utilized as a multifunctional microbial fertilizer following inoculation into fertilizers for agricultural production (Boubekri et al., 2022). Furthermore, studies indicate that this bacterium exhibits heterotrophic (Zhang M. Y. et al., 2020) and autotrophic capabilities (Elkarrach et al., 2021), denitrification processes, degradation of pesticides (specifically Atrazine herbicide) (Zhang et al., 2022), phenol degradation, cold tolerance, and Paccumulation (P removal from wastewater) (Zhang T. et al., 2020). Research also demonstrates that microalgal bacteria exhibit significant responses to exogenous C organic acids addition, leading to marked increases in soil phosphatase activity (Sandra et al., 2020; Nadia et al., 2017). Thus, *Actinobacteria* microorganisms can enhance biological P activity in soil while effectively reducing both P fixation and leaching.

The soil fungal community represents a diverse assemblage within ecosystems, playing a crucial role in various ecological processes and influencing both plant growth and soil health. In this study, the predominant fungal species identified as *Sordariomycetes*

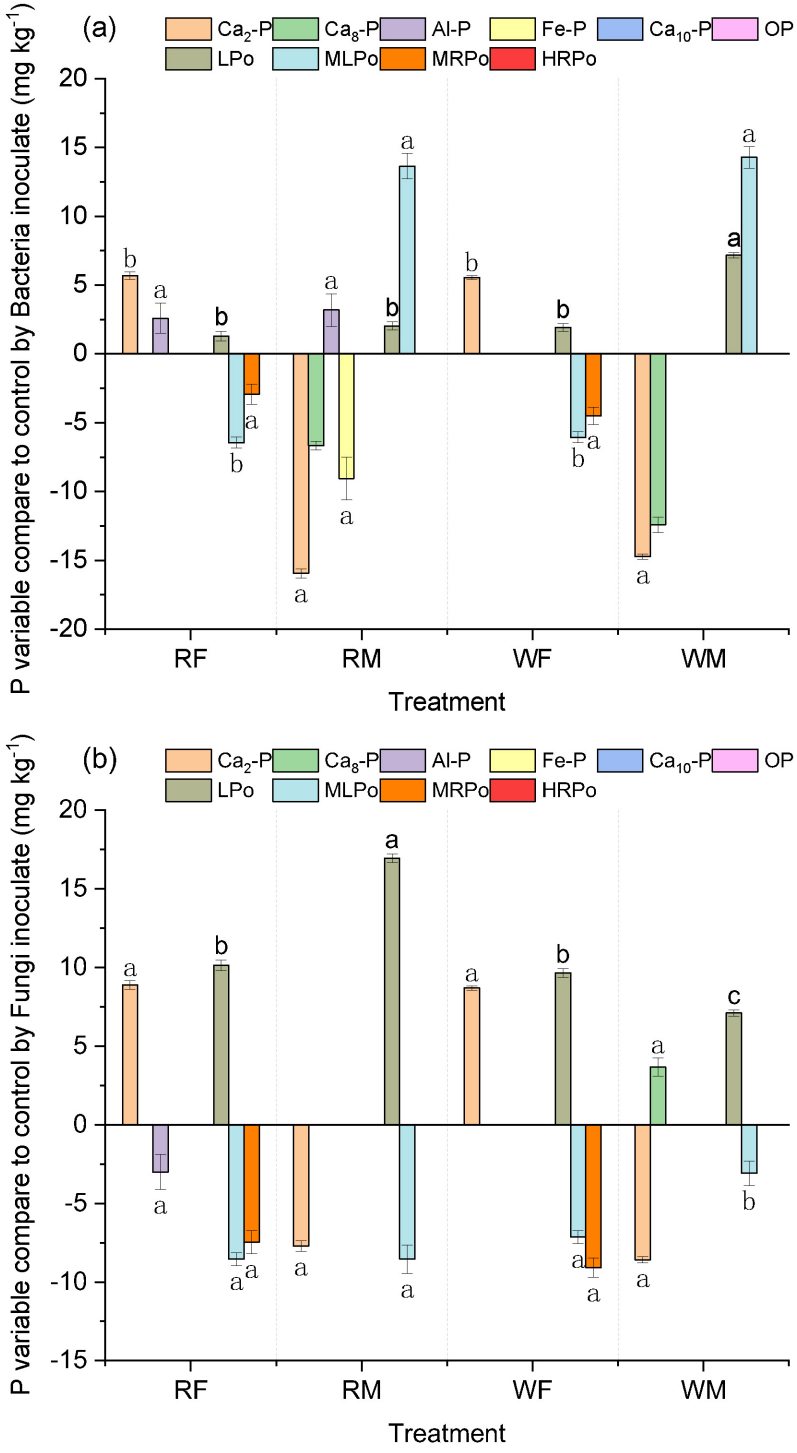


FIGURE 8
P fractions changes in soil inoculated by bacteria and fungi compared with control. **(a,b)** Show the changes of P components in soil inoculated by *Arthrobacter* sp. M4 and *Sordariomycetes 2 MS-M4*, respectively. Different lowercase letters represent significant difference in the change value of the component under different treatments. We do not show that the treatment has not changed the P fractions.

2 MS-M4, extracted from the soil, is classified as a subclass genus of *Ascomycetes*—the most prevalent fungal community found in agricultural soils (Luisa et al., 2024). The medium *Ascomycetes* present in the soil exist in three forms: saprotrophic, parasitic, and symbiotic—interacting with either soil or plant residues—and have been shown to enhance microbial enzyme activity within the soil (Dean et al., 2012; Huang et al., 2021; Xu et al., 2019). The primary metabolic pathway for these fungi involves ATPase catalyzing the hydrolysis of ATP into inorganic phosphate (Luisa et al., 2024; Ondřej et al., 2006). Several studies indicate that *Ascomycete* fungi exhibit a significant positive correlation with available P levels while demonstrating a negative correlation with phosphatase activity

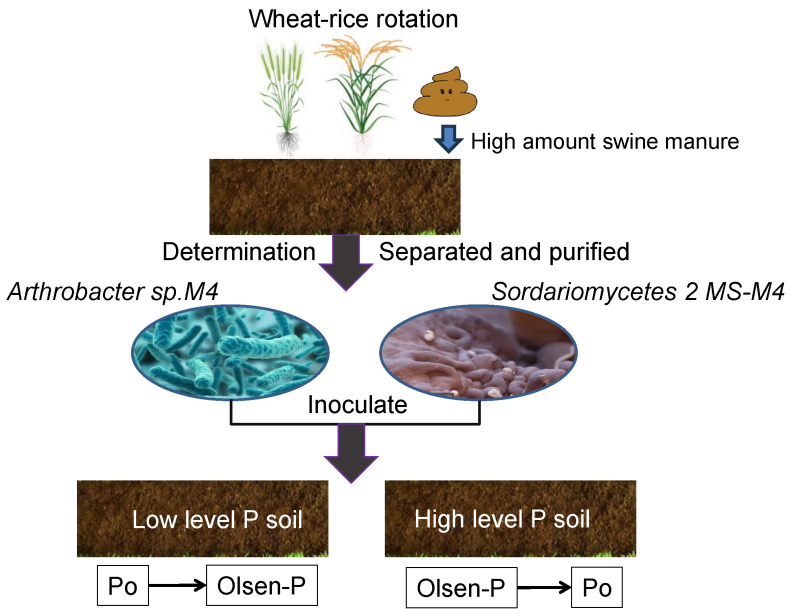


FIGURE 9
Effect of P solubilizing bacteria isolated from soil by adding excess swine manure on soil P. The P-related microorganisms *Arthrobacter* sp. M4 and *Sordariomycetes 2 MS-M4* extracted from soils with high P availability were confirmed to have the key functions of enhancing the fixation of Olsen P into organic P (high-C and high-P condition) or promoting the activation of organic P into olsen P (low C and low P level).

