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Plants surviving in extreme environment: Harnessing soil-plant–microbial relationship to enhance crop health and productivity

Edited by
Priyanka Chandra, Dilfuza Egamberdieva, Nirmalendu Basak and Arvind Kumar Rai

Coordinated by
Bandeppa Sonth

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Plants surviving in extreme environment: Harnessing soil-plant–microbial relationship to enhance crop health and productivity

Topic editors
Priyanka Chandra — Central Soil Salinity Research Institute (ICAR), India
Dilfuza Egamberdieva — Leibniz Center for Agricultural Landscape Research (ZALF), Germany
Nirmalendu Basak — Central Soil Salinity Research Institute (ICAR), India
Arvind Kumar Rai — Indian Council of Agricultural Research (ICAR), India

Topic coordinator
Bandeppa Sonth — Indian Institute of Rice Research (ICAR), India

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EDITED AND REVIEWED BY
Paola Grenni,
National Research Council, Italy

*CORRESPONDENCE
Priyanka Chandra
priyanka.chandra@icar.gov.in
Nirmalendu Basak
nirmalendu.basak@icar.gov.in
Arvind Kumar Rai
ak.rai@icar.gov.in

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Editorial: Plants surviving in extreme environment: harnessing soil-plant–microbial relationship to enhance crop health and productivity

Priyanka Chandra [1]*, Dilfuza Egamberdieva [2,3], Nirmalendu Basak [1]*, Arvind Kumar Rai [1]* and Bandeppa Sonth [4]

[1]Division of Soil and Crop Management, ICAR-Central Soil Salinity Research Institute, Karnal, Haryana, India, [2]Faculty of Biology, National University of Uzbekistan, Tashkent, Uzbekistan, [3]Institute of Fundamental and Applied Research, National Research University TIIAME, Tashkent, Uzbekistan, [4]Division of Soil Science, ICAR-Indian Institute of Rice Research, Hyderabad, Telangana, India

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plant-microbes interaction, abiotic and biotic stresses, plant growth-promoting rhizobacteria (PGPR), soil health, sustainable agriculture

Editorial on the Research Topic
Plants surviving in extreme environment: harnessing soil-plant–microbial relationship to enhance crop health and productivity

Globally, the sustainability of food production is challenged by biotic and abiotic stressors acting alone or in combination in different agricultural systems. Environmental factors such as high temperatures, droughts, waterlogging, excessive salinity, and other pollutants are the key stressors that limit crop choice and cause significant losses in agricultural productivity. However, crop plants vary in their adaptive response to harsh environmental and edaphic circumstances, but the mutualistic association of the microorganisms with crop plants in diverse environments exhibits enormous metabolic capabilities to mitigate abiotic stresses (Chandra et al., 2022). Microbial partners regulate the systemic and defense mechanisms of crop plants in response to unfavorable external conditions, as they are an essential component of the living ecosystem (Verma et al., 2023). Many rhizobacteria possess the ability to produce 1-aminocyclopropane-1-carboxylate (ACC) and deaminase enzymes to transform ACC into α-ketobutyrate and ammonium, which are responsible for plant growth promotion and stress tolerance. The beneficial rhizobacterial partner mobilizes and/or solubilizes minerals through the production of hydrolyzing enzymes and organic acids. Additionally, they also favor the partner by preventing infection by phytopathogens. The changes in phytohormone, antioxidative enzymes (catalase, superoxide dismutase, and guaiacol peroxidase) and osmolyte (proline and glycine betaine) production by the plant –microbe interaction also provide an adaptive advantage in stressed ecologies.

The aim of this Research Topic was to develop an understanding of plant–microbial relationships under extreme environmental conditions through selected studies. This Research Topic provides a comprehensive and mechanistic view of the microbial response

to environmental changes by bringing together 20 studies that illustrate molecular techniques to understand the mechanisms and function of microbial communities through integrated ecological and biogeochemical approaches. In total, 19 original research studies (including field surveys and laboratory or field experiments) and one review article were contributed to this research area. In addition to focusing on niche differentiation in the bacterial microbiomes of the rhizosphere and endosphere, differential metabolite profiles, and the link among soil microbes, this Research Topic also presents new findings on the variables that influence microbial communities, the type and intensity of reactions, and the differences in responses among microbial groups. Yuan et al. offered thorough empirical proof for the significance of silicon fertilizer in agriculture. The phylogenetic and chemotaxonomic studies established the lack of transmissible resistance mechanisms for safe agronomic usage of *Pseudomonas mercuritolerans* under mercury stress conditions (Robas Mora et al.). Singh et al. outlined the taxonomic composition and functional diversity of the potential role of the *Indica* rice rhizome in the development of next-generation microbiome-based technologies for yield enhancement. A review paper by Kumar et al. emphasized the importance of scientific discoveries and policy support for pulse-based cropping systems to achieve the United Nations' Sustainable Development Goals. Two studies reported on the structural features and diversity of rhizosphere bacterial populations of wild *Fritillaria przewalskii* Maxim. (Cui et al.), and *Festuca rubra* subsp. *pruinosa* (Toghueo et al.). Some studies highlighted the importance of soil biological health (Chandra et al.; Chaganti et al.; Rai et al.; Li, Wang et al.; Lu et al.; Yuan et al.) and endophytic bacteria (Badran et al.) for improving productivity, disease resistance and sustainability of different cropping systems and heavy metal contaminated soils (Shahzad et al.).

The response of the microbial flora to different edaphic parameters determines the mitigation capabilities of their mutualistic association with crop plants. The improved performance of wheat and ryegrass in association with halotolerant bacterial and fungal consortia (Marghoob et al.) and *Gracilibacillus dipsosauri* (Li, Zheng et al.), respectively, highlighted the importance of the microbial formulations in reducing salt stress. Adaptation of the pathogenic fungus *Fusarium oxysporum* f. sp. *pisi* by genetic or mutational alterations under the changed temperature, pH, and nitrogen levels are also important factors affecting the disease occurrence and associated management strategies (Chakrapani et al.). The beneficial effect of the bio-stimulants is also modified by the nature of the interaction with other associated microbial communities (Wang et al.; Wei et al.) and the composition of the rhizosphere exudates (Zhao et al.). Although this topic adequately highlights the potential of soil-plant-microbial interaction in extreme environments, further investigations in this domain will enhance the current comprehension of plant-microbial interaction and its effectiveness from agricultural practice perspectives. These studies further validate the importance of plant-microbial interaction, which can play a significant role in boosting agricultural productivity and stress tolerance.

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EDITED BY
Priyanka Chandra,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Kilaza Samson Mwaikono,
Dar es Salaam Institute of Technology, Tanzania
Devendra Singh,
Dr. Rajendra Prasad Central Agricultural University, India

*CORRESPONDENCE
Zhaonian Yuan
yuanzn05@163.com

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Silicon fertilizer mediated structural variation and niche differentiation in the rhizosphere and endosphere bacterial microbiome and metabolites of sugarcane

Zhaonian Yuan[1,2,3]*, Ziqin Pang[1,2], Nyumah Fallah[1,2], Yongmei Zhou[2,4], Fei Dong[4,5], Wenxiong Lin[2,5] and Chaohua Hu[1,2]

[1]Key Laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture, Fujian Agriculture and Forestry University, Fuzhou, China, [2]College of Agricultural, Fujian Agriculture and Forestry University, Fuzhou, China, [3]Province and Ministry Co-sponsored Collaborative Innovation Center of Sugar Industry, Nanning, China, [4]Center for Genomics and Biotechnology, Fujian Agriculture and Forestry University, Fuzhou, China, [5]College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou, China

The microbiomes of plant are potential determinants of plant growth, productivity, and health. They provide plants with a plethora of functional capacities, namely, phytopathogens suppression, access to low-abundance nutrients, and resistance to environmental stressors. However, a comprehensive insight into the structural compositions of the bacterial abundance, diversity, richness, and function colonizing various microenvironments of plants, and specifically their association with bioactive compounds and soil edaphic factors under silicon (Si) amendment remains largely inconclusive. Here, high-throughput sequencing technology and nontargeted metabolite profiling method were adopted to test the hypotheses regarding microbiome niche abundance, diversity, richness, function, and their association with bioactive compounds and soil edaphic factors within different ecological niches (leaf, stem, root, rhizosphere, and bulk soils) under Si amendment during cane growth were we addressed. Our results demonstrated that Si correspondingly increased sugarcane theoretical production and yield, and remarkably enhanced soil nutrient status, especially Si, AP, and AK. It was also observed that bacterial diversity demonstrated tissue-dependent distribution patterns, with the bulk soil, rhizosphere soil, and root endosphere revealing the highest amount of bacterial diversity compared with the stem and leaf tissues. Moreover, Si exhibited the advantage of considerably promoting bacterial abundance in the various plant compartments. Co-occurrence interactions demonstrated that Si application has the potential to increase bacterial diversity maintenance, coexistence, and plant–soil systems bacteria connections, thereby increasing the functional diversity in the various plant tissues, which, in turn, could trigger positive growth effects in plants. Network analysis further revealed that metabolite profiles exhibited a strong association with bacterial

community structures. It was also revealed that Si content had a considerable positive association with bacterial structures. Our findings suggest that the dynamic changes in microbe's community composition in different plant and soil compartments were compartment-specific. Our study provides comprehensive empirical evidence of the significance of Si in agriculture and illuminated on differential metabolite profiles and soil microbe's relationship.

KEYWORDS

sugarcane, compartment niches, co-occurrence networks, silicon fertilization practices, plant-microbe interactions, soil-plant continuum

Introduction

Plant rhizosphere has been widely regarded as a hot spot for different microorganisms and is known as one of the complex ecosystems. A great number of microbes, namely, bacteria and fungi, colonize rhizosphere soil and these microbes play a crucial role in both soil fertility and health, and plant growth and development (Hayat et al., 2010). Plant root systems secrete a series of organic secretions that can promote rhizospheric microbe's growth (Walker et al., 2003), thereby forming a community of microorganisms in a rhizosphere microenvironment. The variations in plant root system rhizosphere deposition and secretion of plant species tend to influence rhizosphere bacterial abundance and community distribution patterns (Lamb et al., 2011; Lundberg et al., 2012). There is clear evidence that a vast majority of these rhizosphere microbes are associated with a complex food web that may use a significant portion of nutrients produced by the plants, thereby influencing their abundance and community distribution pattern (Mendes et al., 2013). In a study conducted by Cordero et al. (2019), it was demonstrated that bacterial abundance and composition in the wheat and canola rhizosphere compartments exhibited distinct distribution patterns, potentially driven by *Acidobacteria*, *Gemmatimonadetes,* and *Firmicutes*. They further postulated that these microbes were crop and organ-specific. Similarly, Ling et al. (2022) revealed that phyla Bacteroidetes and Proteobacteria peaked significantly in the rhizosphere soil, which was largely due to the fact that these bacteria are predominantly adapted to C-rich environment (e.g., rhizosphere soil) to enhance fast growth, propagation, and metabolic activity. Mounting evidence has also suggested that different plant compartments, namely, plant roots are colonized by various microbes. These microbes are capable of enhancing plant resistance to abiotic and biotic stresses, and can play a valuable role in agriculture husbandry. It is, therefore, crucial to have a better insight into how plant root systems influence microbes composition communities and diversity in plant root systems.

Plants endospheres are generally colonized by diverse microbes communities, which inhabit the plant internally during their growth and development and are characterized as endophytes (Giri and Dudeja, 2013; Dudeja et al., 2021). A number of microbial tend to enter plant roots system as endophytes and establish a mutual relationship. Endophytes can not only promote the growth and development of host plants, but also enhance the plants resistance through antagonism induced by pathogenic bacteria (Afzal et al., 2019; Dubey et al., 2020). Additionally, endophytes can promote plants to synthesize a number of plant growth hormones, enhance host plants ability to absorb soil nutrients, promote the growth and development of host roots and absorb various inorganic ions through a synthesis of iron carriers and nitrogen-fixing bacteria. Changes in root rhizosphere deposition and secretion and plant species could influence the distribution pattern of microbes abundance, community, and functional diversity in rhizosphere soil (Dennis et al., 2010), soil fertility (Yang et al., 2022), and plant growth and development (Shen et al., 2018). Moreover, a vast majority of these bacteria can carry genes that facilitate biological nitrogen fixation, possibly enabling them to transform dinitrogen gas (N_2) into forms that are available to plant, namely, nitrate and ammonium within the occupied plant. Therefore, it is crucial to have a broader insight into the endophytes-plant relationship, which is vital to enhancing crop productivity.

Generally, different plant tissues can harbor a diverse group of microbial, and these microbes are component-specific (Wu et al., 2020). For instance, Beckers et al. (2017) observed distinct alterations in bacteria in the different plant tissues (soil, roots, stems, and leaves), and each part had specific bacterial composition. Their finding also revealed that the bacterial diversity diminished with the selection of plants, specifically the bacterial diversity and abundance in the rhizosphere zone were more pronounced than those in the plant. Similarly, Xiong et al. (2020) evaluated bacterial communities of 684 samples from rhizosphere and bulk soils, rhizoplane, root endosphere, phylloplane, and leaf endosphere in maize, wheat, and barley rotation system under soil amendments at two different sites. It was evident that microbiome assembly along the soil–plant continuum was altered primarily by host species and compartment niche compared with soil amendments or sites.

Silicon (Si) is widely deemed as the second richest element in the soil and earth's crust. A large number of studies have

demonstrated that Si is capable of promoting plant growth and enhancing plant-insect and disease resistance, and cold and drought resistance. On the other hand, the vast majority of Si in soil environments exists in the form of precipitates or silicate crystals, and the concentration of Si in soil solutions is usually limited. Moreover, the amount of plant-available Si in soils is deficient, hence utilizing Si properly can increase plant resistance against abiotic and biotic stresses, thereby improving soil health and fertility (Matychenkov and Ammosova, 1996), boosting crop growth and increasing crop yield (Meyer and Keeping, 2000). Despite these findings, our understanding of microbial community composition and diversity in multiple compartment niches (root, stem, leaf, rhizosphere soil, and non-rhizosphere) is still limited, especially under fertilization practices, such as Si amendment. Therefore, this study took sugarcane (ZZ6) as the material to evaluate the changes of bacterial community composition, diversity, and richness in the root, stem, leaf, rhizosphere soil, and non-rhizosphere soils of sugarcane after Si application, combined with the previous metabolomic data. This study aims to answer the following questions: (i) What is the effect of Si fertilizer on soil physiological, bacterial community, diversity, richness, and function during sugarcane growth? (ii) Does Si application has the potential to enhance the association among soil properties, core metabolites, and bacteria in the various soil and plant niches, and the co-occurrence network in plant–soil systems?

Materials and methods

Experimental design and sample collection

This study was conducted in Qumeng Village, Fusui County, Chongzuo City, Guangxi Province, China (22°49′N, 107°76′E). The area has an annual average temperature of 21.7°C and a rainfall of 1,121 mm. The basic soil properties of the site were assessed, including pH (4.22), organic matter (20.07 g/kg), total nitrogen (1.43 g/kg), total phosphorus (1.25 g/kg), and total potassium (1.18 g/kg). The field experimental plot was laid out in a randomized block design consisting of two treatments, and three replicates. The entire field contained 6 plots, with a total area of 1,800 m^2 (6 m × 50 m × 6 plots). In mid-March 2019, rotary tillage was used to plow the soil (30 cm depth). Subsequently, sugarcane variety ZZ6 obtained from Guangxi University was cultivated at the rate of approximately 83,333/hm^2, followed by fertilizer application. On each plot, the line between sugarcane was 1.2 and 0.1 m row spacing. The experiment consisted of two treatments, namely, (1) control, CK: compound fertilizer (NPK151515), and (2) silicon fertilizer, Si (NPK18-18-18 + Sikg/hm^2). Si fertilizer was applied uniformly once, while the compound fertilizer was applied in different doses, and at two intervals. The first dose (40%) and second dose (60%) were applied during the seedling stage and elongation stage, respectively. The compound fertilizer was applied at the rate of 1,500 kg/hm^2, while Si was applied at the rate of 1,250 kg/hm^2. Si fertilizer contained SiO_2 content greater than 10%. These fertilizers were obtained from Hebei Silicon Valley Academy of Agricultural Sciences.

Sampling and preparation of samples

Samples of the rhizosphere soil, roots, stems, and leaves were collected on 28th November 2019, following the approach adopted by Beckers et al. (2017). Briefly, we shook the root on a platform (15 min, 110 rpm) to remove the soil from the root. The dislodged soil particles from the plant roots were considered rhizosphere soil. Soil 30 cm away from the root system was described as bulk soil. Then, the root, stem, and leaf were collected and washed with: (i) sterile Millipore water (30 s), (ii) 70% (v/v) ethanol (2 min), (iii) sodium hypochlorite solution (2.5% active Cl − with 0.1% Tween 80) (5 min), and (iv) 70% (v/v) ethanol (30 s). Finally, the samples were rinsed five times using sterile Millipore water. A sterile scalpel was used to separate the samples into small fragments and then soaked in a sterile phosphate saline buffer (PBS; 130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH_2PO_4, pH 7.4) by employing a Polytron PR1200 mixer (Kinematica A6). Later, sterilization and homogenization of the samples were conducted under aseptic conditions in laminar airflow. Lastly, quadruplicate aliquots of each sample (1.5 ml) of the homogenized plant material were stored at −80°C awaiting DNA extraction.

Measurement of sugarcane agronomic traits

The measurement of cane agronomic traits, namely, sugarcane heights, sucrose content, and stalk weight (kg $stalk^{-1}$), followed by the theoretical production and yield parameters, was conducted in November 2019 using the method we adopted in our previous studies (Fallah et al., 2021; Pang et al., 2022).

Assessment of soil nutrient

We investigated soil Si content by adopting the method used by Babu et al. (2016) study. In summary, an exact amount of filtrate was placed into a plastic centrifuge tube. Later, 10 ml of ddH_2O and 0.5 ml of 1:1 hydrochloric acid (HCl) were added to the solution, followed by 1 ml of 10% ammonium molybdate solution (pH 7.0). Then, 1 ml of 20% tartaric acid solution was added, followed by 1 ml of the reducing agent amino naphthol n-sulphonic acid (ANSA). The solution was later mixed thoroughly at 2-min intervals after 5 min. UV–Vis spectrophotometer (Hach DR 5000) was used to estimate absorbance at 630 nm after 5 min. Finally, a UV–Vis spectrophotometer was concurrently used to assess Si content standard series absorbance readings (0, 0.2, 0.4, 0.8, 1.2, 1.6, and

2.0 mg L^{-1}) organized in the same matrix. Soil physicochemical properties such as organic matter (OM), available potassium (AK), and available nitrogen (AN) were evaluated using methods leveraged by Fu et al. (2015) and Kwon et al. (2009), respectively. Whereas available phosphorus (AP) was assessed by adopting the approach employed by Pansu and Gautheyrou (2006), while soil pH was calculated using the method we leveraged in our previous studies (Bi et al., 2010).

DNA extraction, PCR amplification, and illumina sequencing

We extracted total DNA from 500 mg of each sample (bulk soil, rhizosphere soil, root, stem, and leaf) using the PowerSoil DNA Isolation kit (Mo Bio Laboratories, Carlsbad, CA, United States) and Invisorb Spin Plant Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany) following the manufacturer's instructions. The quantity and quality of the DNA were assessed using the ratios of 260 nm/280 nm and 260 nm/230 nm. The DNA was later stored at −80°C until further processing.

The bacterial 16S rRNA gene V3–V4 region was amplified using a primer pair (Forward primer, 5′-ACTCCTACGGGA GGCAGCA-3′; reverse primer, 5′-GGACTACHVGGGTWTCT AAT-3′) combined with adapter sequences and barcode sequences. PCR amplification was performed in a total volume of 50 μl, which consisted 10 μl Buffer, 0.2 μl Q5 High-Fidelity DNA Polymerase, 10 μl High GC Enhancer, 1 μl dNTP, 10 μM of each primer, and 60 ng genome DNA. Below were the thermal cycling conditions: initial denaturation at 95°C for 5 min, followed by 15 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products obtained from the initial step of the PCR were purified using VAHTSTM DNA Clean Beads. The second round of PCR was then conducted in a 40 μl reaction, which consisted of 20 μl 2 × Phusion HF MM, 8 μl ddH_2O, 10 μM of each primer, and 10 μl PCR products obtained from the initial step. Thermal cycling conditions were as follows: an initial denaturation at 98°C for 30 s, followed by 10 cycles at 98°C for 10 s, 65°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min. Lastly, Quant-iT™ dsDNA HS Reagent was employed to quantify all PCR products and pooled together. Illumina Hiseq 2500 platform (2 × 250 paired ends) at Biomarker Technologies Corporation, Beijing, China, was employed to conduct a high-throughput sequencing analysis of bacterial rRNA genes on the sample. Finally, the raw data were submitted to the NCBI Sequence Read Archive (accession no. PRJNA848385).

FLASH was adopted to conduct merge paired-end reads of the DNA fragments, using a sample-specific barcode appropriated to each sample. The sequences were then clustered at the same operational taxonomic unit (OTU) using 97% similarity. Later, sequences were accordingly chosen for each OTU to conduct the annotation of the taxonomic information for each sequence by employing the Ribosomal Database Project (RDP) (Cole et al., 2009). We also removed sequences with low quality if they did not correspond to the primer and barcode or if they did not exceed 200 nucleotides consisting of a high average quality score ($Q \geq 20$) or no ambiguous base pairs. Sequences were then clustered at 97% nucleotide similarity. Finally, SILVA database (SILVA Release 138, Bacterial) was leveraged for the taxonomic classification of the bacteria respective sequences (Quast et al., 2013).

Preparation of metabolic samples

Sugarcane tissue samples were extracted by adopting the method used by Chenkun et al. (2021) and Chen et al. (2013). In summary, the samples were ground into powder for 1.5 min at 30 Hz using a mixer mill (MM400, Retsch) after they were freeze-dried. The powder was later weighed (100 mg), followed by extraction during the night at 4°C with 0.8 ml 70% aqueous methanol (methanol: H_2O_2, 70:30, v/v) and pure methanol, followed by 10 min centrifugation at 10,000*g*. Supernatants were collected separately and mixed, and later filtrated (SCAA-104, 0.22 mm pore size; ANPEL Shanghai, China, www.anpel.com.cn/). Samples were mixed into five different tissue samples, namely leaf, stem, root, bulk, and rhizosphere soils to investigate the inter-tissue alterations in metabolites using nontargeted metabolomics analysis. Finally, we correspondingly mixed the samples into various quality control to conduct instrument stability.