(Luisa et al., 2024; Zhang H. et al., 2024). Conversely, Geng et al. (2023) reported that *Ascomycete* fungi were significantly negatively correlated with both Olsen P and TP content, this discrepancy may be attributed to the diversity and functional variability among different species of *Ascomycete* fungi along with their specific roles. Compared to well-known phosphate-solubilizing bacteria like *Pseudomonas* and *Bacillus*, *Arthrobacter* sp. M4 exhibits unique dual functionality—activating organic P in low-P soils while immobilizing inorganic P in high-P soils. This contrasts with *Penicillium* fungi, which primarily enhance inorganic P availability (Correa et al., 2023). However, *Sordariomycetes 2 MS-M4*'s adaptability to flooded conditions surpasses that of *Mortierella* species, highlighting its potential for rice-dominated systems. The dominant fungal species identified herein, along with its secretions, could substantially influence P cycling within the soil ecosystem. To confirm this hypothesis, further investigation is required to elucidate the precise mechanisms involved.

4.3 The impact of crucial P-Associated microorganisms on soil P bioavailability

In this study, the bacterium *Arthrobacter* sp. M4, which was isolated and characterized, significantly enhanced soil MBC and MBP content as well as related enzyme activity following its inoculation into the soil (Figure 6). Concurrently, the active organic C components of soil DOC and ROC exhibited a significant decrease, suggesting that the inoculation of *Arthrobacter* sp. M4 improved microbial activity in the soil while markedly enhancing respiration and metabolic processes. These findings align with those reported by Boubekri et al. (2022). Additionally, *Arthrobacter* sp. M4 demonstrated a more pronounced influence on soil indicators under wet conditions than under flooded conditions, indicating heightened activity in aerobic environments. Furthermore, the notable increase in Olsen P content within NPK-treated soils alongside a reduction in Olsen P levels within high nutrient soils (M) suggests that *Arthrobacter* sp. M4 exhibited distinct P functionalities contingent upon varying nutrient levels: it facilitated the conversion of inactive inorganic P to active inorganic P in relatively low-nutrient NPK-treated soils while promoting the fixation of active inorganic P into microbial biomass within high-nutrient soils (M).

The inoculation of the fungus *Sordariomycetes 2 MS-M4* into soil resulted in a significant increase in MBC, MBP content, and ALPase activity, suggesting that the extracted fungi and bacteria may exhibit analogous functional characteristics. This enhancement notably improved soil respiration and microbial activity, contrasting with the outcomes observed when bacterium *Arthrobacter* sp. M4 was introduced into the soil. In this study, while soil ROC content decreased, DOC content increased (Figure 7), potentially due to the preference of *Sordariomycetes 2 MS-M4* for decomposing and utilizing high molecular weight organic matter. A related investigation demonstrated that Mortierella elongata significantly enhanced neutral phosphatase activity in soil while reducing Olsen P levels (Li et al., 2021), which aligns with our findings in M soil, however, *Sordariomycetes 2 MS-M4* exhibited superior efficacy. Compared to *Arthrobacter* sp. M4, *Sordariomycetes 2 MS-M4* displayed greater adaptability with minimal variation in indicators under both flooding and moist conditions, as well as a more pronounced effect on enhancing P availability in NPK-treated soils. Nevertheless, it is important to note that members of the subclass *Ascomycota* may act as plant pathogens leading to rot issues affecting root and stem health (Huang et al., 2021), necessitating further exploration

and validation for agricultural applications. The introduction of P-related microorganisms into soils treated long-term with N, P, and K fertilizers yielded a more substantial enhancement of P availability compared to those inoculated into soils subjected to excessive swine manure over extended periods, this indicates that microorganisms sourced from high-P-availability environments possess stronger capabilities for promoting P release within low-P-soil contexts. While this study did not measure oxalate-extractable P or Mehlich-3 P, the observed reduction in Olsen P under high-P conditions (M soil) aligns with the concept of phosphorus saturation. Future studies should integrate DPS (Degree of P Saturation) analysis to quantify threshold P levels where microbial immobilization becomes dominant. Nevertheless, our functional validation demonstrates that microbial inoculation effectively modulates P availability, suggesting their potential role in mitigating saturation-related environmental risks.

Microbial inoculation significantly elevated the concentrations of high-activity inorganic and organic P fractions, namely Ca_2-P and LPo, in the soil, while simultaneously reducing the levels of MLPo and MRPo (Figure 8). This finding indicates that microbial inoculation promotes the interconversion of active P species, which is intricately associated with the energy requirements of soil microorganisms as well as their capacity for decomposing and utilizing soil organic matter (Zhang H. et al., 2024). In contrast to soils characterized by higher C and P content (M), microbial inoculation had a more pronounced effect on C-related enzyme β-glu activity and soil ROC in low C and P environments (F) (Figures 6, 7). This suggests that microbial inoculation enhances both the decomposition and utilization of organic C within low-C, low-P soils (F), thereby facilitating the transformation of organic P into bioavailable inorganic fractions. Consequently, following microbial inoculation coupled with long-term excessive application of swine manure to the soil—resulting in surplus C and P—microorganisms preferentially utilize greater amounts of inorganic P fraction Ca_2-P while concurrently increasing levels of organic P fraction LPo. Conversely, under sustained application of NPK fertilizers in conditions where C and P are relatively deficient, microorganisms decompose organic P fractions MLPo and MRPo while assimilating available organic matter; this process leads to an increase in both Ca_2-P and LPo concentrations within the soil matrix. Overall, *Sordariomycetes 2 MS-M4* inoculation exerts a more substantial influence on soil dynamics compared to *Arthrobacter* sp. M4 inoculation; it particularly affects P fractions—especially those that are biologically active—in NPK-treated soils (Figure 9). Translating laboratory findings to field applications requires addressing challenges such as microbial survival under fluctuating temperatures, competition with indigenous microbiota, and scalability of inoculum production. For instance, *Arthrobacte* inoculants showed reduced efficacy in field trials due to predation by protozoa (Boubekri et al., 2022). Pilot studies are essential to optimize formulation and delivery methods for real-world conditions.

5 Conclusion

The long-term application of high-volume swine manure significantly enhanced the content and proportion of active P fractions in the soil, while microbial-mediated biological fixation of inorganic P markedly increased the organic P fraction. Specific functional microorganisms involved in P cycling were isolated and characterized from the soil following prolonged high-volume swine manure application. The bacterium *Arthrobacter* sp. M4 and the fungus *Sordariomycetes 2 MS-M4* were identified as key players in this process. Upon reintroducing these P-involved microorganisms into the soil, *Arthrobacter* sp. M4 and *Sordariomycetes 2 MS-M4* effectively utilized substantial amounts of organic C within a high-C, P-rich soil matrix (M), leading to significant reductions in DOC and ROC levels. Consequently, Olsen P concentrations decreased significantly, while MBC, MBP, ALPase, and β-glu notably increased, thus facilitating the conversion of active inorganic P into MBP, thereby enhancing biological fixation processes for this nutrient element. In a low-C environment with limited available P (F treatment), inoculation with *Arthrobacter* sp. M4 and *Sordariomycetes 2 MS-M4* improved utilization rates of soil organic carbon, elevated β-glu enzyme activity along with fluctuations in ROC content, promoted decomposition of organic forms of soil-bound P fractions, converting MLPo and MRPo into more bioavailable forms such as Ca_2-P and LPo, thereby activating previously unavailable organic P sources to enhance overall nutrient efficacy. The findings suggest that both *Arthrobacter* sp. M4 and *Sordariomycetes 2 MS-M4* possess promising applications for agricultural practices focused on effective P management.

Data availability statement

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

Author contributions

CZ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. SZ: Data curation, Methodology, Writing – review & editing. XT: Writing – review & editing. BZ: Formal Analysis, Writing – review & editing. DL: Data curation, Writing – review & editing. ZY: Formal Analysis, Methodology, Writing – review & editing. RH: Formal Analysis, Writing – review & editing. YW: Conceptualization, Writing – review & editing. QT: Data curation, Formal Analysis, Writing – review & editing. YL: Methodology, Data curation, Writing – review & editing. CW: Data curation, Methodology, Resources, Writing – review & editing. BL: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Supplementary material

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Bioprospecting of novel silica solubilizing bacteria as bioinoculants for sustainable silica management

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Silicon (Si) is important quasi-essential element, important for growth and productivity in plants by abetting abiotic and biotic stresses. In the recent times intensive cultivation in India has led to depletion of available Si in soils leads stagnation in the crop productivity. In this study, out of 88 rhizobacterial isolates, 24 potential isolates having significant silica solubilizing capability and exhibited plant growth-promoting characteristics were characterized at biochemical and molecular level and further to study their effect on plant growth stimulation and augment the absorption and accumulation of active silica in plants. In qualitative method, all 24 SiS-RB isolates were able to form clear zone of silica solubilization with the solubilizing index (SSI) in the range of 1.05–3.40 cm, whereas in quantitative silica solubilization the solubilized silica was observed in a range of 1.29–43.29 ppm. The 24 SiS-RB isolates further demonstrated plant growth promoting activities. Subsequently, these isolates were evaluated for their capacity to solubilize various minerals, including biotite, calc silicate, feldspar, muscovite, orthoclase, and quartzite, revealing that only six isolates had significant solubilization ability. The six potent isolates *viz.* SSB-2, SSB-8, SSB-11, SSB-12, SSB-21, and SSB-24 showed a considerable enhancement in maize plant development under *in vitro* conditions, including improved antioxidant properties such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia lyase (PAL) activities. All 24 SiS-RB were subsequently analyzed for genetic diversity using amplified ribosomal DNA restriction analysis (ARDRA) analysis, and findings revealed that considerable higher genetic diversity exists among SiS-RB isolates. The integrated dendrogram exhibited similarity indices between 0.11 and 0.90, with a mean of 0.51. All potent silica-solubilizing plant growth-promoting rhizobacterial isolates were identified using 16S rDNA sequencing and belongs to *Enterobacter* sp., *Serratia surfactantfaciens*, and *Klebsiella* sp. These influential isolates would significantly enhance silicate management through

Si based biofertilizer development for plant growth promotion under Si deficient soils.

KEYWORDS

silica solubilizing rhizobacteria, mineralization, phyto-stimulation, antioxidants, ARDRA, 16S rDNA

1 Introduction

Silicon (Si) is considered a "quasi-essential" or advantageous element, and it has a notable function as a micronutrient for plants (Pavlovic et al., 2021; Thakral et al., 2024). Silicon is primarily found on Earth in numerous forms, including wollastonite, feldspar, Si dioxide, quartz (pure SiO_2), and other clay minerals such as kaolinite, mica, and silicates that include elements like aluminum, magnesium, calcium, salt, potassium, or iron (Tayade et al., 2022; Thakral et al., 2024). The use of Si in agricultural fertilization has become more popular due to its non-corrosive nature and sustainability (Thakral et al., 2024).

Plants assimilate Si from the soil as monosilicic acid (H_4SiO_4), which is then transported throughout the plant's tissues, primarily the cell wall and epidermis (Zexer et al., 2023). This integration boosts the rigidity of the cell wall, promotes the organization of the leaf, and may increase the ability to perform photosynthesis in some species (Zexer et al., 2023). Moreover, Si has a crucial function in augmenting plant immunity against diseases and pests, minimizing water loss via transpiration, and improving water use efficiency (Yang et al., 2022). The presence and dispersion of Si in soils are affected by variables such as soil origin, climate, texture, and the extent of soil erosion. Severe weathering may result in the depletion of Si from soils, resulting in the formation of fewer plant-accessible compounds (Katz et al., 2021). In addition, contemporary agricultural methods such as widespread farming and the use of phytosanitary substances and NPK fertilizers might exacerbate the decline of Si levels in soil (Kovács et al., 2022).

Silicon plays a multifaceted role in plant defense, offering protection against viruses, fungi, and herbivores at various growth stages. It acts by repelling pests, blocking their penetration, and lowering the harm they cause. Si not only creates physical barriers, but also plays a role in stimulating systemic resistance in plants (Singh et al., 2020). This process involves the activation of defense-related enzymes such as peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL). These enzymes have essential functions in the plant's reaction to pathogen invasion, such as the production of lignin and the formation of phenolic compounds via the phenylpropanoid pathway (Alhousari and Greger, 2018; Singh et al., 2020).

Silicon exists in soils in both amorphous and crystalline states, and may be found in minerals such as kaolin, smectite, vermiculite, and quartz. While Si makes up a considerable part of the Earth's crust, it is usually insoluble, which limits the amount of Si that plants can absorb (Etesami and Maheshwari, 2018). Silicon is released by weathering processes or the actions of soil microbes and plants, which breakdown it into soil water. Nevertheless, soils in tropical climates, which are known for their extensive weathering, sometimes suffer from a scarcity of plant-accessible Si (Khan, 2025). Continuous cultivation of Si-demanding crops may result in significant depletion of soil Si, especially in sandy soils used for crops such as sugarcane. Plant tissues, including as husks, leaves, and stems, are the main sites of Si accumulation (Katz et al., 2021). The advantages of this extend to mitigating the impact of both biotic stressors, such as diseases and pests, and abiotic stressors, such as drought, salt, and heavy metal toxicity. Enhancing the absorption of Si in crops is seen as a sustainable approach to improve production under challenging environments. Silicon enhances the structural integrity of plant cell walls, hence enhancing the ability of crops such as rice, barley, wheat, and cucumbers to withstand different types of stress (Zargar et al., 2019). On the other hand, a lack of Si makes plants more susceptible to assaults from pests and pathogens (Islam et al., 2020).

Microorganisms, namely bacteria from the genera *Bacillus*, *Pseudomonas*, and *Burkholderia*, have the potential to dissolve silica and silicate minerals, which in turn increases the availability of Si for plants (Bist et al., 2020). Hence, the presence of silica-solubilizing bacteria (SiS-B) can enhance plant health, soil fertility, and defense systems. Biofertilizers containing SiS-B are becoming increasingly recognized as a sustainable and eco-friendly substitute for traditional Si-fertilizers, which may pose environmental risks and lead to higher production expenses. Silica-solubilizing-bacteria based biofertilizers transform insoluble silicates in the soil into soluble forms that plants may easily take up, providing a cost-efficient method to enhance Si accessibility and agricultural output. Rhizospheric bacteria have silica-solubilizing abilities and may produces phytostimulants, biocontrol agents, and other compounds that promote development in plants, which are together, termed silica-solubilizing plant growth-promoting rhizobacteria (SiS-PGPR) (Chaganti et al., 2023). SiS-PGPR significantly contribute to the mineralization of silica from sequestered silica in the soil (Etesami and Schaller, 2023). The use of local SiS-RB isolates specific to geographic locations will provide the advantage of quick adaptation and less competition when introduced in rhizosphere will make them ideal choice to mitigate environmental stresses. Consequently, this research aimed to (i) isolate and screen silica solubilizing rhizobacteria (SiS-RB), (ii) examine additional characteristics such as mineral solubilization and plant growth-promoting attributes, (iii) investigate the impact of effective SiS-PGPR on maize plant growth performance, and (iv) conduct molecular diversity and identification of potent SiS-PGPR.

2 Materials and methods

2.1 Sample collection and rhizobacterial isolation

Soil samples from the rhizosphere of maize were collected from different sites of Kumbhalgarh district. At each site, 100 g

of root-adhering soil were carefully collected in sterile plastic bag and stored at 4°C in lab. For the isolation of rhizobacteria, root-adhering soil was serially diluted and 10^{-4} and 10^{-6} dilution was used for rhizobacteria isolation by using Nutrient Agar, King's B, and Jensen N-Free media to obtained morphologically different rhizobacteria as previously outlined by Upadhyay et al. (2009).