LC–MS/MS analysis

UHPLC system (1290, Agilent Technologies) with a UPLC BEH Amide column (1.7 μm 2.1 mm × 100 mm, Waters) combined with TripleTOF 5600 (Q-TOF, AB Sciex) was used to conduct LC–MS/MS analyses. The mobile phase comprised of 25 mM NH4OAc and 25 mM NH4OH in water (pH = 9.75) (A) and acetonitrile (B) was conducted using the following elution gradient: 0 min, 95% B; 7 min, 65% B; 9 min, 40% B; 9.1 min, 95% B; 12 min, 95% B, which was conveyed at 0.5 ml min^{-1}, with an injection volume was 3 μl. To acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment, Triple TOF mass spectrometer was adopted.

Data preprocessing and annotation

We converted the MS raw data (.d) files to the mzXML by employing ProteoWizard, and later processed it using R package XCMS (version 3.2). Later, we generated a data matrix that contained the mass-to-charge ratio (m/z) values, retention time (RT), and peak intensity. After XCMS data processing, R package CAMERA was used to conduct peak annotation. Finally, metabolites identification was conducted by employing an in-house MS2 database.

Data analysis

We used Quantitative Insight into Microbial Ecology (QIIME2 v.1.9.1) (Caporaso et al., 2010) and R software (version 3.6.1) (Team, 2014) to examine bacterial community diversity (Shannon) (Keylock, 2005) and richness (ACE) (Chao and Lee, 1992). Bacterial community composition unique and overlap genera were visualized by constructing Venn diagrams (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Principle coordinate analysis (PCoA) with Bray-Curtis distance was used to explore and visualize bacterial composition similarities or dissimilarities in the different plant tissues under both treatments. Source-tracking analysis was leveraged to identify the potential sources of observed bacterial communities in each host niche using Source Model of Plant Microbiome (SMPM) (Xiong et al., 2020). We further employed ADONIS analysis to have an in-depth understanding of how these bacterial community compositions in each plant tissue were altered under both treatments. To test the expression patterns of the abundant bacteria, both Bioconductor (http://www.bioconductor.org/) package 'Mfuzz' and R software (http://www.r-project.org/) based on fuzzy c-means were employed. Then, the fuzzification parameter was adjusted to $m = 2$ and the number of clusters to $c = 12$ to retain the soft clustering of all bacteria. PICRUSt2 was conducted to test soil bacterial metabolic function profile (KEGG) (Langille et al., 2013). Later, STAMP differential analysis was used to evaluate KEGG by comparing two plant tissues under the same treatment (Parks et al., 2014). Co-occurrence networks were further constructed to investigate the associations among soil bacteria, metabolite, and soil properties by following the descriptions in Zhang et al. (2021a) and Chen et al. (2020) studies. The connections between the abundant bacteria and metabolites in the network were visualized by leveraging a correlation matrix. Then, all potential pairwise Spearman's rank was computed by employing Cytoscape version 3.6.1 (Shannon et al., 2003). A correlation among the abundant bacteria, metabolites, and soil properties was deemed statistically significant if Spearman's correlation coefficient (p) was greater than 0.6 and the p-value was less than 0.05 (Junker and Schreiber, 2011). Lastly, the test data were calculated using ANOVA, which were then displayed by DPS software (version 7.05, www.dpssoftware.co.uk), and the differences between mean values of each treatment, plant, and soil compartment were compared using Tukey's HSD test ($p < 0.05$).

Results

Assessment of sugarcane agronomic traits under Si utilization

Compared with CK, Si overwhelmingly promoted ($p < 0.05$) cane production and height, but revealed no significant difference for the rest of the parameters assessed. Although the sugarcane sucrose content did not increase significantly under the Si fertilizer treatment, it increased by 0.73 percentage points (Table 1).

Response of soil physiochemical properties to Si amendment

Compared to CK treatment, soil AN marked a little difference in the rhizosphere soil compared with CK. We also noticed that soil AP in the rhizosphere soil peaked considerably ($p < 0.05$) under Si treatment than CK. Furthermore, soil AK in both the bulk and rhizosphere soils significantly increased ($p < 0.05$), whereas Si in the bulk soil significantly increased ($p < 0.05$) under Si treatment relative to that under CK. On the contrary, Si had no considerable impact on soil pH and soil OM compared with CK treatment (Table 2). MANOVA analysis further revealed that Si had a significant impact on soil AK and Si content, whereas different soil regions induced a significant change in soil AP, followed by soil AN and soil Si, while the interaction of CK and the various soil regions had a significant effect on soil AP, Si, and AK (Table 2).

Bacterial community diversity

With the adoption of high-throughput Illumina sequencing, a total of 2,399,532 raw reads were generated, and a total of 2,241,717 were obtained after quality assessment. The reads were clustered into 1,147 OTUs at a 97% sequence similarity level. The remaining numbers of reads ranged from 49,250 to 73,750 among samples, and we rarefied each sample to the minimum size (49,250) (Supplementary Table S1). The rarefaction curves of OTU richness and Shannon diversity per compartment reached a saturation plateau, thus suggesting that we have sampled most of the diversity in the soil and sugarcane microbiome. The shape of the curves demonstrated that the OTU richness (ACE) and diversity (Shannon) were consistently high in the rhizosphere, followed by the root endosphere (Supplementary Figure S1). We also examined bacterial ACE and the Shannon in each sample (Figures 1A,B). There were obvious differences in bacterial diversity and richness in different locations. OTU richness was highly dependent on plant compartment ($p < 0.05$), consisting of high

TABLE 1 Effect of Si fertilizer on sugarcane agronomic traits.

	CK	Si
Stalk height (cm)	252.16 ± 3.61 b	**275.44 ± 4.50 a**
Stalk diameter (cm)	3.08 ± 0.05 a	3.14 ± 0.09 a
Sucrose content (%)	12.85 ± 0.29 a	13.58 ± 0.26 a
Available stalk number (hm^2)	44,557 ± 1896 a	50,130 ± 1,256 a
Production (kg/hm^2)	73523.6 ± 1703.9 b	**95612.6 ± 5227.6 a**
Single stalk weight (kg)	1.66 ± 0.10 a	1.91 ± 0.12 a

Sugarcane agronomic traits response to silicon fertilizer (Si) and control (CK). Different lowercase letters indicate a significant difference at $p < 0.05$. Values in bold means statistical significant.

TABLE 2 Effect of Si fertilizer on physical and chemical properties of sugarcane rhizosphere soil and bulk soil.

	AN	AP (mg/kg)	AK (mg/kg)	pH	OM (g/kg)	Si (mg/kg)
CK_Bulk	37.27 ± 6.67 ab	73.73 ± 6.07 c	42.00 ± 3.06 b	4.28 ± 0.08 a	23.32 ± 0.39 a	182.10 ± 3.53 b
Si_Bulk	21.53 ± 6.69 b	92.87 ± 3.82 bc	**154.33 ± 16.25 a**	4.25 ± 0.06 a	24.00 ± 0.86 a	**231.30 ± 5.16 a**
CK_Rhi	32.94 ± 10.38 ab	152.13 ± 16.96 b	38.67 ± 8.82 b	4.19 ± 0.12 a	24.53 ± 0.33 a	154.10 ± 2.57 c
Si_Rhi	52.38 ± 9.55 a	**312.67 ± 40.07 a**	**157.33 ± 44.76 a**	4.21 ± 0.04 a	23.62 ± 0.20 a	192.30 ± 2.77 b
Treatment (T)	NS	NS	***	NS	NS	**
Soil region (Sr)	*	**	NS	NS	NS	*
T*Sr	NS	***	**	NS	NS	***

***$p<0.001$, **$p<0.01$ and *$p<0.05$. Values in bold means statistical significant.

richness values of 1017.7 ± 66.0 (bulk soil) and 859.4 ± 100.5 (rhizosphere soil), while bacterial richness in the root samples (828.1 ± 11.0) and stem samples (645.4 ± 5.0) consistently decreased. OTU richness indices of the leaf samples (573.8 ± 68.8) were comparable with the stem samples. In the rhizosphere, OTU richness was more pronounced in the Si treatment compared with CK treatment (Figure 1A). For bacterial diversity, we also observed an obvious separation between the soil samples and endosphere samples ($p<0.05$). Higher diversity was observed in the bulk soil, rhizosphere soil, and root endosphere as compared to the samples of the stem and leaf tissues (Figure 1B).

The Venn diagram results further showed that the number of OTUs was significantly high (1073), but decreased considerably in the leaf endosphere (643), of which 437 OTUs were present in other compartments (Figure 1C). This suggests that most of the bacteria may have migrated from the soil to the different sugarcane compartments. However, except for the root endosphere, the number of bacterial OTUs in the different compartments increased by different degrees under Si treatment. There were 485 OTUs in the different plant and soil compartments. This further showed that compared with the CK treatment, Si can improve the diversity and connectivity of bacteria in different parts (Figure 1C).

Source-tracking analysis was conducted to identify the potential sources of observed bacterial communities in each host niche. The Source Model of Plant Microbiome (SMPM) suggested that crop-associated bacterial communities were mainly migrated from the bulk soils and gradually filtered in different plant compartment niches. Only 49.2% of the bacteria in the rhizosphere soil entered the roots, indicating that the bacteria were strongly selected at the soil–plant interface (Figure 1D). The root endosphere, stem endosphere, and leaf endosphere potentially accommodated the majority of taxa from a nearby species pool, with the known source value of approximately 84.17%, exceeding that in the soil–plant interface. This distribution pattern was further validated by cluster analysis, where bacteria in the aboveground tissues were distinctly separated from the belowground (Figure 1E).

We evaluated beta diversity at the OTU level (OTUs defined at a 97% similarity cut-off). Principle coordination analysis (PCoA) reinforced the distribution trend observed in the bacterial community structures dissimilarities among the various compartments. The results further showed that PCo1 explained 58.95% and PCo2 9.74% of the total variation. The subsurface bacteria (bulk/rhizosphere soil and root endosphere) and the aboveground bacteria (stem and leaf endosphere) were separated on the first main axis. We also observed that Si treatment had little impact on the bacterial community than in the different compartments (Figure 1F).

Abundant bacterial in each compartment

It can be seen from Figure 1G that there were obvious differences in the composition of the bacterial phyla in the different locations. The belowground bacteria were mainly composed of *Proteobacteria*, *Actinobacteria, Acidobacteria,* and *Chloroflexi*, accounting for 80.72% of the total abundance, while the aboveground bacteria were mainly composed of *Cyanobacteria* and *Proteobacteria*, representing 90.94%. Furthermore, we used analysis of variance (LSD) to evaluate the effect of Si and the diffident compartments on bacteria whose relative abundance was greater than 1% (Supplementary Table S4). Virtually all identified bacterial phyla displayed a significant compartment effect, except for *Bacteroidetes*. In the bulk soil, rhizosphere soil, and roots endosphere samples, we observed a significant enrichment ($p<0.05$) of *Actinobacteria* (relative abundance = 21.3%, 16.9%, 27.7%), *Acidobacteria* (relative abundance = 12.5%, 13.2%, 11.9%), and *Chloroflexi* (relative abundance = 14.1%, 7.0%, 5.2%), while *Cyanobacteria* were significantly depleted ($p<0.05$) as compared to the stems and leaves compartments. The abundance of *Proteobacteria* was significantly lower in the cane leaf compared to the other compartments. In the bulk soil and rhizosphere soil samples, WPS-2 relative abundance accounted for 2.35%, followed by *Planctomycetes* (1.90%) as compared to the endosphere compartments. For the genus level, we found a total of 381 genera (Supplementary Figure S2; Supplementary Table S2; Supplementary Sheet 1), we defined the core bacterial as the 20 most abundant genus in each of the compartments, thus resulting in 71 genera (Supplementary Table S2; Supplementary Sheet 2). The percentages of the total community covered by the core genus ranged from 72.9% (bulk soil) to 77.4% (rhizosphere soil), and from 76.1% (root) to 71.7% (stem), and 72.0% (leaf). We observed significant compartment effects across all core bacterial genera. In the bulk soil, genera such as c_AD3 (5.54%), 7,703 (1.21%), *Lactobacillus* (0.78%), and *Romboutsia* (0.38%) were more pronounced ($p<0.05$) as compared to the rhizosphere soil,

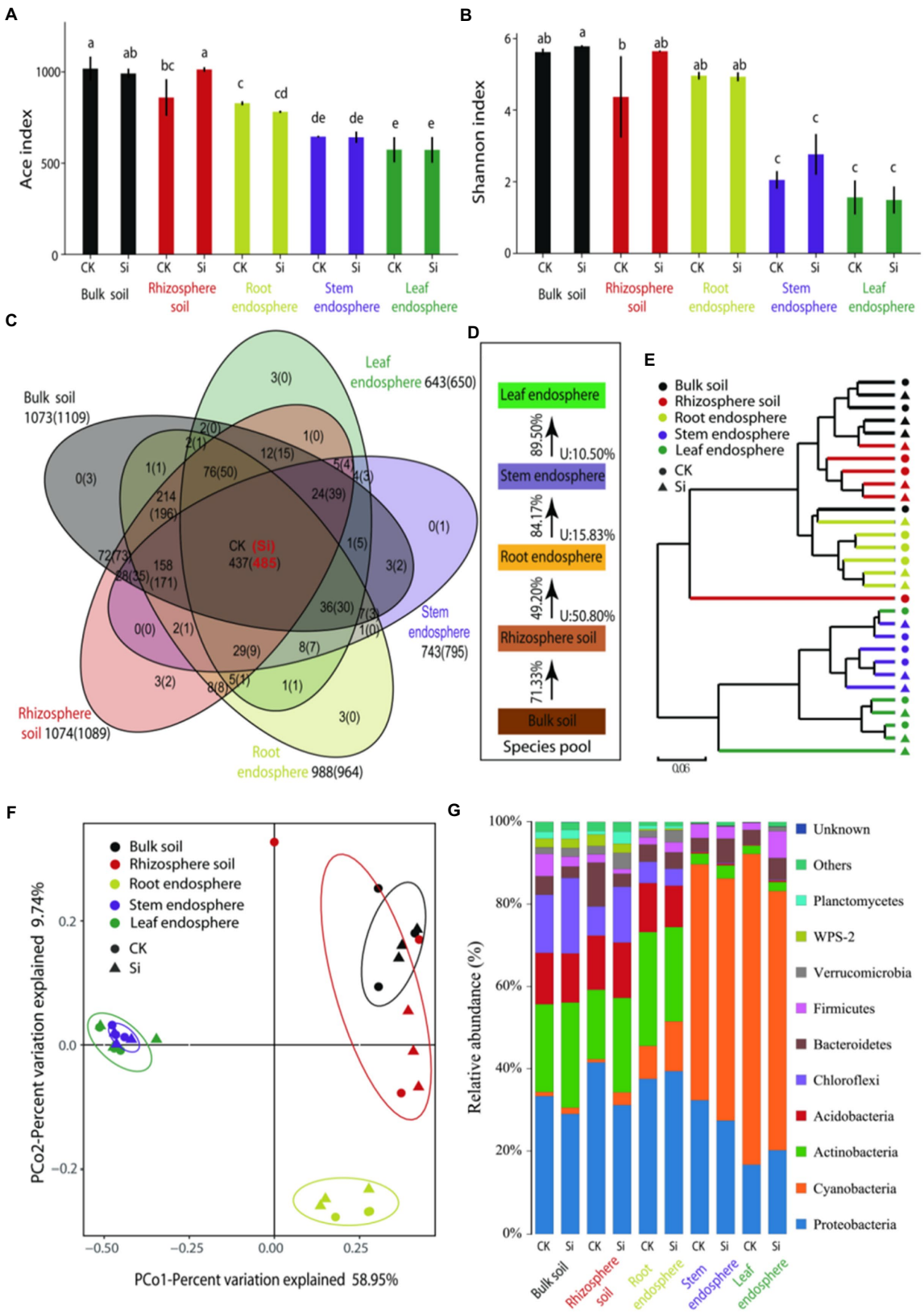

FIGURE 1
ACE index: bacterial richness index **(A)**, Shannon index: bacterial community diversity index **(B)**, a Venn diagram illuminating overlap and unique enriched bacteria OTU in the various plant and soil compartments **(C)**, Source Model of Plant Microbiome (SMPM) illustrating the potential sources

(Continued)

Figure 1 (Continued)
of bacterial community composition in the different plant and soil compartments under both treatments, "U" denotes the proportion of bacterial community composition from unknown source **(D)**. Followed by hierarchical clustered heatmap of all the bacteria in the different soil and plant compartments under both treatments **(E)**, principal coordinate analysis (PCoA) with Bray-Curtis distance showing similarities and dissimilarities of bacterial phyla under the both Si and CK and various soil and plant compartments **(F)**, and the relative abundance of bacterial community composition in the entire sample **(G)**. Different lowercase letters signify significant differences between various compartment under both treatments based on the LSD test (p<0.05).

root, stem and leaf compartments. In the root samples, *Pajaroellobacter* (3.30%), *Salix_integra* (1.30%), and *Thermosporothrix* (1.83%) marked significant enrichment ($p<0.05$) as compared to the other compartments. We also notice that *Aegilops-tauschii* and *Triticum-aestivum-bread-wheat* peaked considerably in stem and leaf samples compared with the soil and root samples. However, *Conexibacter, Acidibacter*, *Acidothermus*, *Singulisphaera, Bradyrhizobium, Burkholderia-Caballeronia-Paraburkholderia, Catenulispora, Mycobacterium, Roseiarcus, Actinospica,* and *Dyella* were significantly ($p<0.05$) depleted in the stem and leaf samples. In addition, it was noticed that Si had significant effects on all the core bacterial genera, particularly in the rhizosphere, bulk soil, root, and stem endosphere.

To statistically support the visual clustering of the bacterial communities in the PCoA analysis (Figure 1F), different plant and soil compartments and Si treatment were examined using ADONIS method (Table 3). Under Si treatment, no significant difference in bacterial composition between bulk soil and rhizosphere soil was observed, but a difference in bacterial function ($p=0.046$) was observed. The result revealed that all the different compartments rendered bacteria significantly dissimilar from each other at the phylum, genus, and functional levels. Compared with the control, there was no difference observed in bacterial composition and function in the bulk soil and sugarcane leaves under Si. However, there were differences in bacterial composition and function in the rhizosphere soil ($p<0.05$). On the other hand, the function of endophytic bacteria (genus level) in the roots and stems changed significantly under Si treatment (Table 3).

TABLE 3 Adonis showed the difference in bacterial composition in two treatments and different compartments.

ADONIS output	Phylum		Genus		Functional	
	R	*P*	*R*	*P*	*R*	*P*
Bulk soil vs. rhizosphere soil	0.143	0.343	0.157	0.307	0.223	0.046
Bulk soil vs. root	0.5	0.02	0.533	0.016	0.446	0.031
Bulk soil vs. stem	0.92	<0.001	0.765	0.003	0.97	0
Bulk soil vs. leaf	0.939	<0.001	0.561	0.013	0.935	<0.001
Rhizosphere soil vs. root	0.502	0.02	0.263	0.133	0.312	0.09
Rhizosphere soil vs. stem	0.654	0.006	0.508	0.019	0.877	<0.001
Rhizosphere soil vs. leaf	0.76	0.003	0.376	0.054	0.83	0.001
Root vs. stem	0.928	<0.001	0.821	0.002	0.979	0
Root vs. leaf	0.946	<0.001	0.583	0.011	0.945	0.001
Stem vs. leaf	0.592	0.01	0.216	0.192	0.485	0.023
CK vs. Si (bulk soil)	0.229	0.098	0.206	0.061	0.169	0.178
CK vs. Si (rhizosphere soil)	0.217	0.041	0.215	0.012	0.228	0.033
CK vs. Si (root)	0.088	0.358	0.199	0.064	0.335	0.031
CK vs. Si (stem)	0.227	0.127	0.233	0.039	0.233	0.073
CK vs. Si (leaf)	0.018	0.725	0.057	0.599	0.026	0.681

Expression patterns of bacterial abundance in compartments

We conducted a cluster analysis to interpret the expression patterns of bacterial abundance in different plant and soil compartments (Figure 2; Supplementary Table S5). The analysis demonstrated that the expression patterns of bacterial communities could be divided into 12 categories. In clusters 4 and 7, the abundance of bacteria peaked significantly in the bulk soil compared with the other compartments, mainly occupied by *Lactobacillus*, *Alcaligenes*, *Bacillus*, *Streptococcus,* and *Nitrospira*. In cluster 12, it was also revealed that the abundance of bacteria in rhizosphere soil was higher than that in the other compartments, particularly driven by *Gemmatimonas*, *Flavisolibacter,* and *Streptomyces*. Furthermore, *Blastococcus*, *Rhizomicrobium,* and *Aquisphaera* expression patterns peaked in cluster 9 in the rhizosphere and bulk soils. In clusters 2 and 8, the analysis also showed that the abundance of bacteria in the soil and roots was higher than that in the stems and leaves, potentially driven by *Sphingomonas*, *Mesorhizobium,* and *Burkholderia-Caballeria-Paraburkholderia*. While *Cetobacterium*, *Amnibacterium*, *Akkermansia,* and *Ruminococcus_UCG-002* abundance in sugarcane stems were more pronounced in cluster 10 than that in the other compartments. Whereas clusters 1 and 6 were predominantly driven by *Lysobacter, Caulobacter,* and *Allorhizobium-Neorhizobium-Pararhizobium-rhizobium* in leaves tissue compared with the other compartments.