2.2 Silica, other mineral solubilizing attributes, including phyto-stimulation analysis

Qualitative investigation of silica solubilization was conducted using 2.5 µl of pre-incubated bacterial culture disseminated over Bunt and Rovira agar supplemented with 0.25% magnesium trisilicate (w/v). The plates were incubated in darkness at 28 ± 2°C for 72 h, following which a clear zone around the bacterial colony was detected (Bunt and Rovira, 1955). The solubilizing capability was evaluated using the Solubilizing Index, computed as the ratio of the overall diameter of the colony. Quantitative silica solubilizing activities were assessed using 100 ml of bacterial culture cultivated in Bunt and Rovira broth, supplemented with 0.25% magnesium trisilicate (w/v), for 7 days, followed by centrifugation at 10,000 rpm for 15 min. One milliliter of the supernatant was combined with reagents and evaluated with the silicic acid-molybdate technique as described by Santi and Goenadi (2017).

Various minerals, including Biotite, Calc-silicate, Feldspar, Muscovite, Orthoclase, and Quartzite, were used to assess the solubilization capability of bacteria. The silica solubilization concentration of various SiS-B isolates was measured after 5 and 10 days. Phosphate solubilization was assessed using 2.5 µl of pre-incubated bacterial culture disseminated over Pikovskaya's agar enriched with 0.5% calcium phosphate. A transparent halo zone around the bacterial colony was noticed after 24 h (Pikovskaya, 1948). Zinc solubilization was evaluated by Krithika and Balachandar (2016), potassium solubilization was analyzed by method outlined (Saheewala et al., 2023), and siderophore production was measured using the method outlined by Schwyn and Neilands (1987). The biochemical tests, including catalase test, gelatin liquefaction, starch hydrolysis, oxidase test, and citrate utilization, were assessed using conventional protocols. The synthesis of phytohormones, namely IAA and Gibberellic Acid, was assessed using the methodologies of Gordon and Weber (1951), Berríos et al. (2004), respectively. The formation of hydrogen cyanide and ammonia, together with ACC deaminase activity, was evaluated in research conducted by Bakker and Schipper (1987), Penrose and Glick (2003), respectively.

2.3 Pot experiments

The pot studies were conducted under net house conditions using a complete random design (CRD) with triplicates for each treatment, outlined by Sukhwal et al. (2023), Upadhyay and Chauhan (2022). Each pot included 250 g of dirt from the Kumbhalgarh district (25°8′56″N 72°34′49″E), Rajasthan. The seeds of the cultivable maize variety were surface sterilized and inoculated on a 0.8% agar plate to facilitate germination, as outlined by Saheewala et al. (2023). Following germination, seeds of comparable size were transferred into pre-treated pots. Each pot containing germinated seeds received 2 ml of silica-solubilizing rhizobacterial culture, which had been pre-incubated for 24 h at 38 ± 2°C, as per Saheewala et al. (2023). The treatments T1 = SSB2, T2 = SSB8, T3 = SSB11, T4 = SSB12, T5 = SSB21, and T6 = SSB24, together with a control group (without SiS-PGPR inoculation), were formulated using effective SiS-plant growth-promoting rhizobacteria. Plant growth metrics, such as root and shoot fresh weight, shoot length, root length, and chlorophyll content, were assessed after 14 and 28 days, respectively. The chlorophyll-a content was quantified using a UV-visible spectrophotometer (Arnon, 1949). Furthermore, stress-related enzymes including Catalase (CAT), Superoxide Dismutase (SOD), Peroxidase (POD), Polyphenol Oxidase (PPO), and Phenylalanine Ammonia Lyase (PAL) were evaluated by methodologies delineated in our prior publication (Upadhyay et al., 2012; Jain et al., 2017).

2.4 Genetic fingerprinting, Molecular characterization, and phylogenetic analysis

The total genomic DNA of SiS-RB was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma, United States). The 16S rDNA region was amplified using the universal primers (Forward 5′-AGAGTTTGATCCTGGCTAG-3′and Reverse 5′-AGGAGGTGATCCAGCCGCA-3′) (Edwards et al., 1989). To assess genetic similarity, ARDRA (Amplified Ribosomal DNA Restriction Analysis) banding patterns were analyzed using Jaccard's coefficient. The resulting similarity coefficient matrix is then processed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm to generate clusters. This analysis is performed using the NTYSYS 2.02 PC program. The amplified 16S rDNA products were then subjected to restriction digestion with endonucleases such as *Alu*I, *Hae*III, *Hin*fI, and *Taq*I. PCR amplified 16S rDNA was purified using a QIA-PCR purification kit (Qiagen) and sequenced on an Applied Biosystems (ABI) prism automated DNA sequencer (3,130 × 1). The obtained nucleotide sequences were aligned with the GenBank using NCBI BLAST, and the partial 16S rDNA sequences were submitted to NCBI GenBank. Genomic sequences of rhizobacterial isolates were subjected to molecular evolutionary studies, and a phylogenetic tree was constructed using BEAST software.

2.5 Statistical analysis

The study used triplicate experimental data for reliability and repeatability, and analyzed statistically using SPSS and OriginPro software. A dendrogram was created using NTYSYS software to understand linkages and evolutionary patterns. A phylogenetic analysis was conducted using the online BLAST program, which offers advanced computational techniques for Bayesian phylogenetic inference.

TABLE 1 Quantitatively and qualitatively estimation of silica solubilization attributes of silica solubilizing rhizobacteria (SiS-RB) isolates.

SiS-RB isolate	Solubilization index (cm)	*In vitro* silica solubilization (ppm)	Gram staining*	Shape
SSB-1	1.87 ± 0.26	3.12 ± 0.9	Gm-ve	Cocci
SSB-2	2.6 ± 0.3	18.32 ± 2.1	Gm-ve	Cocci
SSB-3	1.14 ± 0.36	18.14 ± 2.1	Gm-ve	Cocci
SSB-4	1.22 ± 0.31	8.54 ± 1.3	Gm-ve	Cocci
SSB-5	1.076 ± 0.025	5.69 ± 1.2	Gm-ve	Cocci
SSB-6	2.09 ± 0.01	1.29 ± 0.6	Gm-ve	Cocci
SSB-7	1.09 ± 0.15	4.83 ± 1.1	Gm-ve	Cocci
SSB-8	3.4 ± 0.15	15.97 ± 2.3	Gm-ve	Cocci
SSB-9	1.42 ± 0.20	7.691 ± 1.2	Gm-ve	Cocci
SSB-10	1.1 ± 0.25	12.03 ± 1.6	Gm-ve	Cocci
SSB-11	2.66 ± 0.25	15.74 ± 2.3	Gm-ve	Cocci
SSB-12	2.85 ± 0.030	15.63 ± 2.5	Gm-ve	Cocci
SSB-13	1.05 ± 0.079	11.17 ± 1.6	Gm-ve	Rod
SSB-14	1.09 ± 0.04	17.63 ± 2.1	Gm-ve	Rod
SSB-15	1.09 ± 0.01	4.32 ± 1.1	Gm-ve	Rod
SSB-16	1.2 ± 0.173	14.83 ± 1.5	Gm-ve	Rod
SSB-17	2.16 ± 0.25	4.32 ± 0.5	Gm-ve	Rod
SSB-18	1.1 ± 0.15	12.66 ± 1.3	Gm+ve	Rod
SSB-19	1.14 ± 0.020	6.6 ± 0.6	Gm-ve	Cocci
SSB-20	2.01 ± 0.15	10.26 ± 1.9	Gm-ve	Rod
SSB-21	2.4 ± 0.2	18.2 ± 2.6	Gm-ve	Rod
SSB-22	1.076 ± 0.25	4.66 ± 1.22	Gm-ve	Rod
SSB-23	1.066 ± 0.03	5.97 ± 1.2	Gm+ve	Cocci
SSB-24	2.8 ± 0.25	43.92 ± 4.35	Gm-ve	Rod

*Gm+ve, Gram positive; Gm-ve, Gram negetive.