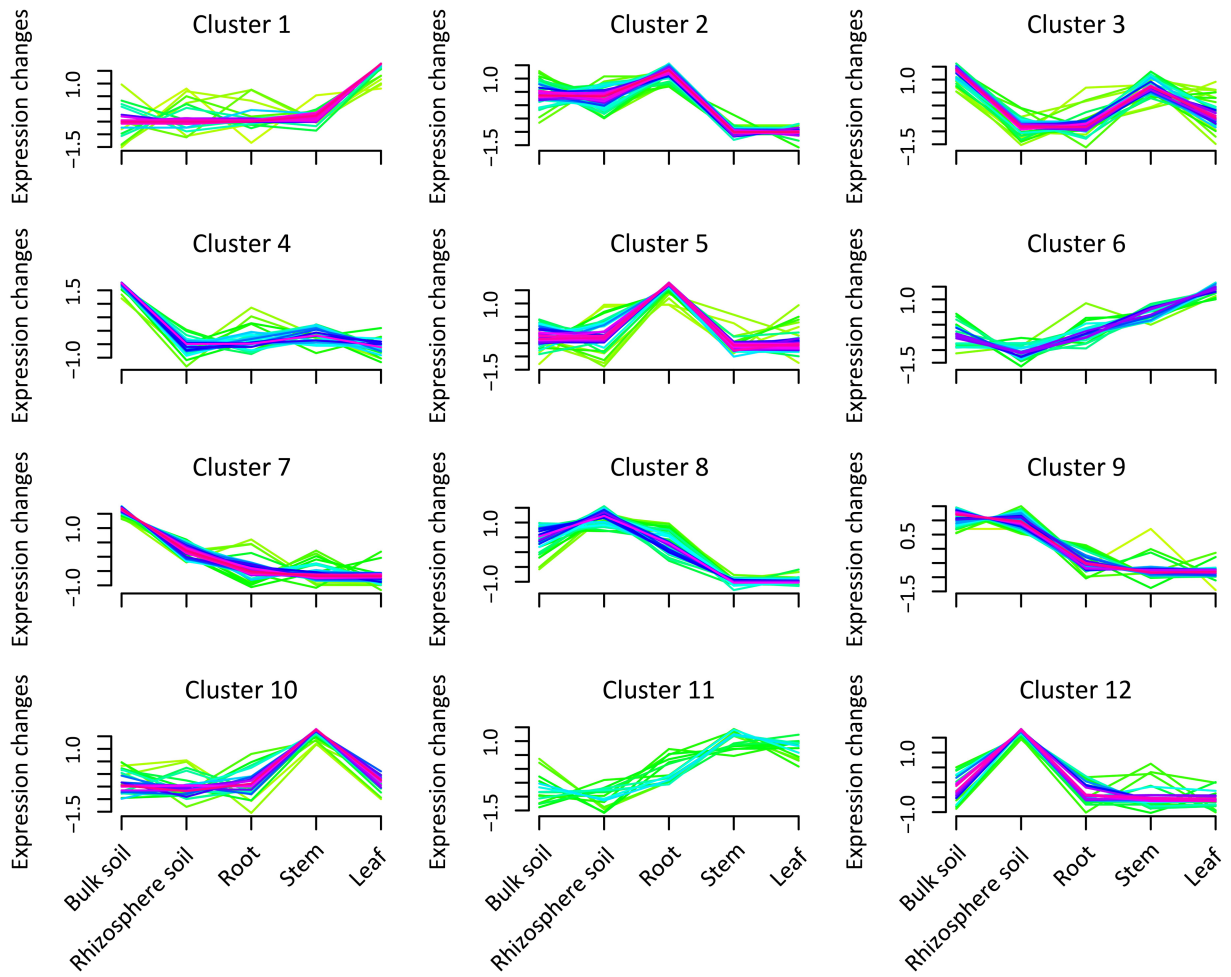

FIGURE 2
Expression pattern of bacteria abundance in the different plant and soil compartments (bulk and rhizosphere soils, leaf, stem, and root). Green indicates low abundance values, and purple represents the cluster expression trend of the abundant bacteria.

Ternary mapping analysis reveals the composition of enriched or depleted bacterial communities in sugarcane and soil compartments

DESeq2 was used to compare the differences of bacterial species in the stem, leaf, and root under CK (CKS *VS* CKL *VS* CKR), and Si treatment (SiS *VS* SiL *VS* SiR) to investigate the enriched or depleted bacteria (Figures 3A–D; Supplementary Table S6). It was observed that the enrichment of endophytic bacteria in different sugarcane compartments was different. For instance, *Allobaculum, Lactobacillus,* and *Bifidobacterium* were significantly depleted in the stem, while *Burkholderia, Caballeronia, Paraburkholderia,* and *Bradyrhizobium* in sugarcane root were significantly higher than that in sugarcane stem and leaf under both CK and Si. It was also noticed that *Desulfovibrio* was significantly enriched only in the stem of the CK, while *Mesorhizobium* revealed the opposite (Figures 3A,B). Moreover, bacterial abundance in the root, rhizosphere soil, and bulk soil exhibited distinct patterns. In the CK and SI treatments, *Lactococcus* in root was significantly higher than that in soil. However, *Sinomonas*, *Gaiella,* and *Ruminococcaceae UCG* abundance significantly diminished relative to those in the soil compartment (Figures 3C,D). In addition, *Bradyrhizobium* in the bulk soil of the CK was significantly lower than that in the rhizosphere soil and roots (Figure 3C). Whereas *Mesorhizobium* was significantly higher in the rhizosphere soil than that in the bulk soil and root. However, *Azospirillum* in the root demonstrated the opposite compared with the bulk soil of Si treatment (Figure 3D).

Changes of bacteria in various parts under Si treatment

Later, the result was visualized using a Manhattan plot to have a comprehensive insight into the effect of Si on bacteria abundance in each of the various plant and soil regions. The results showed that Si significantly improved *Cetobacterium, Bacteroides, Erysipelatoclostridium, Plesiomonas,*

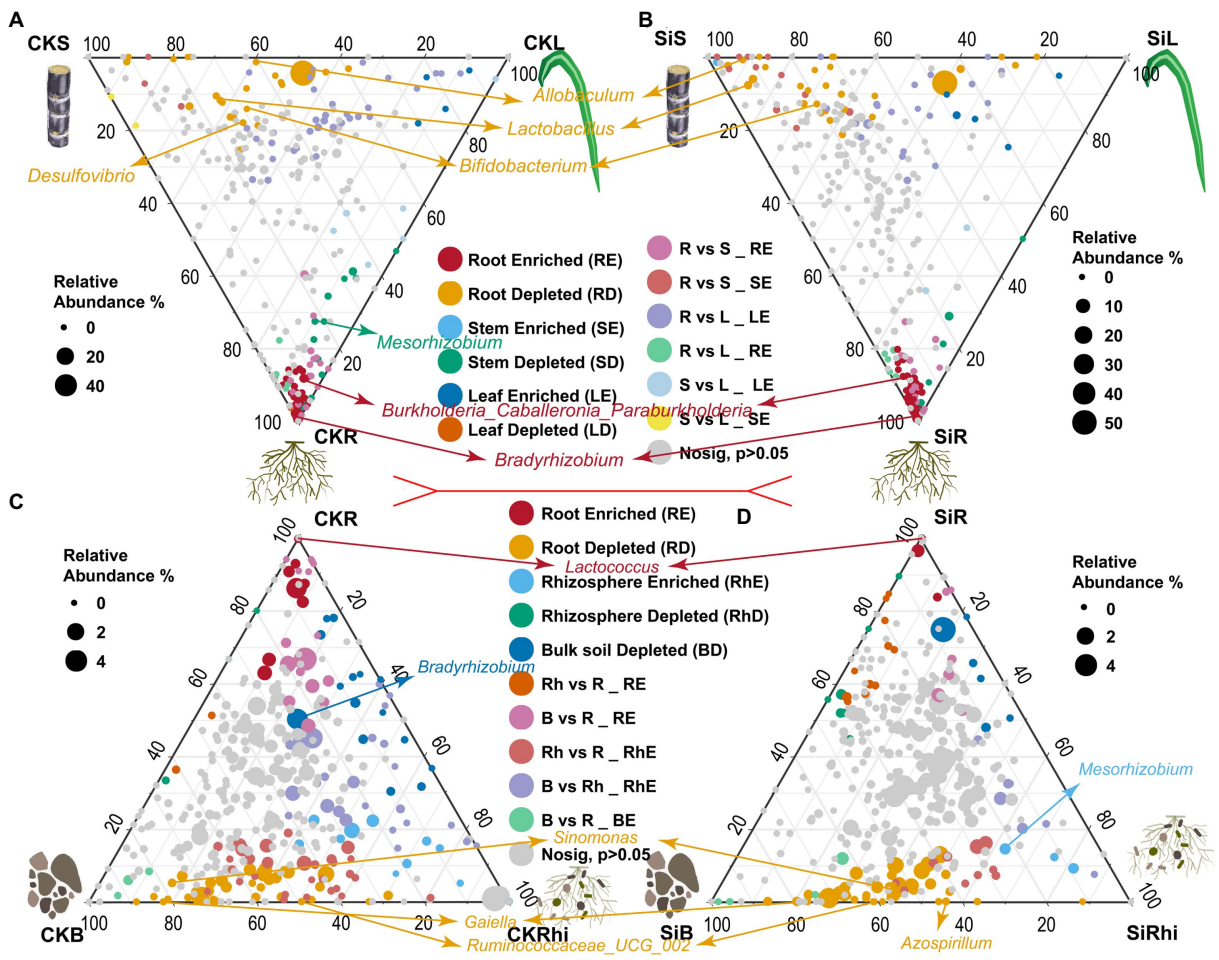

FIGURE 3
Ternary plot illustrating depleted and enriched bacterial community composition in the different plant and soil compartments. Each point represents the depleted or enriched bacterial community composition. The location of each point symbolizes the relative abundance of bacteria in each region, and its size denotes the average across three compared regions. Each colored circles represent depleted or enriched bacterial community composition in one region relative to the other: CKS, sugarcane stem in CK treatment; CKL, sugarcane leaf in CK treatment; CKR, sugarcane root in CK treatment; CKB, bulk soil in CK treatment, and CKRhi, sugarcane rhizosphere soil in CK treatment. Followed by SiS, sugarcane stem in silicon amended soil; SiL, sugarcane leaf in silicon amended soil; SiR, sugarcane root in silicon amended soil; SiB, bulk soil in silicon amended soil, and SiRhi sugarcane rhizosphere soil in silicon amended soil.

Ruminococcaceae_NK4A214_group, *Brevinema*, *Christensenellaceae_R_7_group*, *Edaphobacter*, and *Aliidongia* in the leaf tissue compared with CK. In contrast, *Labedaea*, *Dokdonella*, *Crossiella*, and *Haliangium* were significantly reduced. It was noticed that Si significantly increased *Lachnospiraceae_UCG_009*, *Blastococcus*, *Erysipelatoclostridium*, *Microbacterium*, *Allobaculum*, *Frondihabitans*, *Kineococcus*, *Ellin6067*, and *Akkermansia* in the stems. However, *Gordonia*, *Yonghaparkia*, *Aquisphaera*, and *Methylovirgula* revealed a decreasing trend. In the sugarcane root, Si significantly increased *Acidiphilium*, *Fusobacterium*, and *Allobaculum*. On the other hand, *Chujaibacter*, *Opitutus*, *Amycolatopsis*, *Haliangium*, *Bauldia*, and *Saccharothrix* significantly diminished. Furthermore, Si significantly increased the *Mesorhizobium*, *Sinomonas*, *Enterobacter*, *Olivibacter*, *Glycomyces*, *Mucispirillum*, and *Methylopila* in the rhizosphere. On the contrary, the analysis also revealed that Si significantly reduced *Ochrobactrum*, *Amycolatopsis*, *Bacillus*, *Haematomicrobium*, *Alcaligenes*, *Aquicella*, *Corynebacterium_1*, *Acinetobacter*, *Brachybacterium*, *Lactobacillus*, *Leucobacter*, *Pseudomonas*, *Aeromonas*, *Brevibacterium*, *Akkermansia*, and *Arthrobacter*. In Bulk soil, Si significantly improved *Blastococcus*, *Glycomyces*, *Kribbella*, *Massilia*, and *Mucispirillum*, but *Christensenellaceae R 7 group* and *Saccharum hybrid cultivar* decreased significantly (Figure 4; Supplementary Table S7).

Variation of bacterial function in different soil and plant compartments under Si amended soil

In the different compartments, we assessed variations of bacterial function under the CK treatment. The analysis demonstrated that energy metabolism increased significantly in

FIGURE 4
Manhattan plot showing bacterial community composition in each plant and soil compartment under Si compared with CK. Each triangle or dot signifies a single genus. Genus enriched in CK or Si are represented by filled or empty triangles, respectively (FDR adjusted $p<0.05$, Wilcoxon rank-sum test). Genus are arranged in taxonomic order and colored according to the phylum, while Proteobacteria was assessed at the class level to have a better insight into their classification.

rhizosphere soil compared to the bulk soil (Figure 5A). By comparing the metabolic functions of endophytic bacteria in the different soil compartments, we also found that the metabolism of cofactors and vitamins, and energy metabolism were significantly enriched in the leaf tissue. We also noticed that amino acid metabolism, metabolism of other amino acids, xenobiotics biodegradation and metabolism, and lipid metabolism in bulk and rhizosphere soils were significantly increased (Figure 5B). We also observed significant differences in bacteria functions after bacteria in the stem and root were compared with those in compartments

FIGURE 5
Extended error bar graphs specifying the significant difference in bacterial functional guilds at level 2 in each compartment under CK **(A)** ($p<0.05$, average proportion, $n=3$). The points illustrate differences between CKBulk, bulk soil in CK treatment and CKRh, sugarcane rhizosphere soil in CK treatment; CKBulk and CKR, sugarcane root in CK treatment; CKBulk and CKL, sugarcane leaf in CK treatment; CKBulk and CKS, sugarcane stem in CK treatment, followed by CKRh, and CKL, and CKRh and CKS, respectively.

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under CK (Supplementary Figure S3A). For instance, energy metabolism, global and overview maps, metabolism of cofactors and vitamins, and nuclear metabolism in stem and leaf tissues were significantly higher than those in the root. Furthermore, bacteria metabolic function in the rhizosphere soil and sugarcane root changed (Table 3) after Si amendment. We also observed a similar pattern after we compared the metabolic function of endophytic bacteria between the CK and Si treatments in sugarcane roots. For instance, nucleotide metabolism was significantly enriched in Si treatment, but amino acid metabolism and metabolism of other amino acids decreased significantly (Supplementary Figure S3B).

Exploring the associations between bacterial community structure and different plant and soil compartments under Si amendment

We also explored the topological properties of the symbiotic network composed of microbial communities in the different plant and soil compartments (Table 4). It was observed that the total number of nodes and links, including the network of rhizosphere soil showed greater complexity compared with the other compartments. We further constructed an interactive network of plant–soil systems and bacteria to better visualize the relationship between the bacterial community in each treatment (Figures 6A,B), and in each compartment (Figure 6C). The analysis showed that the network topological parameters such as the number of network nodes, the number of connections, the aggregation coefficient, the characteristic path length, the network density, and the average connectivity were distinct between CK and Si. In the control group, the interaction network between soil and sugarcane bacteria revealed 381 nodes and 2,315 lines, accounting for 94.64% positive correlation (Figure 6A). In the Si treatment, the number of interaction network nodes of bacteria between the soil and sugarcane compartments revealed no difference compared with CK, but the number of connections increased to 2,776, representing 93.05% positive correlation. It was also revealed that the number of network connections and the positive connection of bacteria interaction network in (soil-sugarcane) system increased under Si amended soil. Compared with CK, the bacteria interaction network of the soil-sugarcane system showed higher network density, aggregation coefficient, and average connectivity under Si treatment (Figure 6B; Table 4). This suggests that Si amendment has the potential to increase plant–soil systems bacteria connections, thereby increasing the functional diversity in the host plant, which, in turn, had a positive growth effect on sugarcane growth. Co-occurrence network results of bacterial genera in the different compartments showed that the number of nodes and the number of significant connections between bacteria was more pronounced in the rhizosphere soil, indicating that the rhizosphere soil may be the most complex area for bacteria activities in the soil–plant system (Figure 6C; Table 4).

TABLE 4 Topological features of networks of the different treatments and soil–plant-associated bacterial communities in the various compartments based on Spearman's correlation method.

	CK	Si	BS	RS	R	S	L
R-Value	>0.8		>0.85				
p-Value	<0.05						
N	15	15	6	6	6	6	6
Nodes	381	381	371	372	368	353	327
Edges	2,315	2,776	2,892	4,071	2,346	2,920	3,481
Positive (%)	94.64	93.05	58.64	67.58	53.84	79.18	80.64
Negative (%)	5.36	6.95	41.36	32.42	46.16	20.82	19.36
Average degree (avgK)	12.152	14.572	15.59	21.887	12.75	16.544	21.291
Diameter	7	7	8	8	8	7	7
Density	0.032	0.038	0.042	0.059	0.035	0.047	0.065
Average clustering coefficient (avgCC)	0.601	0.617	0.577	0.612	0.526	0.565	0.611
Average path distance (GD)	2.179	2.146	3.749	3.449	3.677	3.401	3.412

Assessment of the associations among metabolites, bacterial community structure, and soil properties

To establish potential associations of metabolites and bacteria in the various sugarcane and soil regions, we identified 51 core metabolites (Supplementary Table S3; Supplementary Sheet 2) from the top 20 high abundance metabolites in the various plant and soil regions using the entire sample of metabolome data (Supplementary Table S3; Supplementary Sheet 1). We analyzed the correlation between core metabolic substances and core bacterial composition (Supplementary Table S2; Supplementary Sheet 2) in the different plant tissues (root, stem, and leaf), and visualized them with Cytoscape software (Figure 7A). The results showed that the metabolites in the different plant tissues had distinct effects on bacterial community composition. In the various plant compartments, D-biotin showed a significant positive correlation with the *Pajaroellobacter, Actinospica, Thermosporothrix, Haliangium,* and *Bradyrhizobium*. Furthermore, D-fructose was positively correlated with *Enhydrobacter* and *Akkermansia*, while raffinose was positively correlated with *Enhydrobacter*. It was also noticed that salicylic acid (SA) was positively correlated with *F_micropipesaceae, Dyella, Salix Integra, Burkholderia Caballeria Paraburkholderia,*

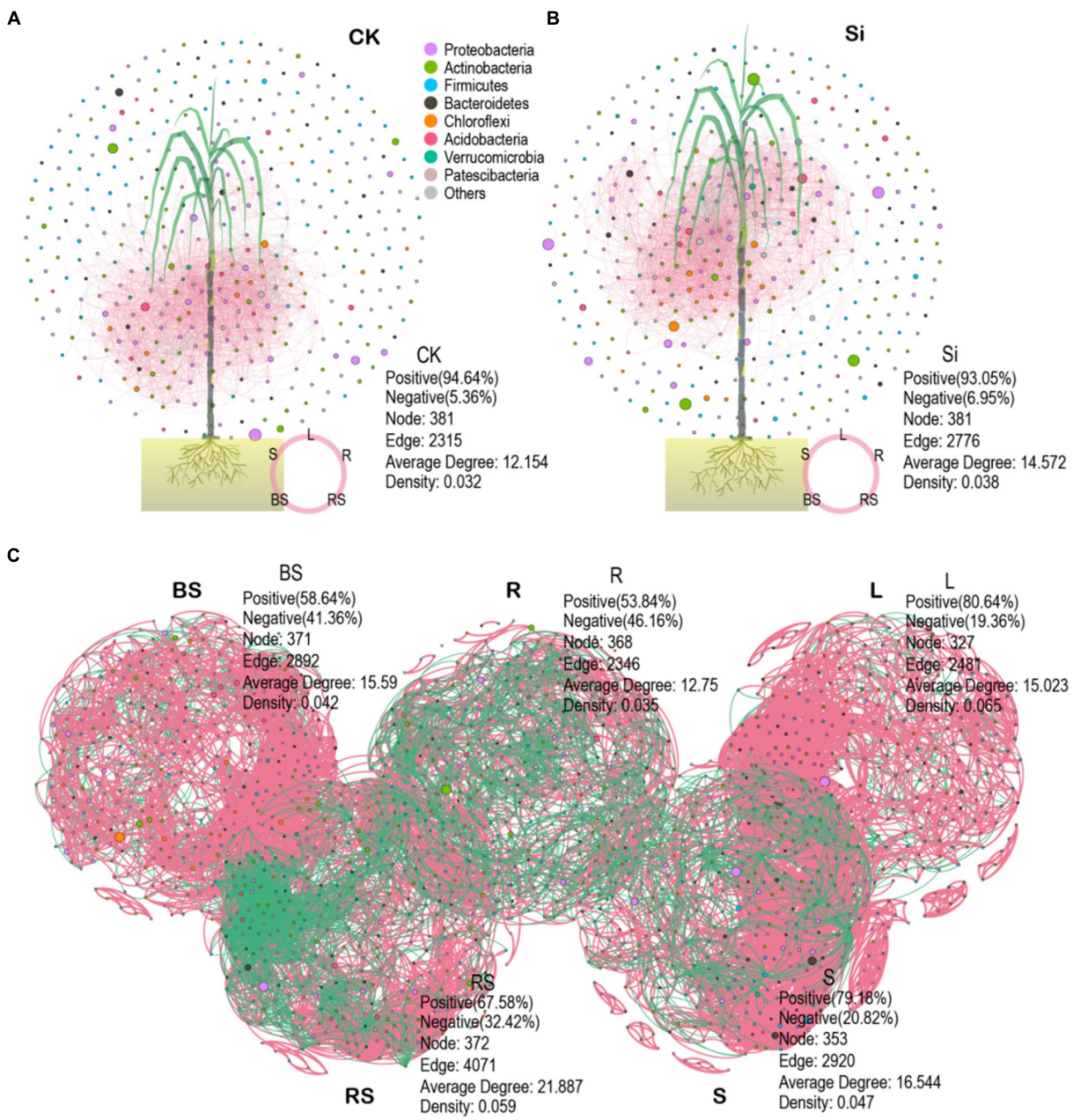

FIGURE 6
Co-occurrence networks depicting the entire bacteria in the different plant and soil compartments under CK **(A)**, Si amendment **(B)**, and bacterial community composition in each compartment (BS, bulk soil; R, root; L; leaves; RS, rhizosphere soil; S, stem) **(C)**. The data in the picture represent the topological role of the microbiome network and nodes. A node represents a genus. The green and red lines indicate negative and positive interaction, respectively.