3 Result and discussion

3.1 Isolation of silica solubilizing bacteria from various sources

The determination of maximal microbial diversity in the rhizospheric zone used several mediums, as previously reported by multiple studies. Various media possess distinct nutrition sources, allowing microbes to proliferate according to their specific nutrient and energy requirements (Upadhyay et al., 2022). This work used several mediums for the isolation of silica-solubilizing rhizobacteria (SiS-RB). Nutrient agar enriched with magnesium trisilicate ($Mg_2O_8Si_3$, ~0.25%) has been used to distinguish silica-solubilizing bacteria from other bacterial species, as shown by Vasanthi et al. (2018). Researchers have often used Modified Bunt and Rovira media containing magnesium trisilicate for the isolation of silica-solubilizing bacteria (Chandrakala et al., 2019). The dominant technique is magnesium trisilicate, which enables the detection of SiSB by the creation of a distinct solubilization zone around bacterial colonies. This research further screened silica-solubilizing rhizobacteria for growth using a modified Bunt and Rovira medium enriched with magnesium trisilicate. Of the 88 rhizobacterial isolates derived from various media, only 24 shown the ability to solubilize silica on Bunt and Rovira medium, and these 24 silica-solubilizing rhizobacterial isolates were subsequently used in this investigation. Similarly, Cruz et al. (2022) identified 130 bacterial strains from field-cultivated sugarcane, rice, wheat, maize, and soybean in diverse locales around Louisiana. Among them, 20 strains were classified as silica-solubilizing bacteria, using various media such as Luria broth (LB) agar, tryptic soy agar (TSA), and silica broth and agar medium.

3.2 Morphological characterization of silica solubilizing bacteria

All 24 screened silica solubilizing rhizobacterial isolates were exposed to morphological characterization for further investigation and results were summarized as shown in Supplementary Table 1. Gram staining is a most useful and important tool to differentiate bacteria based on wall composition, it's helpful beyond the genus level by providing both biochemical information about the composition of bacteria and special information about the distribution of chemicals into the wall (Beveridge, 2001).

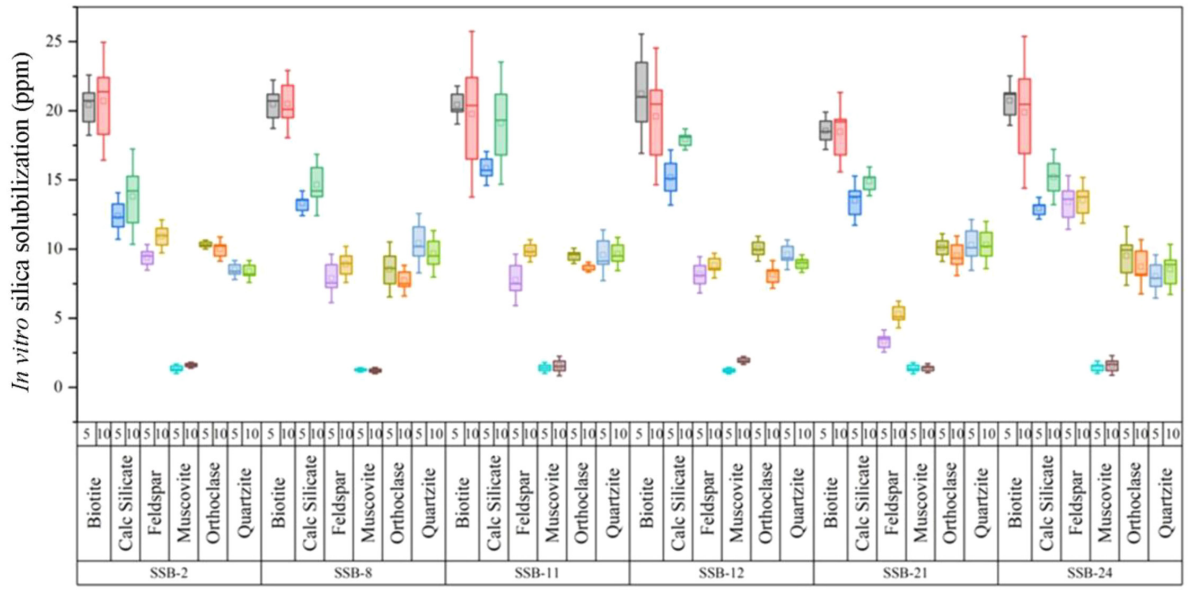


FIGURE 1
In vitro silica solubilization ability of potent rhizobacterial isolates against different silicate minerals after 5 and 10 days, respectively. Error bar reparents standard deviation.

Morphological characteristics of the colony were recorded by gram staining which revealed SiSB as mostly gram negative except SSB-18 and SSB-23. Majority of the SiS-RB isolates were rod in shape with few isolates that are coccoid (Table 1). Similarly, Sulizah et al. (2018) characterized five isolates of silica solubilizing bacteria in terms of morphological characterization, and reported that all the isolates were gram negative.

3.3 Qualitative and quantitative analysis

All 24 SiSB isolates were able to form clear zone or halo zone of silica solubilization on Bunt and Rovira Agar plate supplemented with silica salt. Silica solubilization was measured as Solubilizing Index (SI) which ranged from 1.05 to 3.40 cm as shown in Table 1. Among 24 isolates, SI was maximum recorded for SSB-8 (3.4 ± 0.15) followed by SSB-24 (2.8 ± 0.25) whereas the minimum SI was observed in SSB-13 (1.05 ± 0.079). Babu et al. (2022) reported SI of silica solubilizing bacterial isolates ranging from 2.64 to 4.95. Chopra et al. (2021) reported SI of six solubilizing bacterial isolates were ranging from 1.09 to 2.66. Among them, SSB-24 exhibited the highest solubilization, with 43.92 ppm, while SSB-2 achieved 18.32 ppm, and SSB-6 recorded the lowest at 1.29 ppm. Similar findings were reported by Babu et al. (2022), where SiKPP-1 demonstrated the highest silica content of 2.16 ppm, followed by SiPYY-3 at 2.12 ppm, and SiAGG-1 with 0.52 ppm. Sulizah et al. (2018) reported that OS12 had the highest silicate solubilization of 1.053 ppm in Bunt and Rovira broth.

Variation between qualitative and quantitative screening methods was observed, indicating differences in solubilization of inorganic silicates between plate and liquid assays. Isolates that demonstrated high solubilization index (SI) on solid media did not necessarily exhibit high dissolution in liquid assays reported by Vasanthi et al. (2018). Out of 24, six SiS-RB isolates were solubilized highest silica content in biotite followed by Calc-silicate and feldspar (Figure 1). SSB-8, SSB11, SSB24 were highest solubilizing ability of biotite mineral (Figure 1). Muscovite mineral was least solubilized by all the isolates. Our results were similar to Vasanthi et al. (2018) proportion of SiSB associated with different minerals does not directly correlate with the silica content of the minerals. For instance, muscovite, which contains 21% silica, harbored a higher proportion of SSB compared to phyto-sil, which has 78% silica. In contrast, quartz, with 98% silica, talc with 54%, and feldspar with 45% silica, exhibited lower proportions of SiSB. These findings highlight a significant discrepancy between the total bacterial populations found in soil or silicate minerals and the specific SiSB isolates.

Silica-solubilizing microorganisms have the capability to release soluble silica from insoluble inorganic silicates (such as those containing calcium, aluminum, potassium, and magnesium) and biogenic materials like diatomaceous earth, siliceous earth, rice husk, and rice straw. The formation of a halo zone in agar media can be influenced by several factors, including the type of substrate, the size and volume of the inoculant, the thickness of the agar layer, medium composition, pH, and incubation temperature (Chaganti et al., 2023). Organic acids produced by silica-solubilizing bacteria are key to breaking the Si-oxygen bonds (O-Si-O) in quartz, thereby releasing soluble silica (Etesami and Glick, 2023). In this study, organic acids were analyzed by using HPLC (Data are not shown). These acids, such as acetic acid and citric acid, play a crucial role in the dissolution process by producing hydrogen ions (H^+), which can help dissolve silicate minerals (Figure 1). Organic acids, particularly those containing carboxylate groups, are weak acids that easily ionize, facilitating the release of cations and affecting the pH during silica solubilization (Chaganti et al., 2023). An excess of cations can influence the pH and pull anions like hydroxide

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TABLE 2 Plant growth promoting attributes, biochemical characteristic, and GenBank-accession number of potent silica solubilizing rhizobacteria (SiS-RB) isolates.

SSB isolate	IAA	GA_3	Phosphorus solubilization	Potassium solubilization	Zinc solubilization	ACC	Ammonia	HCN	Starch Hydrolysis	Citrate Utilization	Nitrate Reduction	Gelatin liquefaction	Catalase Activity	Oxidase	GenBank-accession number
SSB-2	1.29 ± 0.3	0.71 ± 0.1	2.5 ± 0.19	3 ± 0.25	5 ± 0.33	+	+	+	+	+	–	–	+	–	PQ157604
SSB-8	8.54 ± 1.2	0.65 ± 0.3	1.6 ± 0.198	3.5 ± 0.26	4.2 ± 0.10	++	–	–	+	+	+	–	+	+	PQ157605
SSB-11	14.83 ± 2	0.62 ± 0.3	1.6 ± 0.18	3.4 ± 0.20	4.25 ± 0.22	+	–	–	+	+	+	–	+	–	PQ157606
SSB-12	4.32 ± 1.1	0.65 ± 0.2	1.16 ± 0.22	3 ± 0.179	5 ± 0.21	+	+	+	+	+	+	+	+	+	MW308551
SSB-21	5.69 ± 1.3	0.61 ± 0.2	1.75 ± 0.21	3.25 ± 0.12	3.75 ± 0.27	+	+	+	–	+	+	–	+	–	PQ157607
SSB-24	4.32 ± 1.2	0.71 ± 0.2	3.0 ± 0.21	6 ± 0.27	4.25 ± 0.29	+	+	+	+	+	+	–	+	–	PQ157608

Data of only potent silica solubilizing plant growth promoting rhizobacteria (SiS-RB).

(OH^-) away from quartz, aiding in its dissolution. The chelation of anions by organic acids results in the solubilization of quartz into a form that can be absorbed by plants, specifically as monosilicic acid $[Si(OH)_4]$, which is taken up by paddy plants through their lateral roots.

3.4 Plant growth promoting activities of potent SSB

All SiS-RB isolates were evaluated for their plant growth-promoting attributes as shown in Supplementary Tables 2, 3. These activities positively impact on plant growth by producing growth regulators, enhancing et al., 2022). nutrient availability, and protecting plants from various abiotic and biotic stresses (Upadhyay In addition to effective silica solubilization and plant growth promoting attributes of SiS-RB, increases their applicability in silica management and plant growth performance, therefore this study plays remarkable role in silica management for plant growth.

Indole 3-acetic acid (IAA) has been reported to play a key role in plant growth promotion (Shoebitz et al., 2009; Upadhyay et al., 2022) and play important role in biotic and abiotic stresses. It is well-known that auxin as important phytohormone enhances the plant growth and development from seedling stage to senescence (Mazzoni-Putman et al., 2021). It is well-known that the presence of auxin enhances the influence of bacteria in the rhizosphere of a plant. All the SiS-RB isolates were IAA producer as sown in (Supplementary Tables 2, 3). Cruz et al. (2022) demonstrated that all silica solubilizing bacterial isolates produced indole IAA with the range of 1.97–77.32 μg/ml. All the six SiS-RB isolates produce GA_3 which ranges from 0.378 to 0.705 μg/ml. SSB-24 produces highest amount of GA_3 (0.711) followed by SSB-2 (0.705) and SSB-12 (0.652) and SSB 8 (0.645) whereas lowest in SSB 22 (0.378) followed by SSB 23 (0.387) (Supplementary Tables 2, 3). Similarly, Upadhyay et al. (2009) investigated the efficiency of salt-tolerant rhizobacteria for gibberellic acid production. Gibberellic acid is a plant hormone and play remarkable role in plant growth (Upadhyay et al., 2022).

Microorganisms enhance the availability of inorganic phosphorus (P) through the production of protons and organic acids, which are commonly found among rhizosphere P-solubilizing microorganisms (Hinsinger et al., 2011). Out of 24 SiS-RB isolates, 21 isolates were found positive for phosphate solubilization. Among 24 isolates, SSB-24 (3.0 ± 0.21) was shown maximum ability for phosphate solubilization followed by SSB-2 (2.5 ± 0.19) (Table 2). Similarly, Cruz et al. (2022) reported that nine out of twenty silica solubilizing bacterial isolates were able to solubilize tricalcium phosphate in Pikovskaya's medium, as evidenced by the formation of a clearing zone around the bacterial colonies. Table 2 shows the potassium solublization ability of six SiS-RB only. Potassium is one of the most important macro-nutrients for plants it plays a significant role in enzyme activation, charge balance, osmoregulation and reduction in the negative effects of stress. Among 24 SiS-RB isolates only 22 were found positive for potassium solubilization, SSB-24 (6 ± 0.27) followed by SSB-8 (3.5 ± 0.26) whereas minimum solubilization was recorded in SSB-16 (1.2 ± 0.058). Zinc is an essential micronutrient necessary in trace amounts for optimal growth, reproduction, and cellular metabolism (Vidyashree and Arthanari, 2021). However,

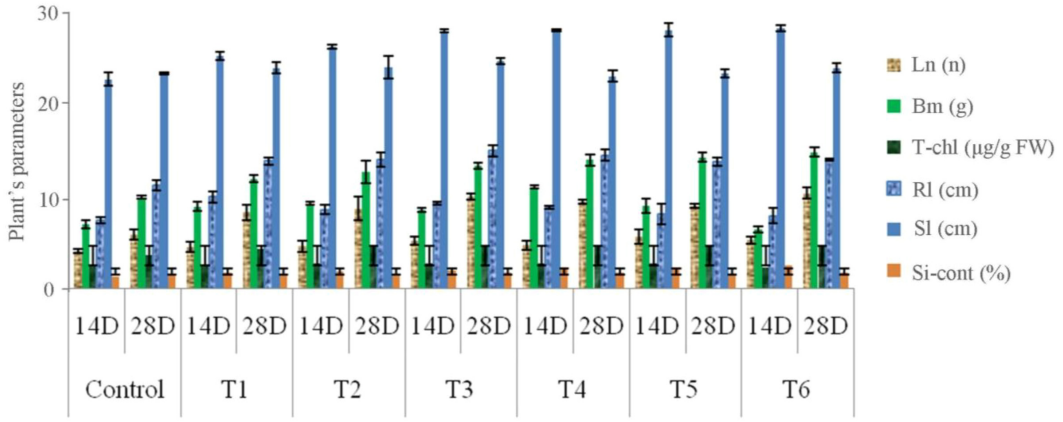


FIGURE 2
Influence of different treatment of silica solubilizing plant growth promoting rhizobacteria on maize plant growth performance after 14 and 28 days. The error bar represents ± Standard deviation values; (T1 = SSB2, T2 = SSB8, T3 = SSB11, T4 = SSB12, T5 = SSB21, and T6 = SSB24), Sl, shoot length; Rl, root length; Ln, leaf number; Bm, biomass (g); T-chl, total chlorophyll content; Si-cont, Silica content in plant.

TABLE 3 *In vitro* seed bacterization studies of silica solubilizing plant growth promoting rhizobacteria on stress related enzymes of maize after 28 days.