Catenulispora, *f_chitinophagaceae*, and *Thermosporothrix*. However, SA was negatively correlated with *Akkermansia* and *Allobaculum*. Our analysis also revealed that S-adenosyl-l-homocysteine was negatively correlated with *Pajaroellobacter*, *Thermosporothrix*, *Longimycelium*, *Bradyrhizobium*, *Haliangium*, *Catenulispora*, *Dyella*, *and Actinospica*. Additionally, sucrose showed a significant negative correlation with *Catenulispora*, *f_Chitinophagaceae*, *Thermosporothrix*, *Acidipila*, *Conexibacter*, *f_Ktedonobacteraceae*, *f_micropipesaceae*, and *Burkholderia-Caballeria-Paraburkholderia*. Sucrose also exhibited a significant negative correlation with *Acidipila*, *Burkholderia-Caballeronia-Paraburkholderia*, *Catenulispora*, *Conexibacter*, *Thermosporothrix* (Figure 7A; Supplementary Table S8).

Later, we elucidated the association among bacterial community, key metabolites and soil properties in the different

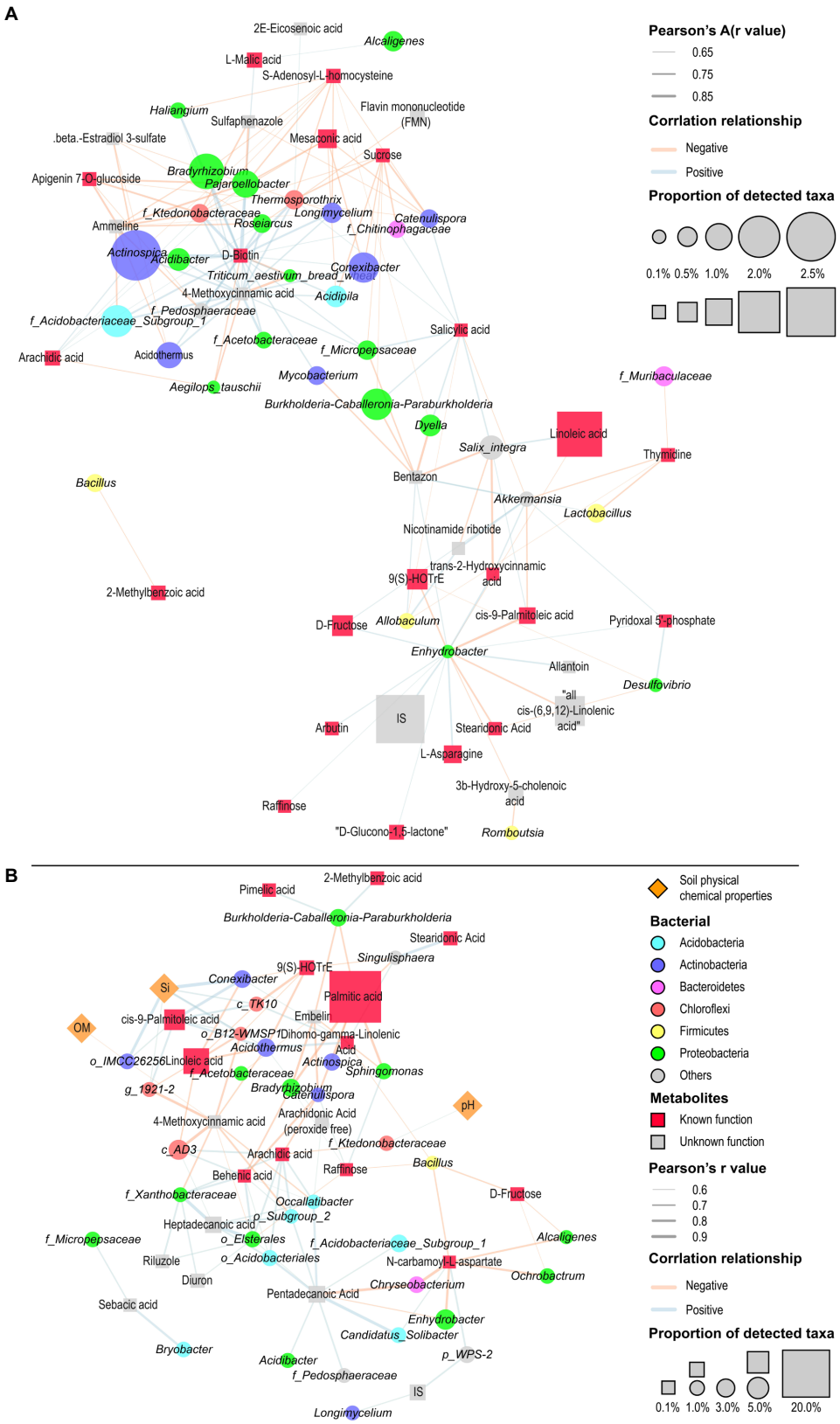

FIGURE 7
Correlation network illuminating the association between bacterial community composition and metabolites in the different sugarcane tissues (root, stem, and leaf) **(A)**, and among soil biochemical properties, metabolites, and bacterial community composition in the different soil regions

(Continued)

Figure 7 (Continued)
(bulk and rhizosphere soils) **(B)**. Blue and red lines depict positive and negative correlations, respectively. Square boxes with orange and red color represent soil physiochemical properties and metabolites, respectively, while circles in different colors represent bacteria. Different colored circles represent different bacterial phyla. Different colored squares represent metabolites classified according to the known Human Metabolome Database. Different sizes of squares and circles indicate relative abundance. The thickness of the connecting line indicates the absolute value of the correlation *R*-value.

soil regions (rhizosphere and bulk soils) (Figure 7B). It was shown that D-fructose, raffinose, dihomo-gamma-linolenic acid, and N-carbamoyl-l-aspartate were negatively correlated with *bacillus* abundance ($R < -0.6$). Moreover, there was a significant negative correlation between the rich palmitic acid, *Actinospica*, *Bradyrhizobium*, *Burkholderia-Caballeronia-Paraburkholderia*, *Catenulispora, Singulisphaera,* and *Sphingomonas*. Linoleic acid and CIS-9-Palmitolic acid were negatively and positively correlated with *Acidothermus* and *Conexibacter*, respectively. The analysis also revealed that arachidic acid was negatively correlated with *Catenulispora* and *f_Ktedonobacteraceae*. However, arachidic acid exhibited a positive correlation with *f_Xanthobacter* and *Occallatibacter*. Whereas dihomo-gamma-linolenic acid was positively correlated with *Acidothermus*, *Conexibacter,* and *Singulisphaera*. While raffinose exhibited a positive association with *Bradyrhizobium*, *Catenulispora* and *Sphingomonas*. Correlation network analysis also revealed that soil physicochemical properties had a significant association with metabolites community, but revealed a minimum relationship with the bacterial community. For example, the content of soil Si was positively correlated with the *Acidothermus*, *Conexibacter,* and *OM o_IMCC26256*. The result also showed that soil pH demonstrated a significant positive correlation with *f_Ktedonobacterae* (Figure 7B; Supplementary Table S8).

Discussion

Our understanding of the impacts of Si application on soil physicochemical properties, microbial composition, metabolisms, and sugarcane productivity is limited. Therefore, our findings could broaden our understanding of how Si influences soil fertility and productivity, as well as microbial and metabolisms communities in different soil and sugarcane compartments. Here, we observed that the application of Si not only improved sugarcane agronomic parameters, but also promoted its yield, which is consistent with previous findings, where Si application enhanced the yield and quality of rice (Song et al., 2021) and sugarcane (Meyer and Keeping, 2000). It was also observed that Si amended soil remarkably enhanced soil nutrient status, especially Si, AP, and AK, which is in agreement with the finding documented by Zhang et al. (2021b) and Matychenkov and Ammosova (1996). We, therefore, concluded that supplementing the soil with Si could enhance soil nutrient status, which, in turn, are made available to the plant, evident by the significant increase in cane agronomic parameters.

A number of studies have documented that soil ecosystems and plant tissues, including root, stem, leaf, flower, and fruit are heavily colonized by different microbes (Beckers et al., 2017). The colonization of these microbes vary distinctly from one compartment to another (Tardif et al., 2016; Xiong et al., 2020). Likewise, we observed that OTUs richness was highly dependent on plant compartment, where OTUs richness consistently decreased in value from 1017.7 ± 66.0 (bulk soil), to 859.4 ± 100.5 (rhizosphere soil), followed by the root samples (828.1 ± 11.0), the stem samples (645.4 ± 5.0) and the leaf samples (573.8 ± 68.8). Similarly, bacterial diversity demonstrated tissue-dependent distribution patterns, with the bulk soil, rhizosphere soil, and root endosphere revealing the highest amount of bacterial diversity compared with the stem and leaf tissues. These findings have been previously suggested for many plants such as maize (Xiong et al., 2021), sugarcane (de Souza et al., 2016), and agaves (Coleman-Derr et al., 2015), wherein it was observed that variations in microbes diversity and richness were compartment-dependent. For bacterial diversity, we also observed a similar pattern, where bacterial diversity in the bulk soil, rhizosphere soil and root endosphere peaked significantly compared with the stem and leaf tissues under both treatments. Interestingly, these distinct variation patterns in the microbial community structure were further validated by PCoA, Adonis, and DESeq2 analyses. Thus suggesting that the distinct distribution patterns observed in the bacterial community structure were component-specific. We, therefore, assumed that the gradual decrease in the OTUs richness and diversity from belowground to aboveground suggests that these bacteria might have initially originated from the soil and moved to the aboveground components of the plant, as displayed by the Source Model of Plant Microbiome (SMPM) analysis. Another reason for this phenomenon could be largely ascribed to the host's selection of bacteria, such as the expression of a series of genes related to the resistance of plant innate immune response (Jones and Dangl, 2006; Hardoim et al., 2008), as well as the exposure of the ground parts to harsh conditions, such as ultraviolet radiation and diurnal temperature fluctuations. These immune responses and environmental conditions may have eventually led to the decrease in the diversity index and richness index of endophytic bacteria in the leaf and stem tissues.

Although Si application exhibited no significant regulatory effect on the endophytic bacteria richness and diversity in different plant and soil compartments, Si exhibited the advantage of considerably promoting bacterial abundance in the various plant compartments. For instance, *Cetobacterium*, *Erysipelatoclostridium*, *Ruminococcus_NK4A214_group,* and *Christensenellaceae_R-7_group* were more prevalent, especially in the leaf compartment. *Cetobacterium* is a Gram-negative, non-spore-forming, pleomorphic, non-motile, and rod-shaped

genus of bacteria from *Fusobacteriaceae* family. Moreover, *Cetobacterium* is a kind of *Brevibacterium* that can oxidize sugar and alcohol into acetic acid and other products, which suggests that Si may have been involved in regulating microorganisms to improve sugar metabolism in sugarcane leaves (Ramírez et al., 2018). Studies have proved that a proper amount of *Erysipelatoclostridium* and *Ruminococcaceae NK4A214* group can improve intestinal health (Zhang et al., 2020; Milosavljevic et al., 2021). *Christensenellaceae R-7 group* can regulate lipid metabolism and could reduce the incidence of obesity (Goodrich et al., 2014).

In sugarcane stems, Si treatment significantly increased *Lachnospiraceae_UCG_009*, *Allobaculum,* and *Akkermansia* compared with the other compartments. It has been documented that *Lachnospiraceae* belong to the core of gut microbiota colonizing the intestinal lumen from birth and increasing. Huang et al. (2019) employed reductive soil disinfestation (RSD), also known as anaerobic soil disinfestation or biological soil disinfestation, an environmentally sustainable approach used to mitigate soil-borne diseases. They observed that *Lachnospiraceae* was one of the prevalent bacteria in the entire sample. Tasnim et al. (2021) mentioned that *Allobaculum* is beneficial bacteria that can produce short-chain fatty acids in the intestine. Moreover, it plays an important role in anti-inflammation, protecting intestinal barrier function, regulating human metabolism and immunity directly or indirectly by increasing the content of short-chain fatty acids in intestines, especially in the process of preventing or treating metabolic diseases such as obesity, insulin resistance, and diabetes. *Akkermansia* is a new generation of probiotics and is considered the most abundant mucolytic bacteria in the intestines of healthy people. It has also the potential to protect the intestines from pathogens (Derrien et al., 2004; Belzer and de Vos, 2012). These findings further conformed that a number of microbial tend to enter plant roots system as endophytes and establish a mutual relationship with their host, which in line with previous studies (Afzal et al., 2019; Dubey et al., 2020).

In the rhizosphere soil of sugarcane, Si treatment significantly increased the abundance of *Mesorhizobium* and *Methylopila*. Studies have documented that rhizobacterial genera, e.g., *Rhizobium*, *Bradyrhizobium,* and *Mesorhizobium* are capable of promoting legumes growth and making nitrogen available to plants in metal-contaminated environments (Drinkwater et al., 2017; Shahid et al., 2021). *Methylotrophic* bacterial composition plays a crucial role in sustainable agriculture by enhancing soil fertility and health, thereby boosting plant growth promotion (PGP) and crop yield, evident by the significant increase in sugarcane traits, namely, stalk height and theoretical production (Kumar et al., 2019).

Co-occurrence interactions are environmentally essential patterns that reveal niche processes that trigger diversity maintenance within biological communities and coexistence (Williams et al., 2014; de Vries et al., 2018). However, little is known about how co-occurrence networks within these communities in different soil and plant compartments respond to agriculture practices such as fertilization, specifically Si amendment. Here, we adopted co-occurrence network to decipher potential communications of community assembly in the different soil and plant tissues. We noticed that Si amendment has the potential to increase bacterial diversity maintenance, coexistence, and plant–soil systems bacteria connections, which, in turn, had a positive growth effect on sugarcane growth compared with the CK treatment. This finding partly conformed with Zheng et al. (2018) study, wherein it was mentioned that soil management practices, namely, cover crops potentially increased bacterial community co-occurrence networks, but not within the fungal community.

A number of studies have reported that the extractable pool of metabolites has the potential to interact with the soil microbial communities (Swenson et al., 2015; Song et al., 2020). For instance, Jones et al. (2004) documented that plant exudates comprising predominantly of sugars, organic acids, and amino acids stimulated microbes' activity in soils. Hence, it is important to have a broader understanding of the interaction between extractable pools of metabolites and microbial communities. In this study, we explored the relationships between the differential microbes and differential metabolites data generated from the different plant tissues (root, stem, and leaf) by constructing an interactive network. Generally, the analysis demonstrated that there were more negative and positive correlations than correlations in the network within the metabolites and microbes communities. However, SA demonstrated a significant positive association with a majority of bacteria in the plant tissues, including *f_micropipesaceae, Dyella, Salix Integra, Burkholderia Caballeria Paraburkholderia, Catenulispora, f_Chitinophagaceae,* and *Thermosporothrix*. We also found that D-fructose displayed a positive correlation with *Enhydrobacter* and *Akkermansia*, whereas raffinose was positively correlated with *Enhydrobacter* in the different plant tissues. While in the different soil regions, raffinose demonstrated a significant positive association with *Bradyrhizobium, Catenulispora,* and *Sphingomonas*. This finding is partly in agreement with Song et al. (2020) work. They reported that differential metabolites, such as sucrose displayed a considerable relationship with the soil bacterial members, including *Rhizobiaceae* family, *Betaproteobacteria* class, *Burkholderiales* order, *Proteobacteria* phylum, and *Ensifer* genus. We, therefore, inferred that bacteria with increased abundances and positive correlations with these mentioned metabolites above especially in the different plant tissues tended to favor the production and yield of sugarcane.

We also assessed the relationship among the differential microbes, differential metabolites, and soil properties. It was noticed that soil Si content demonstrated a considerable positive association with the bacterial communities, namely, *Conexibacter*, *OM o_IMCC26256,* and *Acidothermus*. This result is in agreement with the finding mentioned by Huang et al. (2021), who documented that the nitrogen concentration in soil and the flavonoid content in stalks were markedly associated with the soil microbiome composition.

Conclusion

Taken together, our study demonstrated that Si utilization had a marked positive effect on sugarcane agronomic parameters and soil physicochemical properties. Moreover, the trend of bacterial richness and diversity index was soil > root > stem > leaf. It was also noticed that plant and soil compartments exhibited a modulatory effect on bacterial abundance and community compositions. Co-occurrence interactions showed that Si amendment has the potential to increase bacterial diversity maintenance, coexistence, and plant–soil systems bacteria connections, thereby increasing the functional diversity in the various plant tissues, which, in turn, could trigger positive growth effects in sugarcane. Network analysis further revealed that metabolite profiles exhibited a strong association with bacterial community structures. It was revealed that Si content had a considerable positive association with bacterial communities. This study provides comprehensive empirical evidence of the significance of Si in agriculture and illuminated on differential metabolite profiles and soil microbes relationship.

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

ZY: conceptualization, data curation, funding acquisition, supervision, and project administration. ZP: data curation, formal analysis, investigation, methodology, software, visualization, and writing–review and editing. NF: formal analysis, software, writing–original draft, writing–review and editing, and methodology. YZ and FD: formal analysis and software. WL: conceptualization, funding acquisition, and resources. CH: supervision, funding acquisition, and resources. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1009505/full#supplementary-material

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EDITED BY
Paola Grenni,
Water Research Institute,
National Research Council,
Italy

REVIEWED BY
Jairam Choudhary,
Indian Council of Agricultural Research,
India
Surender Singh,
Central University of Haryana, India

*CORRESPONDENCE
Alok Kumar Srivastava
aloksrivastva@gmail.com;
alok.srivastava@icar.gov.in
Anil Kumar Saxena
saxena461@yahoo.com

†These authors have contributed equally to this work and share first authorship

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Influence of host genotype in establishing root associated microbiome of *indica* rice cultivars for plant growth promotion

Arjun Singh[1,2†], Murugan Kumar[1†], Hillol Chakdar[1], Kuppusamy Pandiyan[1,3], Shiv Charan Kumar[1], Mohammad Tarique Zeyad[1], Bansh Narayan Singh[1], K. T. Ravikiran[2], Arunima Mahto[4], Alok Kumar Srivastava[1*] and Anil Kumar Saxena[1*]

[1]ICAR-National Bureau of Agriculturally Important Microorganisms, Mau, India, [2]ICAR-Central Soil Salinity Research Institute, RRS, Lucknow, India, [3]Ginning Training Center, ICAR-Central Institute for Research on Cotton Technology, Nagpur, India, [4]National Institute of Plant Genome Research, New Delhi, India

Rice plants display a unique root ecosystem comprising oxic-anoxic zones, harboring a plethora of metabolic interactions mediated by its root microbiome. Since agricultural land is limited, an increase in rice production will rely on novel methods of yield enhancement. The nascent concept of tailoring plant phenotype through the intervention of synthetic microbial communities (SynComs) is inspired by the genetics and ecology of core rhizobiome. In this direction, we have studied structural and functional variations in the root microbiome of 10 *indica* rice varieties. The studies on α and β-diversity indices of rhizospheric root microbiome with the host genotypes revealed variations in the structuring of root microbiome as well as a strong association with the host genotypes. Biomarker discovery, using machine learning, highlighted members of class *Anaerolineae*, *α-Proteobacteria*, and bacterial genera like *Desulfobacteria*, *Ca*. *Entotheonella*, *Algoriphagus*, etc. as the most important features of *indica* rice microbiota having a role in improving the plant's fitness. Metabolically, rice rhizobiomes showed an abundance of genes related to sulfur oxidation and reduction, biofilm production, nitrogen fixation, denitrification, and phosphorus metabolism. This comparative study of rhizobiomes has outlined the taxonomic composition and functional diversification of rice rhizobiome, laying the foundation for the development of next-generation microbiome-based technologies for yield enhancement in rice and other crops.

KEYWORDS

***indica* rice, community metagenomics, machine learning, SynComs, PiCRUST**

Introduction

An increase in anthropogenic activities and dependency on inorganic agriculture for obtaining maximum crop yields over time, has led to the deterioration of soil quality. According to a report by Food and Agriculture Organization (FAO), at the current rate of soil erosions and climate change, 20–80% of the crop yield will be affected in the future.[1] Microorganisms are an essential component of the soil ecosystem, and through various biogeochemical cycles provide sustenance to all the food crops consumed worldwide (Kopittke et al., 2019). Crop plants depend on the syntropic interaction with their below-ground microflora or rhizobiome, which provide resistance against an array of stressors such as drought, salinity, heavy metal pollution, and biotic stresses. Next-generation omics approaches have provided new insights into the biodiversity of soil-dwelling microbes and revealed that about 1,000 Gbp of microbial genomic content is available per gram of soil (Vogel et al., 2009; Friedman and Alm, 2012; Azaroual et al., 2022).

Soil microbial communities act in synchrony to maintain soil fertility, biogeochemical cycling of nutrients, detoxification, and carbon sequestration (Banerjee and van der Heijden, 2022). Plant root exudates consist of various signaling molecules, dissolved organic matter, volatile organic compounds (VOCs), etc., which tend to attract diverse groups of microbial communities. These in turn support the growth and performance of the assembling host by acting as hotspot regions of many biogeochemical cycles (Hu et al., 2018; Singh et al., 2020). Advancements in the area of plant-microbe interactions have shown that host varieties influence their associative soil microbial communities, which in turn regulate many of the plant phenetic traits. For instance, rhizobiome can help their host to survive abiotic stresses by changing root morphology, delaying senescence, and reducing heavy metal bioavailability to the host plant (Dodd et al., 2010; Deepthi et al., 2014). Rhizobiome also provides protection against biotic stresses by competitively interacting with the invading pathogenic species and hindering their colonization (Wei et al., 2015; del Carmen Orozco-Mosqueda et al., 2022). Recent studies, using systematic targeted metagenomic and high throughput data analyses, have provided deeper insights into plant-microbiome interactions. The first study conducted on *Arabidopsis thaliana* revealed that microbiota colonizing its rhizosphere has a significant impact on plant growth, productivity, and carbon sequestration (Lundberg et al., 2012). It was also found that the assemblage and composition of root microbiota were conditioned by the *Arabidopsis* variety. Similar outcomes and observations were reported for root microbiome studies conducted on other terrestrial plants (Fitzpatrick et al., 2018). More recently, the impact of agriculturally intensive cereal crops in shaping soil microbiome diversity is also being explored, concerning their influence on physiological growth and development. A study conducted on wheat to understand the effect of nitrogen fertilization on the rhizosphere microbial community showed that an increase in N fertilization level decreases microbial community variations. It was also observed that organic acid secretions present in root exudates promote the assemblage of beneficial bacteria around the root zones (Chen et al., 2019). Another study on root microbiome of maize revealed that genetic makeup of maize strongly influences shaping of the microbiome. Studies have also shown that these microbial communities can impart psychrotolerance to host (Beirinckx et al., 2020). Barley root microbiome study revealed that the assembled microbiome was involved in functions such as detoxification, nutrient mobilization, siderophores production, etc (Bulgarelli et al., 2015). Among fruit crops, studies on grapevine rootstock have shown that underground microflora had activities related to ACC deaminase, P-solubilization, and production of siderophore, and indole acetic acid, which benefit plant growth and development (Marasco et al., 2018). Concerning leguminous crops, the rhizosphere microbiome of *Phaseolus vulgaris* (common bean), *Vigna unguiculata* (cowpea), and *P. lunatus* (lima bean) have also been deciphered. The findings revealed that the cultivation of common beans on native soils increased the rhizosphere bacterial diversity as compared to agricultural soils. The nodule microbiomes of cowpea and lima bean were not affected by the application of compost or prevailing soil physico-chemical characteristics (Rocha et al., 2020).

Rice is a major food crop grown globally that feeds about 50% of the world's population. In India, rice is grown in more than 45.1 Mha with a total production of 127.93 million hectares (Mha) (www.indiabudget.gov.in). Most of its physiological growth period requires submerged conditions, which result in the development of oxic-anoxic zones around rice roots; oxygenated zones around its rhizosphere, and anoxic zone farther to roots (Yuan et al., 2016; Zhao et al., 2019). This oxic-anoxic interface of the rice root rhizosphere supports the colonization of diverse aerobic, anaerobic or facultative anaerobic microbes (Revsbech et al., 1999; Li and Wang, 2013). Since it is an important crop with a vast global presence and peculiar growth requirements, several attempts have been made to comprehend the structural and functional diversity of its rhizosphere-associated microflora. Among the underlying factors which shape the rhizosphere microflora, the age of rice plants and root exudates composition are highly important (Aulakh et al., 2001). Previously, a culture-dependent study revealed that rice root and seed microbiota were comprised of *Bacillus firmus*, *B. fusiformis*, *B. pumilus*, *B. velezensis*, *Caulobacter crescentus*, *Kocuria palustris*, *Nitrobacter* spp. and *Nitrosospira* spp. (Banik et al., 2016; Hwangbo et al., 2016). These bacterial strains were capable of performing biogeochemical cycling of phosphorus and nitrogen, and also had antagonistic properties against various plant pathogens. High throughput sequencing technologies have accelerated these studies presenting new insights into the diversity of non-cultivable bacterial flora associated with rice. Metagenomic studies indicate that the composition of the rice rhizosphere microflora is the interactive effect of rice varieties with their growth environments (Edwards et al., 2015; Oliveira et al., 2022). Functional annotations of

1 https://www.fao.org/statistics/en/

rhizospheric bacterial flora indicate their involvement in the biogeochemical cycling of nutrients, biocontrol, and plant growth promotions (Raaijmakers and Mazzola, 2012). Furthermore, culture-independent studies indicate that rice varieties also influence the flux of methane, and can be categorized into low and high methane emitters based on the abundance of methanotrophs in their rhizobiome (Liechty et al., 2020). The concept of rhizospheric influence was furthered when microbial communities of *indica* rice varieties were tailored into SynCom-based formulations for improving nitrogen use efficiency (Zhang et al., 2019).

India is the world's second-largest producer of rice with current production of 127.93 million tonnes. It is grown in almost all the agro-climatic zones of the country covering a total acreage of 45.1 Mha. In this study, we have characterized the rhizobiome of ten popularly grown *indica* rice varieties (non-Basmati of *indica* ecotype) *viz*., MTU1001, BPT5204, CO52, HUR105, HUR917, MTU7029, SHIATS1, TKM13, Warangal 3,207 and Rajender Sweta. The study is intended to understand the influence of these varieties on the abundance of key bacterial species for regulation of biogeochemical/transformations of plant nutrients, especially carbon and nitrogen, methanogenesis and other plant growth promotional attributes. The results from this study will provide new prospects for the development of microbiome-based technologies for improving rice productivity and sustainability.

Materials and methods

Soil sampling and DNA isolation

For this study, 10 high-yielding, popular *indica* rice varieties, *viz*., MTU1001, BPT5204, CO52, HUR105, HUR917, MTU7029, SHIATS1, TKM13, Warangal 3,207, and Rajender Sweta, were grown during Kharif season, in separate experimental plots at Indian Institute of Seed Sciences, Mau, Uttar Pradesh, India (25.89 °N and 83.48 °E). All the rice varieties were grown following a uniform set of agronomic cultivation practices. Similar doses of fertilizer were applied to fulfil the nutrient requirement of the crop; nitrogen was applied at @ 130 kg/ha, whereas phosphorous @ 70 kg/ha was applied, and potassium @ 60 kg/ha was applied (Kaga et al., 2009). Before the transplantation of the rice seedlings, organic carbon and available nitrogen content of the soil was also estimated following the protocol of Walkley and Black (1934) and Subbaiah (1956). Field soil was having an organic carbon content of 0.51 ± 0.09% and available nitrogen was estimated to be 0.01 ± 0.001%. The rhizosphere soil was sampled at the tillering stage. Plants were uprooted and the soil attached to the roots was collected. The obtained 30 soil samples were air dried and sieved. DNA was isolated from these soil samples using the FastDNA™ SPIN Soil kit (MP Biomedicals, Solon, United States) according to the manufacturer's protocol. For improving DNA yield, the initial bead beating step was extended to 15 min. The quality of DNA was determined on agarose gel (1% w/v) and its concentration was estimated using Qubit Fluorimeter (v.3.0).

Targeted PCR amplification of 16S rRNA and next generation sequencing of soil DNA

Soil DNA was used for 16S rRNA gene amplification using V3 and V4 primer sets (V3F-5' CCTACGGGNBGCASCAG3'; V4R-5' GACTACNVGGGTATCTAATCC3') (Takahashi et al., 2014). The amplified products were checked by running on 2% (w/v) agarose gel. The PCR products were purified for paired-end library preparation. Approximately 5 ng/μL of the PCR amplicon was used for library preparation using the NEB Next Ultra DNA library preparation kit (New England Biolabs, United Kingdom), as per the manufacturer's protocol. Library size and quality were determined using Agilent 2200, TapeStation (Agilent Technologies, United States). High-throughput sequencing was performed using Illumina HiSeq 2500 platform with 2*250 cycle chemistry to obtain the metagenomic data.

Analysis of metagenome sequence data

The raw data obtained from sequencing were processed and analyzed using the QIIME2 pipeline. Briefly, the *de novo* sequence quality filtering, chimera removal, and error corrections were done by deblur. The amplicon sequence variants (ASVs) generated were used to calculate phylogenetic distance based α and β-diversity indices, i.e., Faith phylogenetic diversity indices, and unweighted and weighted Unifrac distances. The relatedness of the groups was estimated by PERMANOVA using non-metric multi-dimensional scaling ordination (NMDS). For taxonomic annotations, the green genes database (version 13–8-99) was used at 99% sequence similarity.

Metagenome reconstruction to ascertain functional relevance of rhizobiome

The PICRUSt2 pipeline was used for functional annotation of the rhizobiome of rice varieties (Douglas et al., 2020). To run this analysis, the PICRUSt2 plugin for QIIME 2 was obtained. As an input, feature tables as well as feature sequences obtained during the analysis were used. A Default NSTI cut-off of 2 was used throughout the analysis. After analysis, the datasets get predicted for KEGG orthologues, EC metagenome predictors, and MetaCyc pathway abundance predictors. The KEGG orthologues were used for further analysis and predictions. The taxonomic to phenotypic mapping of the datasets was done using the Metagenassist pipeline (Arndt et al., 2012).

Identification of taxonomic and functional bioindicators

For the identification of key bacterial features associated with rice rhizobiome, a random forest machine learning approach was used. This involved Classification and Regression Tree to make decision trees for sub-setting the samples and variables of importance as per the rhizosphere conditions of rice varieties. R package was used to run the random forest models such as "randomForest," "plyr," "rfutilities" and "caret." Univariate analysis was used to identify differentially abundant significant archaebacterial genera, whereas Lefse analysis was done to predict the important rhizobiome KEGG orthologues associated with rice varieties. For conducting univariate analysis and Lefse analysis MicrobiomeAnalyst pipeline was used (Dhariwal et al., 2017).

Identification of microbiome network in rice rhizosphere

To elucidate microbe-microbe associations in the rhizosphere of rice and various drivers of ecological functions, co-occurrence networks based on the prevalence of the OTUs were identified. MetagenoNets pipeline was used as a guideline for various steps in this analysis (Nagpal et al., 2020). Firstly, the top 100 biologically relevant bacterial OTUs were identified through a random forest machine learning approach. Then these 100 bacterial OTUs and 24 archaebacterial OTUs were used for the prediction of microbial co-occurrence networks. The settings used for network predictions were:

A. Algorithm: Pearson Correlation.
B. Value of p: ≤ 0.05.
C. Iterations: 500.
D. Correlation cut-off: Based on Critical-R (For creating edges in the network).

Network topology including nodes, edges, diameter, density, and average degrees were calculated. For the identification of keystone species, nodes with the highest betweenness centrality values were extracted. Upon completion of this analysis 10 cultivars exhibited variations in microbial co-occurrence networks and functional guilds, which were discussed as network scenarios.

Statistical analysis and data visualization

The calculation of PCA and multivariate analysis of the microbiome data were done using STAMP software, applying the Tukey–Kramer *post hoc* test and Benjamin-Hochberg FDR corrections (Parks et al., 2014). Box plot was drawn using the ggplot2 R package and heatmaps were drawn using the Clustvis tool (Metsalu and Vilo, 2015). The CIRCOS plot was drawn using the CIRCOS table viewer tool (Krzywinski et al., 2009).

Data submission

The sequence data of this study has been submitted to NCBI, under BioProject accession number PRJNA790010.

Results and discussion

Plant variety influences on its root microbiome assemblage

A total of 30 rhizosphere soil samples, from 10 *indica* rice varieties, were collected. Their eDNA was isolated and sequenced, generating more than 18 million reads, with an average of 5 million reads per sample. The *de novo* sequence quality filtering and error corrections by deblur resulted in about 55,000 to 60,000 ASVs or microbial features (Supplementary Table S1). The faith_pd of rhizobiomes, an indicator of α-diversity, ranged from 69.85 to 74.95. Among the 10 *indica* varieties, phylogenetic diversity was highest for MTU7029 (faith_pd = 74.95 ± 2.08) and least for Rajender Sweta (faith_pd = 69.85 ± 0.343) (Supplementary Table S2; Figure 1). Statistical difference in the root-associated bacterial communities of the rice varieties was estimated by PERMANOVA, based on non-metric multi-dimensional scaling ordination (NMDS) as well as unweighted and weighted Unifrac distances. Based on phylogenetic relatedness of root-associated bacterial communities, both Unifrac distances revealed that rice varieties had a strong influence in recruiting their microbiome (Figure 2) and all the cluster groups had developed a unique microbiome related to their host. The rank-based β-diversity, based on NMDS ordination, also revealed that root microbiome assemblages of rice varieties are strongly linked to their hosts. Distinct clusters of rice varieties were observed along both axes, indicating the uniqueness of the microbial niches (Figure 3). The rhizosphere is a region of the root that acts as a highly transiently active zone of microbial successions and plant microbiome assemblage (Hartmann et al., 2008). It also contains root secretions having essential chemi-signals and nutrition, which attract diverse soil microorganisms to establish a root symbiotic microbial community (Ofaim et al., 2017). Previous studies have indicated that root exudates composition and the microbial community getting influenced, are dependent on the host's variety and prevailing environmental cues (Li et al., 2018; Chen et al., 2019; Imchen et al., 2019). Results of the present study also reflected those significant variations obtained in α and β-diversities of *indica* rice microbiome assemblages are host induced and thus, strongly vary according to the host variety.

Rhizospheric microbiome changes across rice varieties at phylum level

Taxonomic annotation of 16S rRNA reads revealed that 44 bacterial phyla were distributed across all the 10 rice

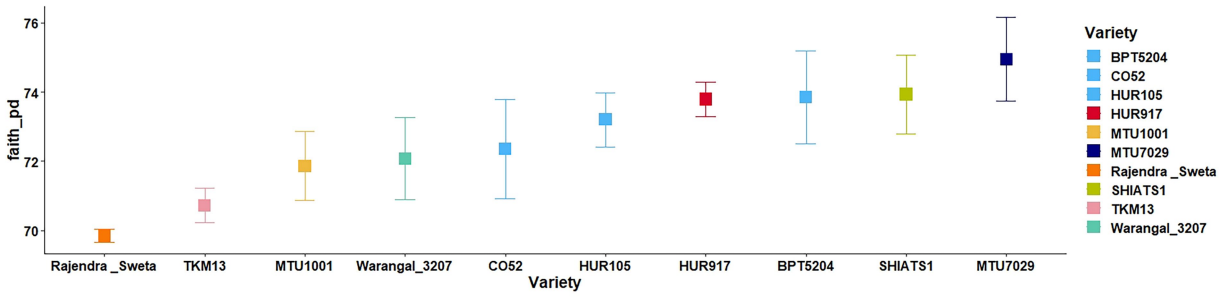

FIGURE 1
Variations in α-diversity indices of the rhizo-microbiome of rice varieties based on faith phylogenetic diversity. The significance of the diversity indices as per the rice varieties' rhizosphere condition was determined based on the Krusal Walis test.

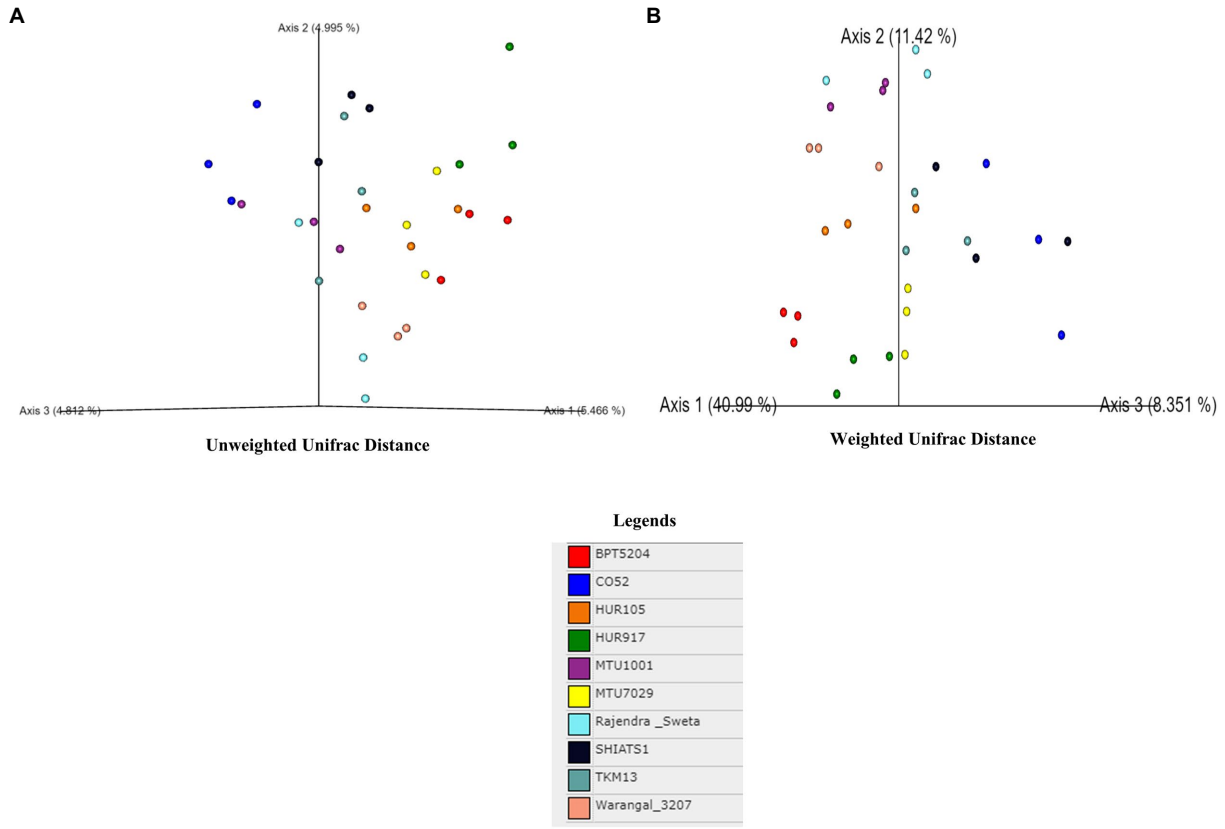

FIGURE 2
Effect of rice varieties on β-diversity of associated rhizo-microbiome as revealed by un-weighted **(A)** and weighted unifrac distances **(B)**.

rhizospheric groups. Statistically, the mean relative abundances of 33 bacterial phyla were found to be significant (Supplementary Data File S1). Among these, the most abundant bacterial phylum included *Proteobacteria*, *Chloroflexi*, *Bacterioidetes*, *Candidatus Phylum OD1 or Parcubacteria*, *Acidobacteria*, *Verrucomicrobia*, *Firmicutes*, and *Gemmatimonadetes* (Tables 1, 2). The mean relative abundance of *Proteobacteria* was found to be highest in rhizobiome of rice variety MTU1001 (22.42 ± 0.94%) and lowest in BPT5204 (17.37 ± 0.81%). Looking at the mean relative abundance of *Chloroflexi*, the rhizobiome of CO52 harbored the lowest population of the phylum (9.21 ± 0.41%), whereas the highest population was recorded for BPT5204 (14.21 ± 0.24%). The distribution of *Candidatus Phylum OD1* ranged from 4.8 to 7.4% in the rhizobiomes, highest was recorded for MTU7029 (7.42 ± 0.71%). Members of phylum *Acidobacteria*, *Verrucomicrobia*, and *Firmicutes* were evenly distributed in all the rhizobiome samples.

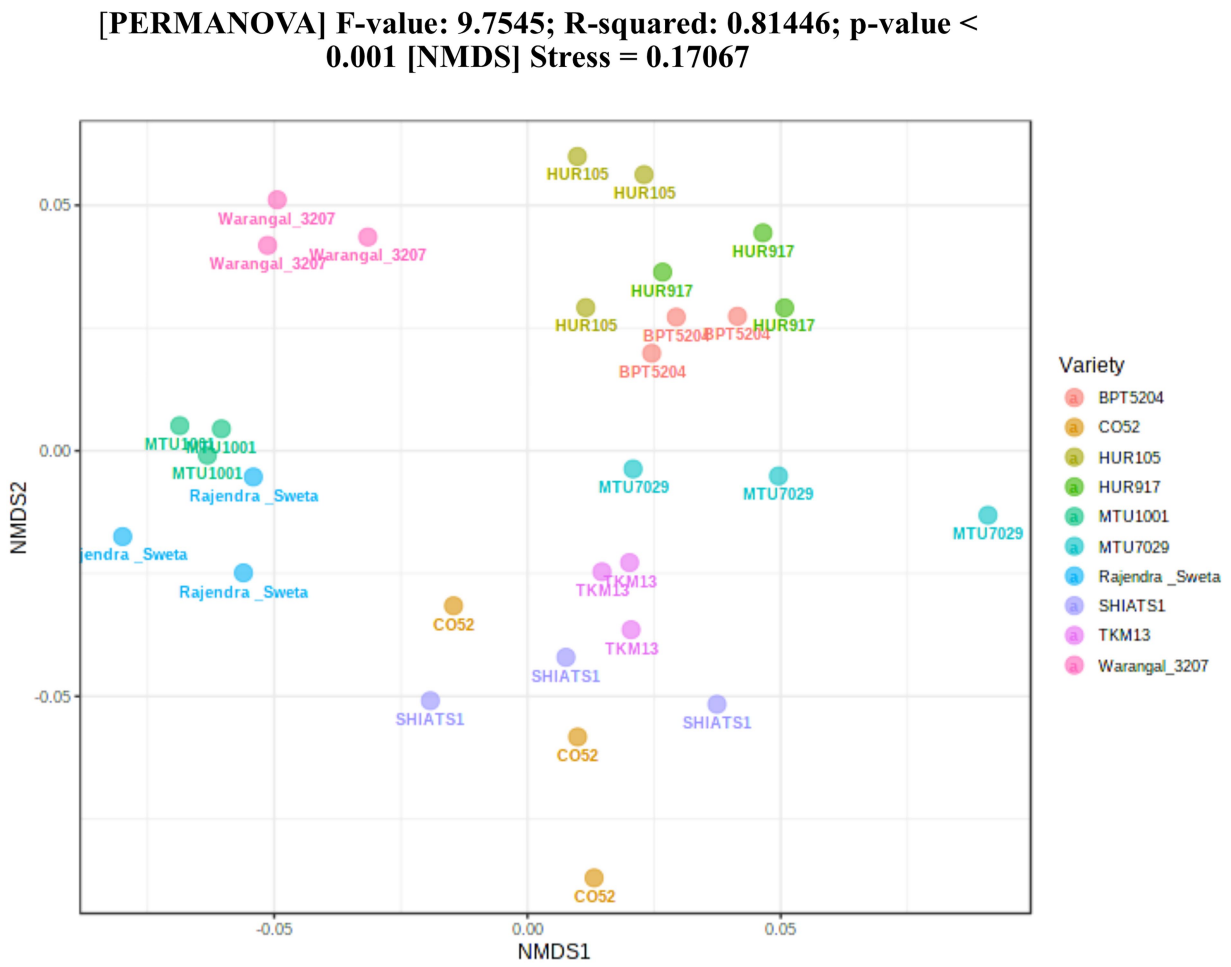

FIGURE 3
Estimation of rank based β-diversity based on NMDS-PERMANOVA to show statistical significance of the effect of rice varieties on the assemblage of rhizo-microbiome (p<0.001).