Treatment	SOD µmol min^{-1} g^{-1}	POD µmol min^{-1} g^{-1}	PPO µmol min^{-1} g^{-1}	PAL µmol min^{-1} g^{-1}	CAT µmol min^{-1} g^{-1}
Control	9.35 ± 0.61^{a}	0.0501 ± 0.0017^{a}	0.09 ± 0.014^{a}	0.214 ± 0.04^{a}	89 ± 9.46^{a}
T1	10.09 ± 0.23^{b}	0.0833 ± 0.0061^{b}	0.12 ± 0.012^{b}	0.248 ± 0.03^{b}	101.5 ± 3.25^{b}
T2	10.64 ± 0.38^{b}	0.0851 ± 0.0093^{b}	0.15 ± 0.018^{c}	0.251 ± 0.07^{b}	119 ± 7.39^{b}
T3	11.06 ± 0.60^{c}	0.0847 ± 0.0102^{b}	0.17 ± 0.011^{c}	0.268 ± 0.04^{b}	120.75 ± 9.87^{b}
T4	11.10 ± 1.04^{c}	0.0889 ± 0.0122^{b}	0.25 ± 0.021^{d}	0.280 ± 0.09^{b}	155.25 ± 12.05
T5	11.20 ± 0.81^{c}	0.0987 ± 0.0142^{bc}	0.28 ± 0.025^{d}	0.299 ± 0.05^{c}	289 ± 9.1^{cd}
T6	11.56 ± 0.61^{c}	0.0991 ± 0.0152^{c}	0.29 ± 0.021^{d}	0.287 ± 0.06^{b}	300.5 ± 11.54^{cd}

Data was collected from triplicate study, DMRT at $p < 0.05$ shows similar letter represent data not significant, while different letters show significant data. T1 = SSB2, T2 = SSB8, T3 = SSB11, T4 = SSB12, T5 = SSB21, and T6 = SSB24.

elevated levels of zinc in soils can pose environmental hazards and threaten sustainable and high-quality food production. The use of zinc-tolerant microorganisms can help mitigate these issues and manage excessive zinc concentrations (Rani et al., 2023). All SiS-RB isolates were found positive for zinc solubilization as shown in Supplementary Tables 2, 3. The maximum solubilization was recorded in SSB-2 (5 ± 0.33) followed by SSB12 (5 ± 0.21) exhibiting the maximum SI. The SSB 20 (1.42 ± 0.12) found to be least solubilizer (Table 2).

Stress can lead to increased production of 1-aminocyclopropane-1-carboxylic acid (ACC), which serves as a precursor to ethylene (Wang and Adams, 1982). It is widely recognized that ACC can be hydrolyzed by the bacterial enzyme ACC deaminase into ammonia and α-ketobutyrate (Sarapat et al., 2020). By reducing the amount of ACC in plants, PGPRs with ACC deaminase activity can promote the development of a more robust root system. In this study, 17 out of 24 SSB strains were found to be positive for ACC deaminase activity, while 7 were negative (Supplementary Tables 2, 3). Similarly, Cruz et al. (2022) reported that 9 out of 20 silica solubilizing bacteria isolates exhibited ACC deaminase activity. Ammonia and HCN production by plant growth-promoting bacteria (PGPB) plays a crucial role in nitrogen supply to host plants, enhancing root and shoot elongation and overall biomass. The ammonia produced by these microorganisms in the soil serves as a valuable nitrogen source for plants. In this study, Table 2 showed the Ammonia and HCN activities by SiS-RB.

3.5 *In vitro* efficacy of SiS-RB in maize

In vitro studies of silica solubilizing bacteria in maize plant growth-performance were described in Figure 2. The inoculation of silica solubilizing plant growth promoting rhizobacteria significantly increased shoot length of maize T5 (7.56 ± 1.28). Average biomass recorded in maize were found to be maximum in T1 (14.33 ± 2.08) compared to control. With the increase of chlorophyll highest silica content was found in T6 (3.68 ± 0.056) compared to control.

Vandevivere et al. (1994) highlight the role of Si-solubilizing bacteria in consistently providing Si, which contributes to enhanced plant growth characteristics. This is due to their ability to release soluble silica, which can be utilized by plants for improved development and stress resistance. The expression of stress related enzymes viz., Catalase (CAT), Superoxide dismutase (SOD), Peroxidase (POD), Polyphenol oxidase (PPO) and Phenylalanine ammonia lyase (PAL) were also studied in 28 days old maize

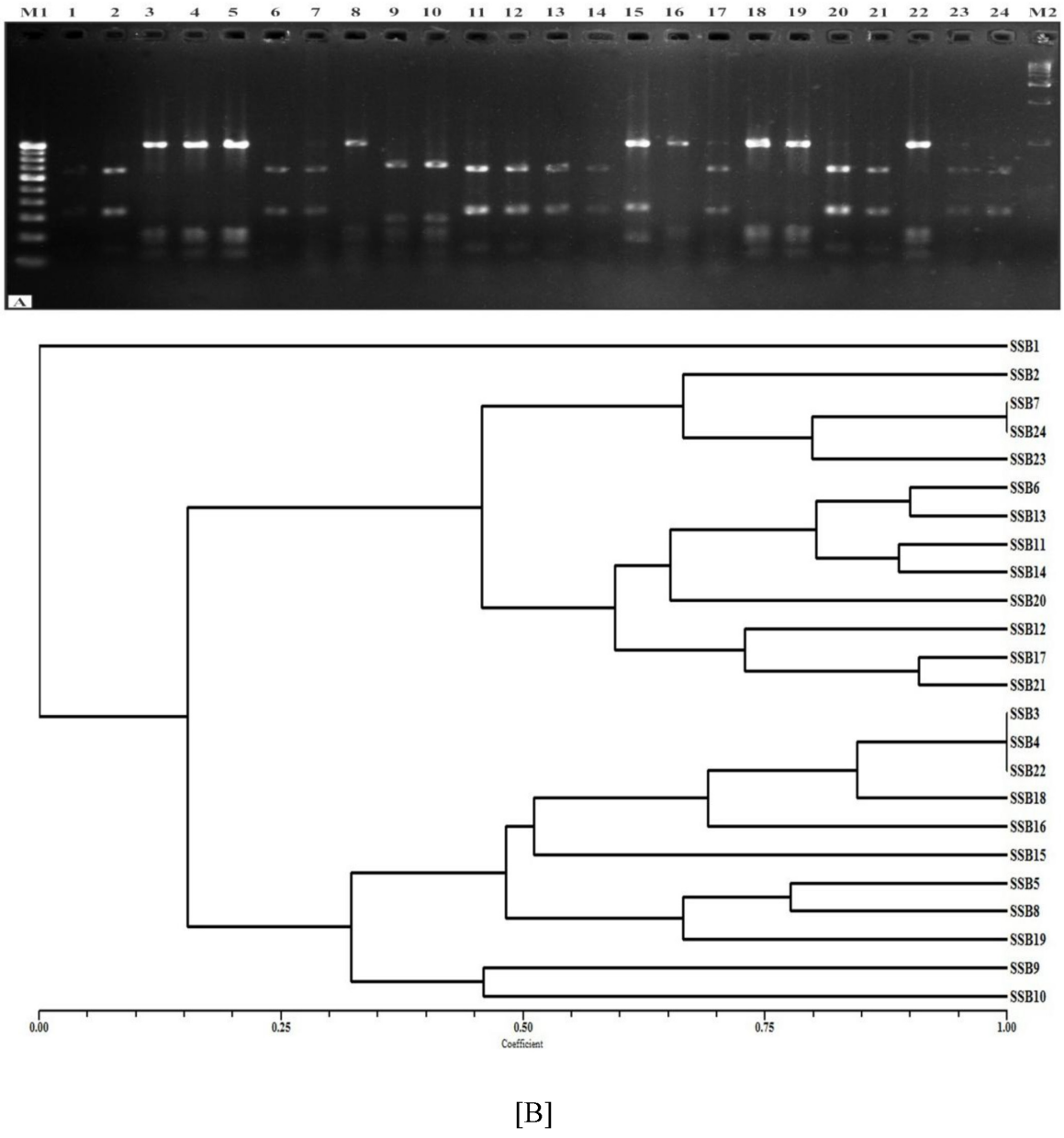


FIGURE 3
(A) Amplification profile based on ARDRA patterns of 24 SiS-RB isolates using restriction endonuclease *Hin*fI. **(B)** Combined dendrogram of 24 SiS-RB isolates based on average similarity coefficients for *Alu*I, *Hae*III, *Taq*I, and *Hin*fI enzyme using the unweighted pair group method with Arithmetic averages (UPGMA).

plantlet and it was significantly influenced by SiS-RB treatment (Table 3). The higher expression of these antioxidant/defense enzyme activities in the maize will not only mitigate stress but also contribute to plant growth.