Previous work for elucidating the composition of the rice rhizosphere microbial community has highlighted the abundance of *Gemmatimonadetes*, *Proteobacteria*, and *Verrucomicrobia* in the rhizosphere of the Koral rice genotype (Breidenbach et al., 2016). In many of the studies, the abundance of *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Acidobacteria* was reported (Edwards et al., 2018; Ding et al., 2019; Imchen et al., 2019). The present findings were consistent with the past reports and similar results were observed at the phylum level.

Important bacterial communities associated with *indica* rice rhizosphere

There were inter-varietal differences at the genera level in rhizobiomes of rice varieties unlike the distributions of phyla. It was seen that due to the anoxic nature of submerged rice soil, there was a prevalence of facultative to obligate anaerobes in the presently studied rice rhizosphere (Figure 4). The submerged condition exhibited in the rice rhizosphere causes hypoxia or anoxia which leads to the enrichment of anaerobic bacterial communities (Liesack et al., 2000). The distribution of the top 20 bacterial taxa (above 0.2% mean relative abundance) among the rice rhizospheres, revealed that bacterial genera belonging to phyla-class *Anerolinea*, *Proteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Verrucomicorbia* and *Firmicutes* (Supplementary Data File S2; Figure 5) were the most dominant genera. Compositionally, unclassified *Anaerolineae* was most predominant phyla in the rhizosphere of all the 10 rice cultivars (2.36 to 4.52%), whereas even distribution of bacterial genus *Anerolinea clone SJA-15* (0.99 to 1.24%), *Anaerolineae_S0208* (0.41–0.75%), *Syntrophobacteraceae* (0.78 to 1.54%), *Sphingomonadaceae* (0.54–1.04%), *Chitinophagaceae* (0.73 to 1.15%), *Flavisolibacter* (0.35–0.62%) and *Pedosphaerales* (0.92 to 1.15%) was observed. The δ-proteobacterial taxa, iron-reducing bacterial genera *Geobacter* was found enriched in the rhizosphere of HUR917 (2.74 ± 0.17%), whereas propionate oxidizing bacterium *Syntrophobacter* was found to be highest in

TABLE 1 Mean relative abundance (%) of top 20 bacterial phylum associated with rhizo-microbiome of the rice varieties.

Phylum	Mean relative abundance (%)									
	BPT5204	CO52	HUR105	HUR917	MTU1001	MTU7029	Rajender Sweta	SHIATS1	TKM13	Warangal3207
*Proteobacteria***	17.37 ± 0.81	19.23 ± 0.92	18.32 ± 0.94	19.4 ± 0.42	22.42 ± 0.94	17.63 ± 0.26	22.4 ± 0.75	17.69 ± 1.09	22.02 ± 0.42	21.47 ± 0.81
*Chloroflexi***	14.21 ± 0.24	9.21 ± 0.41	13.63 ± 0.46	13.52 ± 0.05	11.23 ± 0.53	11.58 ± 0.21	10.77 ± 0.96	12.92 ± 0.95	10.67 ± 0.33	10.71 ± 0.35
*Bacteroidetes***	6.55 ± 0.17	7.52 ± 0.44	6.24 ± 0.18	7.14 ± 0.09	5.16 ± 0.25	6.59 ± 0.32	4.1 ± 0.14	7.22 ± 0.76	7.16 ± 0.24	4.3 ± 0.10
*OD1***	6.43 ± 0.33	6.73 ± 0.21	6.7 ± 0.25	6.72 ± 0.37	4.88 ± 0.29	7.42 ± 0.71	5.73 ± 0.26	7.05 ± 0.44	5.79 ± 0.39	5.16 ± 0.21
*Acidobacteria***	5.01 ± 0.16	3.41 ± 0.06	4.27 ± 0.16	3.95 ± 0.02	3.41 ± 0.16	4.05 ± 0.19	4.02 ± 0.50	3.19 ± 0.18	4.11 ± 0.38	4.54 ± 0.3
*Verrucomicrobia***	3.43 ± 0.05	2.84 ± 0.11	3.17 ± 0.11	3.19 ± 0.1	2.12 ± 0.02	3.51 ± 0.08	2.42 ± 0.16	2.51 ± 0.25	3.48 ± 0.14	2.68 ± 0.25
*Actinobacteria***	2.83 ± 0.19	2.44 ± 0.18	2.25 ± 0.11	2.13 ± 0.15	3.89 ± 0.18	2.39 ± 0.23	3.87 ± 0.08	2.55 ± 0.17	2.68 ± 0.11	3.68 ± 0.24
*Firmicutes***	1.95 ± 0.02	1.78 ± 0.51	2.76 ± 0.26	2.71 ± 0.53	4.69 ± 0.11	1.48 ± 0.23	3.29 ± 0.09	2.43 ± 0.33	1.47 ± 0.09	2.54 ± 0.23
*Cyanobacteria***	0.78 ± 0.04	1.53 ± 0.16	0.73 ± 0.001	0.74 ± 0.06	0.92 ± 0.02	2.07 ± 0.07	1.05 ± 0.13	2.42 ± 0.07	1.1 ± 0.04	0.82 ± 0.04
*Gemmatimonadetes***	1.15 ± 0.09	0.82 ± 0.01	0.94 ± 0.09	0.96 ± 0.05	1.06 ± 0.09	0.97 ± 0.07	1.18 ± 0.05	0.65 ± 0.04	1.06 ± 0.08	1.28 ± 0.03

Statistical significance of the relative abundance of the bacterial phylum was estimated by multiple group ANOVA with Benjamini-Hochberg FDR multiple test correction. The p values <0.05 were considered significant. Values indicates mean ± standard deviation; ** indicates p values<0.05.

TABLE 2 Global network property of rhizo-microbiome of rice varieties.

Topology	HUR917	MTU1001	BPT5204	Warangal3207	TKM13	HUR105	CO52	Rajender Sweta	MTU7029	Co522	TKM133	MTU10014
Nodes	68	68	68	68	68	68	68	68	68	68	68	68
Edges	1,065	979	941	928	912	898	764	734	724	764	912	979
Diameter	3	3	3	3	3	3	3	3	3	3	3	3
Density	0.454	0.417	0.401	0.395	0.388	0.382	0.325	0.313	0.308	0.325	0.388	0.417
Average degree	30.86	28.37	27.28	26.89	26.43	26.02	22.14	21.27	20.98	22.14	26.43	28.37

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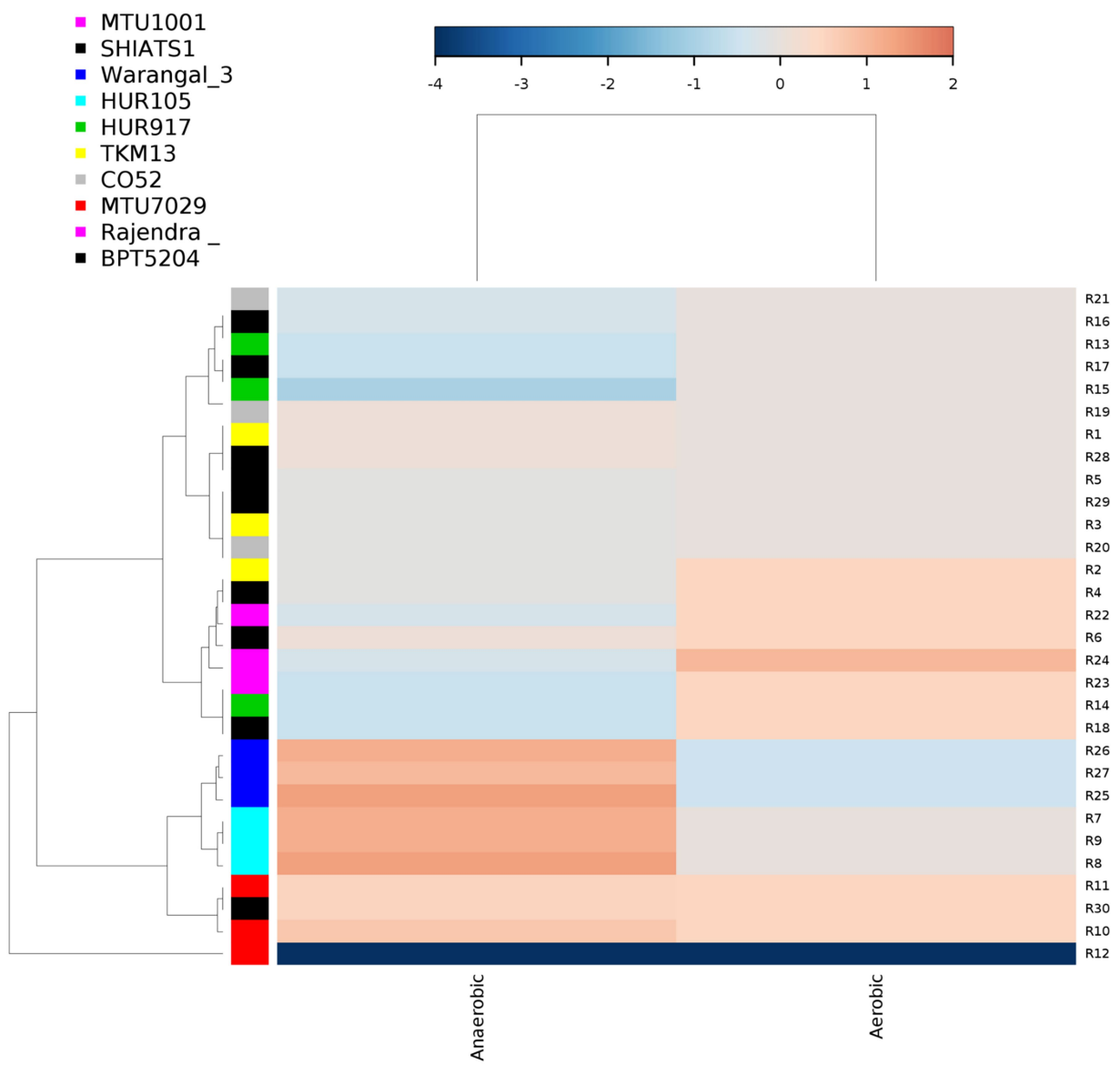
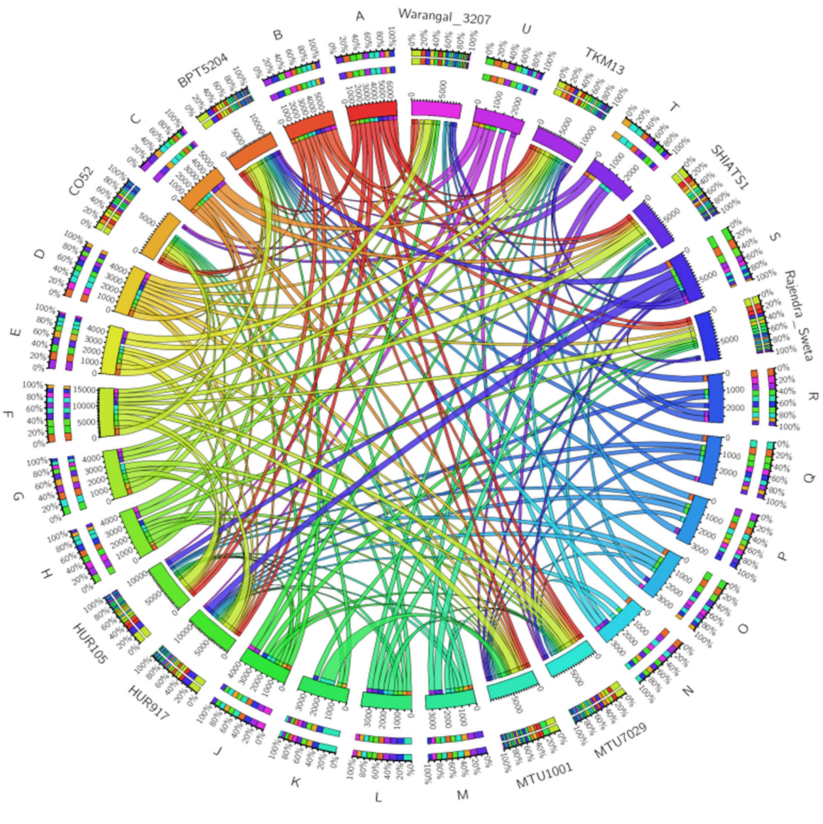


FIGURE 4
Distribution of aerobic and anaerobic bacterial flora in the rhizo-microbiome of rice varieties. For clustering Euclidean distances were estimated, the color gradient toward brown indicates higher abundance whereas lighter shades represent low abundance.

HUR105 (1.02 ± 0.07%). β-proteobacterial taxa, uncultured *Rhodocyclaceae* known for its bioremediation potential, was having highest representation in the rhizosphere of CO52 (0.73 ± 0.02%), whereas uncultured *β-proteobacteria* was most prominent in Rajendra Sweta (0.99 ± 0.1%). Uncultured *γ-proteobacteria* was found to be enriched in SHIATS-1 rhizosphere (0.90 ± 0.08%). *Acidobacterial* taxa *Solibacterales* (0.42–0.73%) and *Acidobateria*-iii1-15 (0.46–0.97%) were well distributed among the rice rhizobiomes. Uncultured *Gemmatomonadetes clone*, *Gemm-5*, known for its abiotic stress ameliorating capabilities was found to be predominant in Warangal 3,207 rhizosphere. *Bacteriodetes* taxa, *Sapropspiracea*, *Cytophagaceae*, and *Flavisolibacter*, were found to be enriched in rhizospheres of TKM13 (1.44 ± 0.02%) and MTU7029 (1.06 ± 0.11%), respectively, whereas uncultured *Bacteroidetes* was found to be enriched in the rhizosphere of CO52 (0.67 ± 0.04%). These bacterial genera are the potential source of a group of enzymes for carbohydrate metabolism. Taxa belonging to *Bacillus* was found to be highest in MTU1001 (1.76 ± 0.06%) rhizospheres. This indicates that the rice cultivars can tailor their rhizosphere microbiome according to their requirement.

A random forest machine learning approach was used to predict key bioindicator taxa among rice rhizospheres. This helped in identifying niche-specific key bacterial genera associated with rhizobiomes of non-aromatic *indica* rice cultivars. The prediction model, utilizing bacterial OTUs predicted with



Labels

Anaerolineae_SJA-15	A
Syntrophobacteraceae	B
Saprospiraceae	C
Sphingomonadaceae	D
Chitinophagaceae	E
Anaerolineae	F
Cytophagaceae	G
Syntrophobacter	H
Acidobacteria-iii1-15	I
Bacillus	J
Uncultured Betaproteobacteria	K
Unculutred Gammaproteobacteria	L
Solibacterales	M
Anaerolineae_S0208	N
Pedosphaerales	O
Flavisolibacter	P
Uncultured Gemmatimonadetes clone-Gemm-5	Q
Geobacter	R
Uncultured Rhodocyclaceae	S
Uncultured Bacteroidales	T

FIGURE 5
Circos plot showing the distribution of top 20 bacterial taxa (relative abundance >0.2%) associated with rhizo-microbiome of rice varieties.

86.6% accuracy. The data for the top 20 key bioindicators have been discussed (Figure 6). Bacterial taxa *Anaerolineae* was predicted as a key bioindicator of rhizospheres associated with rice varieties HUR917, BPT5204, and HUR105. Uncultured bacterial clone *WD2101* was the key feature of HUR917 and BPT5204 rhizospheres. The bacterial genus *Anaerolineae_OTU6* was strongly linked to rhizosphere conditions of HUR917, BPT5204, HUR105, SHIATS1, and MTU7029. *Acidimicrobiales* was having niche specificity for rhizospheres of MTU7029, HUR917, BPT5204, Warangal_3,207, Rajender Sweta, and HUR105. Uncultured bacterial clones BPC076 and *Desulfobacteraceae* were key features of MTU7029 rhizobiomes, whereas *Paracoccus* was a bioindicator for MTU7029 and CO52. Ammonia oxidizing genus *Candidatus Nitrosphaera* was the key feature of rhizospheres of TKM13, CO52, and MTU1001. *Alphaproteobacteria* and *Aeromonas* emerged as the key feature of rice varieties Warangal_3,207, Rajender Sweta and SHIATS1. The uncultured bacterial clone was specifically associated with rhizospheres of Warangal_3,207, Rajender Sweta, and MTU1001. *Pseudoxanthomonas* was the prominent feature of rice rhizospheres of MTU7029, TKM13, and Warangal 3,207. Niche specificity of *Candidatus Entotheonella* and *Methylosinus* was predicted for rhizospheres of TKM13 and CO52. *Desulfobulbaceae* was predicted as a bioindicator for rhizobiomes of TKM13 and SHIATS1. The abundance of *Algoriphagus* and *Clostridiaceae* was marked as an important feature of rhizobiomes of CO52 and SHIATS1. The presence of *OD1* was identified as a key biological indicator of MTU7029, HUR917, CO52, HUR105, and SHIATS1 rhizospheres.

Due to the anoxic nature of rice rhizosphere, most of the niche-specific bacterial taxa were metabolically fermentative to organic acid utilization capability, showing dissimilatory sulphur and nitrogen reduction, hydrogen evolution, chemoorganotrophic to autotrophic bacteria, etc. The abundance of *Chloroflexi* class *Anearolinea*, which thrives on organic acids might be due to the anoxic degradation of polysaccharides. Earlier, the genus was well reported from anaerobic digesters, but its presence in the rice rhizosphere can be no exception due to low redox potential and high organic matter in the niche (Xia et al., 2016; Edwards et al., 2018). Well-distributed populations of dissimilatory sulphate reducing taxa, such as *Desulfobacteraceae*, and *Desulfobulbaceae* were showing niche specificity to rice rhizosphere. This taxon has been found to switch from sulfate to nitrate respiration as an alternative respiratory metabolism under an abundance of nitrate (Wörner et al., 2016; Zecchin et al., 2021). Dissimilatory nitrate reduction to ammonium (DNRA) is a characteristic of genera *Paracocccus* and *Pseudoxanthomonas*, which are reported from soil and groundwater sediments, and rhizosphere of corn, cotton,

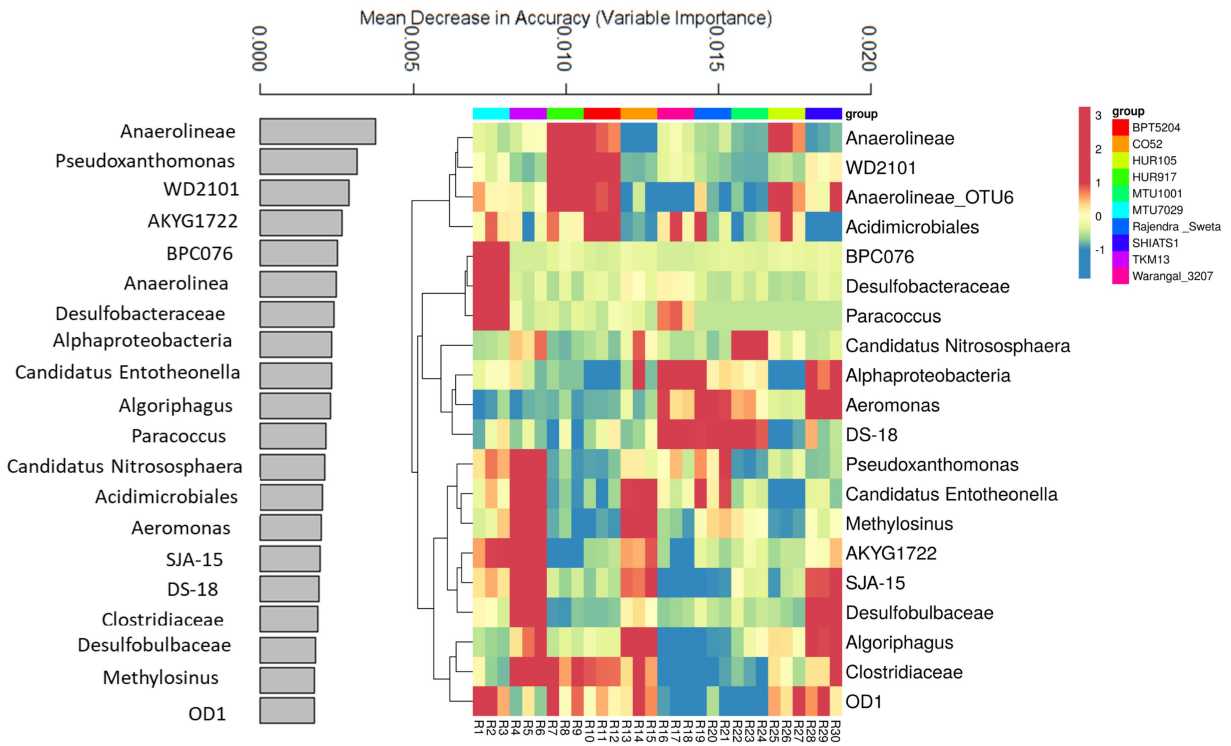


FIGURE 6
Random forest machine learning approach to identify important bioindicator bacterial taxa associated with rhizo-microbiome of rice varieties. The bar plot was drawn for the top 20 important bacterial taxa identified by the random forest machine learning approach, the values indicate mean decrease accuracy, along with heatmap was drawn to show the relative distribution of these bacterial taxa in the rice variety rhizo-microbiome, color gradient toward brown indicates high abundance whereas lighter shades represent low abundance, clustering was done based on Euclidean distances.

soya, and millet (Mohapatra et al., 2018; Pan et al., 2020). The abundance of DNRA activity in the rice rhizosphere is advantageous for transforming loss-prone nitrate ions to soil retainable ammonium ions (Tylova-Munzarova et al., 2005). Apart from DNRA activity, *Pseudoxanthomonas* have been reported to be involved in the reductive transformation of toxic arsenic, thus providing avenues for utilization of the genera for bioremediation of metalloids (Mohapatra et al., 2018). Rice rhizosphere of heavy metal contaminated soil has reported the presence of *Acidimicrobiales* (Huaidong et al., 2017). *Acidimicrobiales*, a photoheterotroph, is generally isolated from the deep photic zone of the marine environment. They assimilate C_2 compounds using the ethyl-malonyl coenzyme A pathway (Chen et al., 2019). The growth condition of the bacterium highly suits the flooded condition of the rice rhizosphere. *Aeromonas* has been reported as the N-acyl homoserine lactone-mediated biofilm-forming bacterium from rice rhizoplane (Balasundararajan and Dananjeyan, 2019). The presence of a biofilm-forming rhizosphere community is involved in quorum sensing mediated colonization and antagonistic responses against the foreign pathogen (Pieterse et al., 2016). The rice rhizospheres were also harboring potential organic matter degraders *Algoriphagus* and *Clostridiaceae*, which have been reported as major players in carbon cycling (Gavande et al., 2021). Type II methanotrophic bacteria *Methylosinus* has been isolated from the rhizosphere of *Oryza sativa Nipponbare*, which not only oxidizes methane but also possesses a nitrogenase complex suggesting its role in maintaining carbon and nitrogen cycling in the root zone of rice (Bao et al., 2014). Environmental bacterial taxon with a large and distinct metabolic repertoire, *Candidatus Entotheonella* was reported from marine sponge *Theonella swinhoe* (Wilson et al., 2014). This is the first report of *Candidatus entotheonella* from the rhizosphere of rice. Metabolically they utilize oxalate and methanol as carbon sources, which are found abundantly in the rice rhizosphere. The taxa is a treasure trove for many important metabolites (Wilson et al., 2014).