3.6 Molecular characterization using ARDRA

Genetic fingerprinting, a key molecular technique, creates a unique profile of microbial communities through the direct analysis of PCR products from individual strain DNA. This process generates a fingerprint based on either sequence polymorphism or length polymorphism. Genetic fingerprinting is both rapid and capable of analyzing multiple samples simultaneously. While it is effective in demonstrating differences or effects on microbial communities, it does not provide direct taxonomic identities (Lendvay et al., 2020). To assess genetic similarity, ARDRA (Amplified Ribosomal DNA Restriction Analysis) banding patterns are analyzed using Jaccard's coefficient. The resulting similarity coefficient matrix is then processed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm to generate clusters. This analysis is performed using the NTYSYS 2.02 PC program.

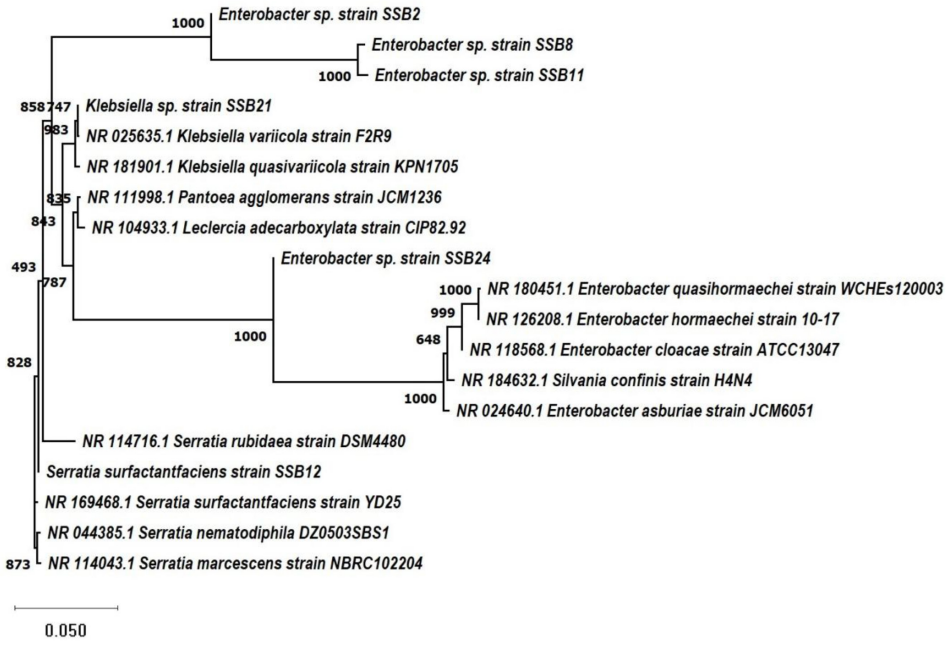


FIGURE 4
Phylogenetic tree constructed by using submitted sequences of potent SSB2, T2 = SSB8, T3 = SSB11, T4 = SSB12, T5 = SSB21, and T6 = SSB24 isolates with retrieved sequences from GenBank of closely submitted sequences of different species.

Four restriction endonucleases viz. *Alu*I, *Hin*fI, *Hae*III, and *Taq*I were used for 16S rDNA RFLP analysis and the banding patterns of the representative SiS-RB with standard molecular weight marker are shown in (Figure 3). In total, 24 bands of varying sizes were observed in all the SiS-RB strains with catalysis by four restriction endonucleases. The *Hin*fI produced 05, *Hae*III produced 08, *Alu*I produced 05, and *Taq*I produced 06 polymorphic bands upon digestion. Jaccard's similarity coefficient-based banding pattern was used for cluster analysis to study genetic relationship. Similarity indices established on the basis 24 bands of 4 restriction enzymes ranged from 0.11 to 0.90 with an average value of 0.51. The pair wise comparison of ARDRA patterns based on both shared and unique amplification products was made to generate a similarity matrix. The dendrogram is a close representation of the values obtained in the Jaccard similarity matrix discriminated all SSB isolates into two major clusters at 0.15 similarity coefficient. The ARDRA revealed moderate molecular diversity among SSB strains studied in the present study.

The Ist (A) cluster consists of twelve strains and it was divided into sub cluster A1 and A2. Sub cluster A1 included total of four strains *viz.*, SSB2, SSB 7, SSB 23 and SSB 24 Sub cluster A2 included four strains and comprises of eight strains *viz.*, SSB 6, SSB 13, SSB 11, SSB 14, SSB 20, SSB 12, SSB 17, and SSB 21. The second cluster (B) included total eleven strains and it was divided into sub cluster B1 and B2. Sub cluster B1 included total of nine strains *viz.*, SSB3, SSB 4, SSB 22, SSB 18, SSB 16, SSB 15, SSB 5, SSB 8 and SSB 19 Sub cluster B2 included two strains *viz.*, SSB 9 and SSB 10. SSB1 was not grouped in any cluster and was kept as independent strain in the dendrogram (Figure 3). The combined dendrogram, generated using UPGMA based on average similarity coefficients, showed similarity indices ranging from 0.11 to 0.90, with an average of 0.51. This dendrogram closely reflects the Jaccard similarity matrix, which classified all SiS-RB isolates into two major clusters at a similarity coefficient of 0.15, comprising 12 and 11 strains, respectively.

In this study, SSB-2 was identified as *Enterobacter sp.* (PQ157604), SSB-8 *was Enterobacter sp.* (PQ157605), SSB-11 was *Enterobacter sp. (*PQ157606), SSB-12 was *Serratia surfactantfaciens (*MW308551), SSB-21 was *Klebsiella sp.* (PQ157607), and SSB-24 was *Enterobacter sp.* (PQ157608). Based on submitted sequences of 16S rDNA of six SiS-RB were used to construct phylogenetic tree (Figure 4), which raveled the close relationship between silica solubilizing microbes. Chandrakala et al. (2019) utilized 16S rDNA gene sequencing to identify the silicate solubilizing IIRI-1 isolate as *Rhizobium sp.* Well-known root nodulation bacteria. Similarly, Raturi et al. (2022) performed comprehensive genome sequencing of *Enterobacter* sp. LR6 of Gram-negative *Enterobacteriaceae* family which also represents the potent isolates of the present study and reported that this family provided a landscape of highlighting genes responsible for silicate solubilization, stress tolerance, and growth-promoting activity.

4 Conclusion

This study underscores the considerable potential of rhizospheric bacteria in solubilizing silicate minerals and enhancing plant development. The discovery and characterization of silica-solubilizing plant growth-promoting rhizobacteria (SiS-PGPR), comprising distinct isolates such as *Enterobacter* sp., *Serratia surfactantfaciens*, and *Klebsiella* sp., provide significant insights into sustainable agriculture techniques. These bacteria exhibited significant capacities, including the enhancement of silica solubilization, the promotion of plant growth, and the

increase of silica absorption in maize plants. 24 rhizobacterial isolates had robust silica-solubilizing and plant growth-promoting characteristics, while six isolates exhibiting outstanding efficacy in mineral solubilization, plant growth promotion, and enhancement of antioxidant enzyme activities in maize. Further, the dedicated field studies for these SiS-PGPR need to conducted in order to assess its efficacy and mechanism under different soil ecosystems including silicon deficiency conditions. Utilizing the capabilities of these effective SiS-PGPR for the development of effective biofertilizer formulations, agricultural systems may get improved crop yield and silica management while decreasing dependence on chemical fertilizers. This research underscores the significance of biofertilizers in advancing sustainable agriculture while minimizing environmental effect, in accordance with the global sustainable development goal.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PQ157604, PQ157605, PQ157606, MW308551. PQ157607, PQ157608.

Author contributions

EM: Data curation, Methodology, Writing – original draft. SC: Formal Analysis, Methodology, Writing – original draft. SMa: Formal Analysis, Methodology, Software, Writing – review and editing. SU: Data curation, Resources, Software, Writing – review and editing. SRM: Funding acquisition, Resources, Visualization, Writing – review and editing. AA: Writing – review and editing, Funding acquisition, Software. RS: Writing – review and editing, Formal Analysis, Visualization. DJ: Conceptualization, Project administration, Resources, Supervision, Writing – review and editing.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1556406/full#supplementary-material

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