Predominant archaebacteria associated with *indica* rice rhizosphere

Archaebacterial OTUs were also widespread among the rice rhizospheres. These OTUs were classified under phylum *Euryarchaeota*, *Parvarchaeota*, and *Crenarchaeota*. Univariate analysis was performed to predict differentially abundant archaebacterial taxa of the rice rhizospheres. Six archaebacterial genera *viz.*, *Candidatus Methanoregula* ($p = 0.001$), *Methanosaeta* ($p = 0.002$), *Methanocella* ($p = 0.003$), *Methanomassiliicoccus*

($p = 0.004$), *Methanobacterium* ($p = 0.005$) and *Methanomicrobiales* ($p = 0.01$) were found to be differentially abundant among the rice rhizospheres under study (Figure 7). All the differentially abundant taxa were found to be belonging to *Euryarchaeota*, capable of hydrogenotrophic methanogenesis (Berghuis et al., 2019).

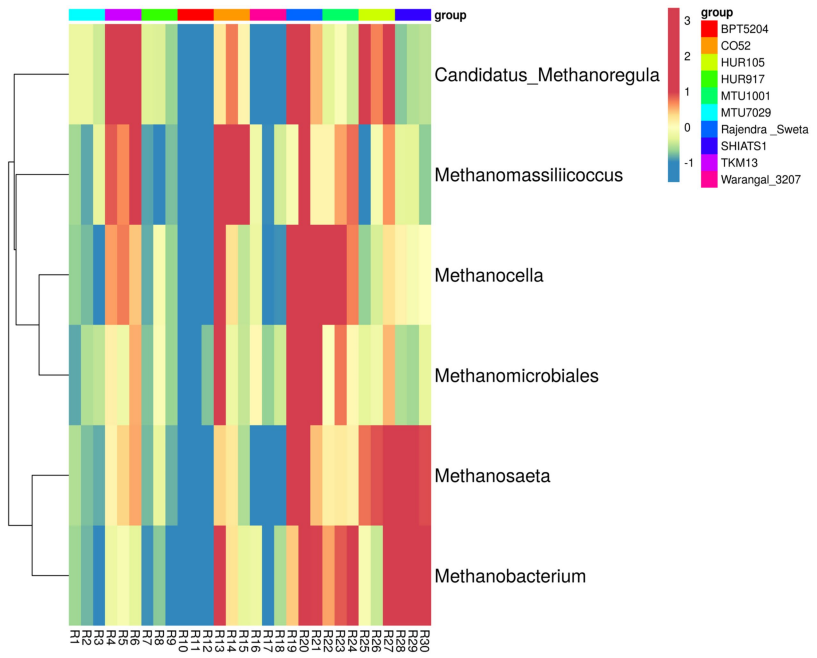
Among the anthropogenic sources of methane emission, paddy cultivation accounts for 25 to 100 teragrams of CH_4 per year (Conrad, 2020). Liechty et al. (2020) have characterized rice cultivars based on the rate of methane emission. Through deep sequencing approaches, they found that syntropic methanogenesis was more prevalent in high emission rice cultivars as compared to low emission cultivars. A high abundance of hydrogenotrophic methanogen throughout the growth period of rice indicates its niche specificity for rice roots (Imchen et al., 2019). Ammonia oxidizing *Crenarchaeota* genera *Candidatus Nitrosphaera* has also been reported as the predominant ammonia-oxidizing archaea from the rice roots (Imchen et al., 2019).

Unveiling the microbiome networks of rice rhizosphere

Root microbiome assemblages play a key role in the sustenance of plant health and productivity. Community metagenomics is the only tool that can be able to decipher how the individual taxa within the microbiome are distributed in the host plant. However, the underlying mechanism for the interaction of microbe-microbe at the trophic level is not revealed. Hence, co-abundance network analysis was performed by taking into account a 100 bioindicator bacterial OTUs predicted by a random forest machine learning algorithm with 24 archaebacterial OTUs. We have generated 10 different co-occurrence microbiome network scenarios specific to microbiomes of rice varieties under study (Supplementary Figure S1; Supplementary Table S2). Global network properties such, as the average number of edges per node (average degree) were calculated to reveal network topology. To define the functionality/ecological role of the microbiome networks we have also predicted the keystone species based on the betweenness centrality values of the nodes.

Scenario 1: Syntropic nitrogen and carbon cycling

Co-abundance network analysis for rhizobiome of HUR917 yielded 1,065 significant correlations (Supplementary Figures S1A, S2; Supplementary Table S3). The network's keystone species was *Crenarchaeota* (ArcOTU_2), a chemoautotrophic ammonia oxidizer (Martin-Cuadrado et al., 2008). It was found that keystone species have shown a positive significant correlation with complex organic matter degrading bacterial taxa *Clostridium* (OTU_60) and *Saprospiraceae* (OTU_10), along with nitrogen-fixing taxa *Syntrophobacteraceae* (OTU_55), *Rhodospirillales* (OTU_49) (McIlroy and Nielsen, 2014; Liu and Conrad, 2017). A strong positive correlation of ammonia



Univariate Test Analysis

Genus	Pvalues	FDR
Candidatus_Methanoregula	0.0014036	0.018976
Methanosaeta	0.0015813	0.018976
Methanocella	0.0029807	0.021048
Methanomassiliicoccus	0.003508	0.021048
Methanobacterium	0.0047397	0.022751
Methanomicrobiales	0.0095641	0.038257

FIGURE 7
Univariate test analysis to identify important archaebacterial taxa associated with rhizo-microbiome of rice varieties, the heat map is drawn taking their relative abundance and clustering was done based on Euclidean distances, color gradient toward brown indicates high abundance, whereas lighter shades represent low abundance.

oxidizers with nitrogen-fixing taxa has been reported in ecosystems of pastures and crops in Amazonia, Brazil (Lammel et al., 2015).

Scenario 2: Multispecies synergistic biofilms conferring biotic stress amelioration

The Microbiome network scenario of MTU1001 had 979 significant correlations (Supplementary Figures S1B, S3; Supplementary Table S4). Rhizobiome of MTU1001 has *Aeromonas* (OTU_87) as the keystone species. Strains of *Aeromonas* have been reported to form multispecies synergistic biofilms through the type 1 autoinducer quorum sensing mechanism (Li et al., 2008). There is a strong possibility that positive interactions with *Bacteroidetes* (OTU_50) and *Rhizobiales* (OTU_37) might be involved in forming biofilms and regulation of chitinase production (Ochiai et al., 2013). Thus, it can be concluded that the bio-filmed microbiome network of MTU1001 might be playing a role in host defense against biotic stresses.

Scenario 3: Diel cycling to synchronize with host circadian rhythm

There were 941 significant correlations in the microbiome network of BPT5204 (Supplementary Figures S1C, S4; Supplementary Table S5). Keystone species was predicted as *Gaiellaceae* (OTU_124). Genomic studies revealed that members of the *Gaiellaceae* family have Calvin–Benson–Bassham (CBB) cycle with a type I RuBisCO and show diel cycling (Severino et al., 2019). Keystone OTU was establishing positive interactions with other anoxic chemoorganotrophic bacterial taxa *Rhodospirillales* (OTU_49) and *Anaerolineae* (OTU_6). These interactions indicate that carbon metabolism in this microbiome was regulated preferably by chemoautotrophs showing diel cycling (Alcamán-Arias et al., 2018). Further, diel cycling also has an added advantage for the rhizospheric community to synchronize their abundances as per the circadian rhythm of the host plant (Staley et al., 2017).

Scenario 4: Syntrophic methanogenesis

The Microbiome network of rice variety Warangal 3,207 had 928 significant correlations (Supplementary Figures S1D, S5; Supplementary Table S6). *Sinobacteraceae*, (OTU_121) was discovered as keystone species of the network. A significant positive correlation was found with *Candidatus* genera JG30-KF-CM45 (OTU_25) of *Chloroflexi* and *Luteolibacter* (OTU_84). Metabolically, the consortia might be involved in the cycling of nitrogen in the rhizosphere as *Sinobacteraceae* carry out ammonia oxidation (Bäumgen et al., 2021), whereas *Candidatus* genera JG30- KF-CM45 possess DNRA activity and the simple sugars are being provided through polysaccharide degradation by *Luteolibacter*. The consortia were showing competitive interaction with *Methanosaeta* (ArcOTU_12). Energetically, the requisite amount of hydrogen and organic acid gets consumed in supporting DNRA activity and ammonia oxidation, thus slowing down the process of methanogenesis.

Scenario 5: Syntrophic methanogenesis

Rhizobiome network analysis of TKM13 yielded 912 significant correlations (Supplementary Figures S1E, S6; Supplementary Table S7). Polysaccharide degrader *Luteolibacter* (OTU_86) was found as keystone species (Cardman et al., 2014). Syntrophic association was observed with fermentative polysaccharide degrader *Clostridiaceae* (OTU_21), biofilm-forming genera *Aeromonas* (OTU_87), forming an organic matter degrading guild. The organic matter degrading guild was also positively correlated with hydrogenotrophic methanogens like *Methanosaeta* (ArcOTU_12), *Methanobacterium* (ArcOTU_6), etc. Anoxic degradation of organic matter results in the production of organic acids such as acetates, lactates, and formic acids. These organic acids are further utilized by hydrogenotrophic methanogens as substrates for methane formation (Shiratori et al., 2006).

Scenario 6: Syntrophic methanogenesis

The microbial network of HUR105 yielded 898 significant correlations (Supplementary Figures S1F, S7; Supplementary Table S8). Cellulose degrading taxa *Algoripahagus* (OTU_95) was identified as the keystone species (Deng and Wang, 2017). Propionate oxidizing bacterial family *Syntrophobacteraceae* (OTU_55) and *Methanocella* (ArcOTU_7) have shown a syntrophic association with *Algoripahagus*. During this interaction, organic acids like propionic acid produced during the degradation of organic matter is utilized by *Syntrophobacteraceae* resulting in the generation of acetate and hydrogen (Liu and Conrad, 2017). These compounds are further utilized by *Methanocella* symbiotically to produce methane (Sakai et al., 2014).

Scenario 7: Syntrophic methanogenesis

Co-abundance microbial network of CO52 yielded 764 significant correlations (Supplementary Figures S1G, S8; Supplementary Table S9). Keystone species was predicted as *Gaiellaceae* (OTU_124). As described above members of the *Gaiellaceae* family have Calvin–Benson–Bassham (CBB) cycle with a type I RuBisCO and show diel cycling. Although the ecological significance of the taxa is unclear (M. et al., 2021). Strong positive correlation with chemoorganotrophic, *Anerolinea*

(OUT_6, OTU_9), chemolithotrophic *Geobacter* (OTU_57), facultatively autotrophic hydrogen-oxidizing bacterium *Hydrogenophaga* (OTU_28) and acetogenic methanogen *Methanocella* (ArcOTU_7) was suggestive of carbon cycling guilds (Li et al., 2015; Liechty et al., 2020). The direct positive correlations of the bacterial OTUs with methanogen are suggestive of up-regulation of methanogenesis (Liechty et al., 2020).

Scenario 8: Syntrophic methanogenesis

Microbial network analysis of rhizobiome of Rajender Sweta had 734 significant correlations (Supplementary Figures S1H, S9; Supplementary Table S10). The keystone species of the network was found as *Aeromonas* (OTU_87). Syntrophic association was seen with *Acidobacterial candidatus* family *Ellin 6,075* (OTU_58), *Anaerolinea* (OTU_6), *Candidatus Entotheonella* (OTU_185), *Bacteroidetes* (OTU_50), and *Methanosaeta* (ArcOTU_12). This particular network was syntrophically forming complex organic matter degradation guilds for the production of acetate and hydrogen and thus supplementing the substrate demand for methane formation (Wrighton et al., 2014; Eichorst et al., 2018). Further, the carbon cycling also favors ammonia oxidation due to the prevalence of *Candidatus Nitrososphaera* (ArcOTU_3) positively correlated node (Sauder et al., 2017).

Scenario 9: Case of anaerobic methane oxidation

The microbial network architecture of MTU7029 revealed 724 significant correlations (Supplementary Figures S1I, S10; Supplementary Table S11). Hydrogenotrophic methanogen, *Methanomicrobiales* (ArcOTU_8) was found as the keystone species. Two Anaerobic polysaccharides degrading fermentative *Chloroflexi* OTU's (OTU_154 and OUT_101), denitrifying α-proteobacteria (OTU_37) and propionate degrading β-proteobacteria (OTU_16) were forming a syntropic association with keystone species. Degradation of polysaccharides and propionic acids yielded substrate and electrons for sustaining methanogenesis. On the contrary, we found that the guild was performing the denitrification process which was earlier known to suppress methane formation, but it was indicated that in the presence of nitrate, the process of methanogenesis process gets reversed to anaerobic methane oxidation (Bulseco et al., 2019). Hence, instead of methanogen abundance, methane flux is getting reduced in this microbiome interaction scenario.

Scenario 10: Syntrophic methanogenesis

The Microbiome network of SHIATS1 had 692 network correlations (Supplementary Figures S1J, S11; Supplementary Table S12). *Candidatus* taxa OPB54 (OTU_168) which co-produces ethanol-hydrogen was the keystone species (Liu et al., 2016). Significant positive correlations were seen with other fermentative bacterial-archeal taxa such as *Geobacteraceae* (OTU_57), *Anaerolinea* (OTU_6), *Parvarchaea* (ArcOTU_23) along with hydrogenotrophic methanogen *Methanobacetrium* (ArcOTU_6) (Hug et al., 2013; Castelle and Banfield, 2018; Zhang et al., 2020). These archaeal bacterial consortia were acting as organic carbon cycling guilds, and organic acids and direct interspecies electron transfer were also being mediated to facilitate methanogenesis.

To conclusively represent various microbiome network scenarios and their ecological significance in the rice rhizosphere, taxonomic to phenotype mapping was performed to infer ecological functions. The mapping revealed 12 ecological functions *viz*., streptomycin production, xylan degradation, degradation of aromatic hydrocarbon, sulfur oxidation, sulphite oxidizer, sulphate reduction, ammonia oxidation, dehalogenation, nitrogen fixation, nitrate reducer, methanogenesis, and chitin degradation. The correlations were drawn between the functions and it was observed that strong positive correlations can be observed in two clusters *viz*., xylan degrader vs. dehalogenation vs. ammonia oxidizer vs. sulphide oxidizer vs. nitrite reducer (Cluster 1) and chitin degrader vs. sulphate reducer and nitrogen fixation (cluster 2) (Figure 8). These functional interactions were well supported by our results on microbiome networks that play an important role in carbon, sulfur, and nitrogen cycling. Methanogens were found to show weak positive correlations with sulfate and nitrate reducers, which was in line with the fact that the addition of nitrate and sulfate to the environment reverses the process of methanogenesis and supports anaerobic methane oxidation.

Prediction of important gene families associated with rice rhizobiome conferring plant growth promotional traits

PICRUSt2 was used to infer gene families associated with important metabolic pathways associated with rice rhizobiomes (Douglas et al., 2020). The tool enables accurate prediction of genomes based on the ASV's data generated during bacterial profiling of the samples. PICRUSt2 works by placing the 16S rRNA gene sequences of the samples in a reference tree of 20,000 full length 16S rRNA gene sequences. Based on the phylogenetic placements of the ASV's genome predictions are performed and metagenome is reconstructed.The pipeline predicted more than 7,000 KEGG orthologous (KO) associated with the rhizobiomes. As the rice rhizobiomes were harboring their bacterial community structure, it was also observed that significant differences in metabolic functions were observed among the rhizobiomes (Figure 9). The differential abundance of various gene families was inferred by performing Lefse analysis, and 50 differentially abundant KOs enriched in rhizobiomes were predicted (Supplementary Figures S12A,B).

The rhizosphere has been designated as the hub for many metabolite exchange networks and signaling channels with soil bacterial communities (Hermans et al., 2017; Sasse et al., 2018). Compositionally root exudates contain many metabolites like flavonoids, strigolactone, organic acids, ions, sugars, phenols, etc., many of them serve as an energy source and many as chemotactic signals to establish rhizobiome. Under the influence of these metabolite, selective overexpression of prokaryotic genes conferring nutrient acquisition, biological nitrogen fixation, environmental stress tolerance, colonization, rhizosphere competence and biotransformation of organic matter has been observed during the cross-talks (Matilla et al., 2007; Dong et al., 2019). The inferred functional predictions of rice rhizobiome suggest the abundance of many KO's involved in rhizosphere competence of bacterial community. Differential abundance of ABC-transporter (K02004, K02003) suggests an increase in activity of uptake of organic acids, amino acids, sugars, phosphorus mobilization, and exchange of sulfur-containing molecules in root zones (Zerbs et al., 2017; Lidbury et al., 2019). Thus, there is a preferential increase in the colonization of bacterial communities having a preference for the root exudate metabolites. Since the rhizodeposits of paddy are carbonaceous and about 1.61–5.24 t ha^{-1} gets released in paddy fields (Wang et al., 2017). Correlating with the nature of rhizodeposits, the majority of the functional orthologous were annotated to carbon cycling and sequestration groups. Li et al. (2018) have found that compositional variations of the dissolved organic carbon in the paddy fields are the major drivers of carbon cycling pathways. As per our results and a previous study performed by Li et al. (2018) on the paddy field regarding methane metabolic pathway

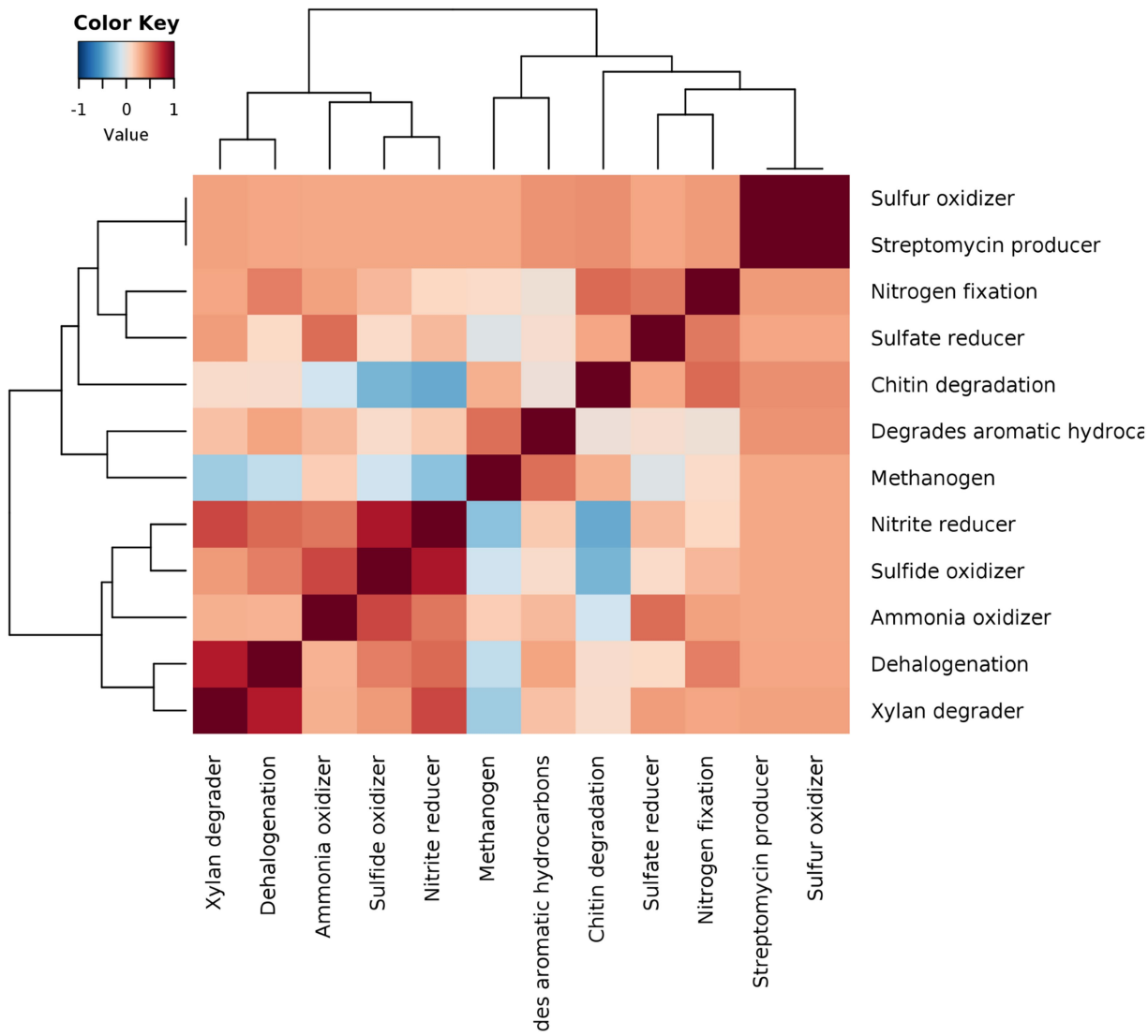


FIGURE 8
Pearson correlation based heatmap showing the distribution of important soil ecological functions associated with rhizo-microbiome of rice varieties, color gradient toward brown represents high positive correlation and toward blue represents negative correlation.

(K05299) it was found that they were non- acetoclastic and were *via* CO_2. Further, it was also observed that an alternate source of electron donor other than H_2 for methanogenesis was observed for instance presence of aldehyde: ferredoxin oxidoreductases (Aor, K03738) which catalyze the conversion of aldehyde to carboxylic acid and thereby supplying reduced ferredoxin (Beulig et al., 2019). Paddy ecosystem was also acting as an active carbon sequestering zone as many of the autotrophic carbon fixation pathways KOs was found abundant, such as 2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase subunit alpha (K00174), 2-oxoglutarate ferredoxin oxidoreductase subunit gamma (reductive citric acid cycle, K00177) and transketolase (Calvin Benson Cycle, K00615). The abundance of these KO's has been reported in *Gaiella* and *Desulfobacter* (Hügler et al., 2010; Severino et al., 2019), these genera were reported as keystone species and bioindicators in the present study. Methanotrophy has been found as one of the features of the paddy ecosystem (Van Bodegom et al., 2001). Particulate methane monooxygenase is the key enzyme involved in methane oxidation, the electron transfer in the whole process is mediated by NADH: Quinone Oxidoreductase, the abundance of KO's NADH-Quinone oxidoreductase subunit N (K00343), NADH-Quinone oxidoreductase subunit M (K00342) and NADPH: quinone reductase (K00344) suggest their role in the regulation of methane monooxygenase (Cook and Shiemke, 2002). A KO involved in alkane biosynthesis was also discovered, the KO (K00257) was annotated to acyl-ACP dehydrogenase. This enzyme in combination with aldehyde decarboxylase converts intermediates of fatty acid to alkanes and alkenes, the pathway is prevalent in *Synechococcus* (Schirmer et al., 2010). The discovery of the alkane biosynthesis pathway in the rice rhizosphere presents an interesting avenue for researchable steps in the direction of biofuel production.

Looking for the distribution of nitrogen regulators, it was found that *NtrC* a phospho-relay sensor kinase (K02428), was prevalent in the rice rhizosphere. Biochemically it catalyzes the phosphorylation of histidine residue in response to changes in the environment. It has been reported that in *Rhodospirillum rubrum*, phosphorylated NtrC regulated the posttranslational regulation of nitrogenase activity (Zhang et al., 2005). Dissimilatory sulphate reduction has been the most important process of microbial respiration in reducing the environment. The membrane complex QrcABCD has been widespread in the sulphate-reducing bacteria

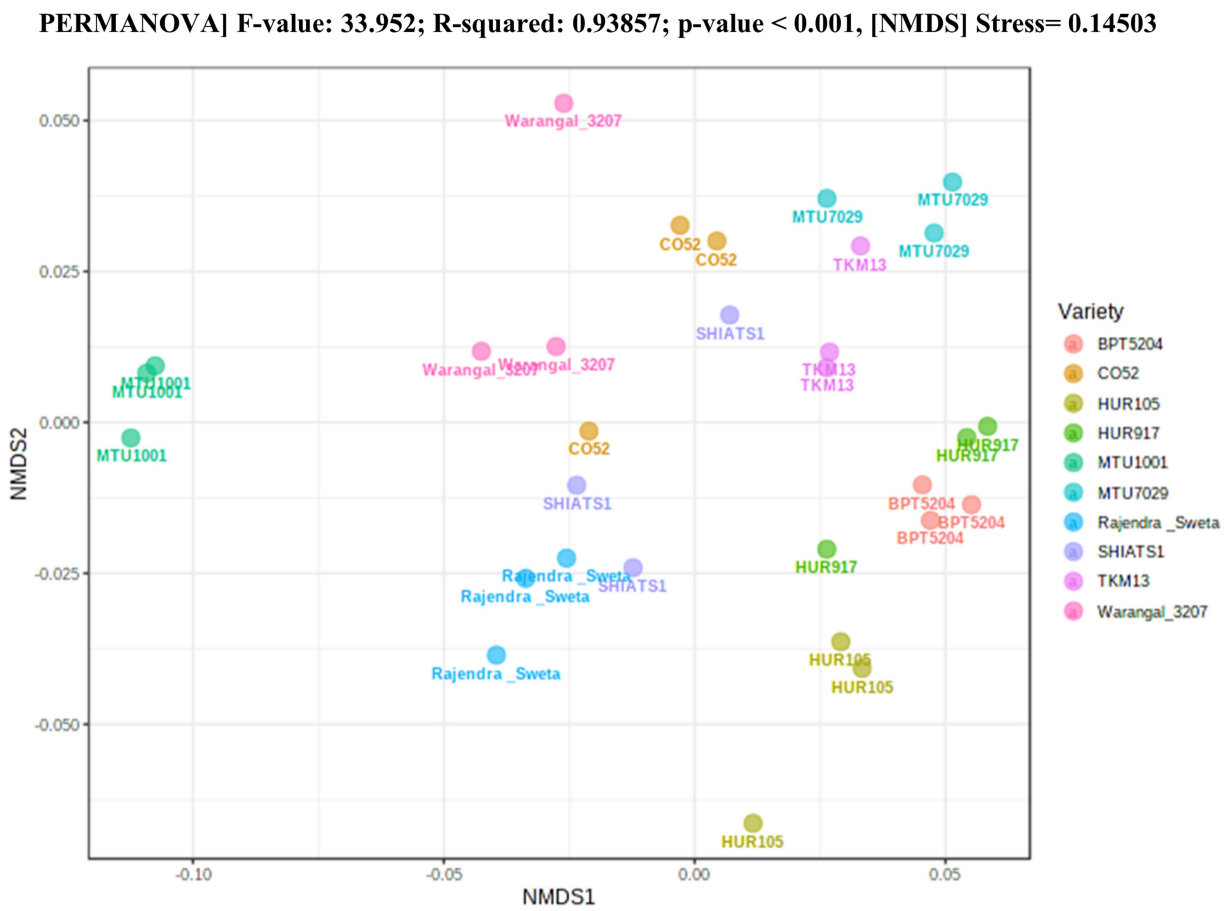


FIGURE 9
Principal coordinate analysis to reveal statistically significant differences in the association of various KEGG orthologues-based functions associated with rhizo-microbiome of rice varieties. To perform the clustering PERMANOVA based on NMDS was used.

Desulfovibrio and is involved in energy conservation during sulfate respiration (Venceslau et al., 2010; Zecchin et al., 2018). The QrcABCD complex is involved with the electron transfer subunit consisting of the DMSO reduction module consisting of dmsB (K0184) and dmsC (K00185) for sulphate reduction, which are found in the present study (Duarte et al., 2018). Sulphate permease (SulP, K03321), acts as a sulphate transporter during the dissimilatory sulphate reduction (Aguilar-Barajas et al., 2011). The overexpression of SulP was observed when there was a drop in sulphate concentration (Kharwar et al., 2021).

Biogeochemical cycling of metalloids, especially arsenic (As) has been gaining importance in the context of paddy cultivation. ArsR family transcriptional regulator (K03892), is a part of the arsenic sensing module. The ArsR represses arsenical resistance operon in the absence of As (III) (Slyemi and Bonnefoy, 2012). Complete phosphate uptake regulon comprising of two-component system response regulator of phosphate assimilation, PhoP (K07658) and phosphate transport system substrate-binding protein, PstS (K02040) was detected in the rhizosphere. Under phosphate limitation, PhoP binds with the promotor region and expresses the synthesis of PhoA Alkaline phosphatase which mineralizes the phosphate, and the inorganic phosphorous binds with PstS which is transported in the cell (Apel et al., 2007; Chhabra et al., 2013). Complete phosphate uptake regulon suggests its role in maintaining adequate phosphate levels in the rice rhizosphere.

Much environmental stress tolerance KO's responsible for imparting tolerance against osmotic and heat stress were also predicted in the paddy rhizosphere. For instance, a two-component system atoC regulator for c Poly-Hydroxybutyrate biosynthesis (cPHB) (K07714) was detected. PHB biosynthesis imparts protection of non-halophilic bacteria under a hyper saline environment (Obruca et al., 2017). The presence of chaperone protein families such as HSP20 family protein (K13993), *DnaK* (K04043), and chaperones and folding catalysts (K03769) confers the role of rhizobiome in imparting heat tolerance to the host plant (Xue et al., 2019).

Regarding biotic stress, KO involved in biofilm formation was predicted. The Phenyl acetate CoA ligases (K01912) are involved in polysaccharide synthesis as a part of the biofilm formation pathway in the rice rhizosphere. The formation of biofilm in the rice rhizosphere is suggestive of its role in inducing antagonism against invasive microbiota thus conferring induction of resistance to the host plant. This KO has also been detected as part of the *nfs* cluster of *Pseudomonas* sp. P482 which confers biocontrol activity against plant pathogens (Krzyżanowska et al., 2021).

Chemotaxis is an important rhizobacterial competence characteristic, which helps the bacterial community to recognize the host and establish itself. Among the chemotactic responsive KO's OmpR family protein (K02483) and flagellin protein (K01426) were detected. The nitrogen-fixing plant symbiont *Sinorhizobium meliloti* chemotaxis and motility are regulated by OmpR-like product Rem which exerts control over flagellin and chemotaxis genes (Rotter et al., 2006). Lrp/AsnC family transcriptional regulator (K03719) is a putative leucine-responsive regulator of the ACC deaminase gene (*acdS*) (Checcucci et al., 2017). The *acdS* gene has been an important feature of the rhizosphere bacterial genome, as the gene product 1-aminocyclopropane-1-carboxylate (ACC) deaminase cleaves plant-derived ACC, which is the precursor of the plant stress-hormone ethylene, thus delaying the stress-induced senescence in the host plant (Glick et al., 2007).

Conclusion

The outcome of the work presented thereupon revealed that the rice root microbiome is greatly influenced by the cultivar types. Results obtained through high throughput *in silico* approaches based on machine learning, microbiome network predictions and metagenomic reconstruction has helped in predicting many new important bacterial taxa co-existing with each other in the rhizosphere zone of the rice plants. Their interplay was also regulating the C-N-P-S cycling, methane emission, and amelioration of abiotic and biotic stresses on the host genotype. The studies also revealed several keystone genera regulating the functional guilds of root microbiome but were still non-culturable, and to utilize them, novel approaches should be devised for *in vitro* culturing. The new technique of culturomics has been widely used for the discovery of new bacterial taxa present in the human microbiome (Diakite et al., 2020), still, its application has not been widely explored in plant research. The amalgamation of the present rice root microbiome results with culturomics protocol will open up the avenue for *in vivo* studies leading to the devising of a novel strategy for the development of SynCom-based microbiome technologies for sustainable rice 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 in the article/supplementary material.

Ethics statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Author contributions

ASa and ASr conceptualized and designed the experiments. SK, MZ, and BS conducted the lab work under the supervision of MK and HC. AS, MK, and HC analyzed the data and wrote the manuscript. KP, KR, and AM improved the manuscript draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

The reviewer JC declared a shared affiliation with the authors AS, MK, HC, KP, SK, MZ, BS, KR, ASr, and ASa at the time of review.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1033158/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Random forest machine learning coupled rhizo-microbiome co-occurrence network analysis of ten rice varieties, the size of the nodes is arranged based on their betweenness centrality values, nodes having the highest radius are depicted as keystone taxa; HUR 917 **(A)**, MTU1001 **(B)**, BPT5204 **(C)**, Warangal 3207 **(D)**, TKM13 **(E)**, HUR 105 **(F)**, CO52 **(G)**, Rajender Sweta **(H)**, MTU 7029 **(I)** and SHIATS 1 **(H)**.

SUPPLEMENTARY FIGURE 2
Correlogram depicting significant microbiome associations ($p < 0.05$) of bacterial taxa representing rhizo-microbiome of rice variety HUR 917, $p < 0.05$.

SUPPLEMENTARY FIGURE 3
Correlogram depicting significant microbiome associations of bacterial taxa representing rhizo-microbiome of rice variety MTU1001.

SUPPLEMENTARY FIGURE 4
Correlogram depicting significant microbiome associations of bacterial taxa representing rhizo-microbiome of rice variety BPT5204.

SUPPLEMENTARY FIGURE 5
Correlogram depicting significant microbiome associations of bacterial taxa representing rhizo-microbiome of rice variety Warangal 3207.

SUPPLEMENTARY FIGURE 6
Correlogram depicting significant microbiome associations of bacterial taxa representing rhizo-microbiome of rice variety TKM13.

SUPPLEMENTARY FIGURE 7
Correlogram depicting significant microbiome associations of bacterial taxa representing rhizo-microbiome of rice variety HUR105.

SUPPLEMENTARY FIGURE 8
Correlogram depicting significant microbiome associations of bacterial taxa representing rhizo-microbiome of rice variety CO52.

SUPPLEMENTARY FIGURE 9
Correlogram depicting significant microbiome associations of bacterial taxa representing rhizo-microbiome of rice variety Rajender Sweta.

SUPPLEMENTARY FIGURE 10
Correlogram depicting significant microbiome associations of bacterial taxa representing rhizo-microbiome of rice variety MTU 7029.

SUPPLEMENTARY FIGURE 11
Correlogram depicting significant microbiome associations of bacterial taxa representing rhizo-microbiome of rice variety SHIATS 1.

SUPPLEMENTARY FIGURE 12
Differential abundance of top fifty gene families associated with rhizo-microbiome of rice variety. As revealed by Lefse analysis **(A)** and Heatmap showing the distribution of the top fifty gene families associated with the rhizo-microbiome of rice, the color gradient towards orange and green represents high and low abundance as indicated in the scale bar.

SUPPLEMENTARY DATA FILE 1
XLSX table containing a tab with the full list of the taxa with taxonomy, prevalence, and relative abundance.

SUPPLEMENTARY DATA FILE 2
XLSX table containing a tab with the full list of the genus and mean relative abundance.

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Paola Grenni,
National Research Council, Italy

REVIEWED BY
Rakesh Kumar Sharma,
Manipal University Jaipur, India
Savitha Santosh,
Central Research Institute for Dryland Agriculture (ICAR), India

*CORRESPONDENCE
Marina Robas Mora
marina.robasmora@ceu.es
Pedro A. Jiménez Gómez
pedro.jimenezgomez@ceu.es

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Oxidative stress protection and growth promotion activity of *Pseudomonas mercuritolerans* sp. nov., in forage plants under mercury abiotic stress conditions

Marina Robas Mora*, Vanesa M. Fernández Pastrana, Daniel González Reguero, Laura L. Gutiérrez Oliva, Agustín Probanza Lobo and Pedro A. Jiménez Gómez*

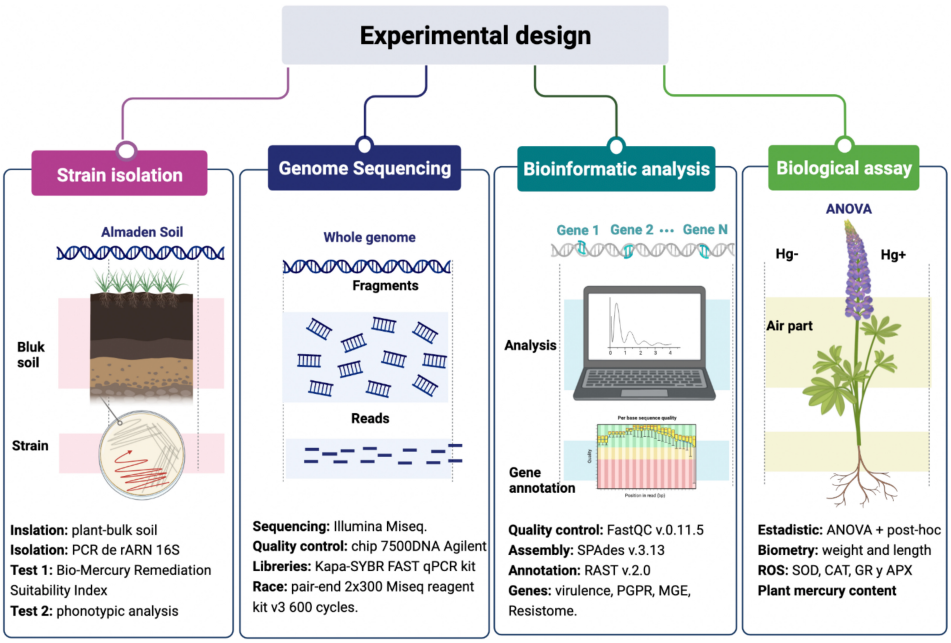
Department of Health and Pharmaceutical Sciences, School of Pharmacy, Universidad San Pablo-CEU, CEU Universities, Madrid, Spain

SAICEUPSM^T strain was isolated from soils in the mining district of Almadén (Ciudad Real, Spain), subjected to a high concentration of mercury. Using the plant model of lupinus, the strain was inoculated into the rhizosphere of the plant in a soil characterized by a high concentration of mercury (1,710 ppm) from an abandoned dump in the mining district of Almadén (Ciudad Real, Spain). As a control, a soil with a minimum natural concentration of mercury, from a surrounding area, was used. Under greenhouse conditions, the effect that the inoculum of the SAICEUPSM^T strain had on the antioxidant capacity of the plant was studied, through the quantification of the enzymatic activity catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and glutathione reductase (GR). Likewise, the capacity of the plant to bioaccumulate mercury in the presence of the inoculum was studied, as well as the effect on the biometric parameters total weight (g), shoot weight (g), root weight (g), shoot length (cm), root length (cm), total number of leaves (N), and total number of secondary roots (No). Finally, in view of the results, the SAICEUPSM^T strain was identified from the phenotypic and genotypic point of view (housekeeping genes and complete genome sequencing). The inoculum with the SAICEUPSM^T strain in the presence of mercury produced a significant reduction in the enzymatic response to oxidative stress (CAT, APX, and SOD). It can be considered that the strain exerts a phytoprotective effect on the plant. This led to a significant increase in the biometric parameters total plant weight, root weight and the number of leaves under mercury stress, compared to the control without abiotic stress. When analyzing the mercury content of the plant with and without bacterial inoculum, it was found that the incorporation of the SAICEUPSM^T strain significantly reduced the uptake of mercury by the plant, while favoring its development in terms of biomass. Given the positive impact of the SAICEUPSM^T strain on the integral development of the plant,

it was identified, proving to be a Gram negative bacillus, in vitro producer of siderophores, auxins and molecules that inhibit stress precursors. The most represented fatty acids were C16:0 (33.29%), characteristic aggregate 3 (22.80%) comprising C16:1 ω7c and C16: 1ω6c, characteristic aggregate 8 (13.66%) comprising C18:1 ω7c, and C18: 1 cycle ω6c and C 17:0 (11.42%). From the genotypic point of view, the initial identification of the strain based on the *16S rRNA* gene sequence classified it as *Pseudomonas iranensis*. However, genome-wide analysis showed that average nucleotide identity (ANI, 95.47%), DNA-DNA *in silico* hybridization (dDDH, 61.9%), average amino acid identity (AAI, 97.13%), TETRA (0.99%) and intergenic distance (0.04) values were below the established thresholds for differentiation. The results of the genomic analysis together with the differences in the phenotypic characteristics and the phylogenetic and chemotaxonomic analysis support the proposal of the SAICEUPSM^{T} strain as the type strain of a new species for which the name *Pseudomonas mercuritolerans* sp. is proposed. No virulence genes or transmissible resistance mechanisms have been identified, which reveals its safety for agronomic uses, under mercury stress conditions.

KEYWORDS

heavy metals, PGPB, oxidative stress protection, *Pseudomonas mercuritolerans*, phytoprotection against mercury, mercury contamination



GRAPHICAL ABSTRACT

Introduction

Up to 9,000 tons of mercury are released annually into the atmosphere, water, and soils (Zhang et al., 2021). Mercury is a global pollutant that causes damage to the environment and can affect animals and people by its transmission through the food chain. Therefore, it has the potential to become a public health and environmental threat (Ballabio et al., 2021). For this reason, there is a growing scientific, technical, and social interest in reducing mercury pollution and mitigating

its effects. Bioremediation techniques have proven to be an effective and environmentally friendly alternative (Bhatt et al., 2021) as well as useful for the recovery of high land extensions (Dary et al., 2010). *In situ* metal phytostabilization is a phytoremediation technique that uses metal-tolerant plants for the mechanical stabilization of the contaminant, preventing its transport to other environments by leaching or air transport. In addition, it reduces the accumulation of pollutants in biological systems, such as plants. Mercury is a heavy metal that has no biological role in plants or animals. For this reason, it tends to bioaccumulate, replacing other metabolically active metals in the body and triggering numerous diseases (Ballabio et al., 2021). Phytostabilization therefore sequesters contaminants in the soil environment in a more cost-effective way, especially in the case of extensive contamination. For this purpose, the use of fast-growing forage plants, such as the *Lupinus* genus, may be useful.

Phytostabilization process can be improved with the use of microorganisms with the ability to degrade contaminants and/or with the ability to promote plant growth (PGPB, plant growth promoting bacteria) under stress conditions (Kuiper et al., 2004; Azaroual et al., 2022). Traditionally, PGPB were used primarily to help plants absorb nutrients from the environment or to prevent plant diseases, in both cases by promoting their development.

Despite the natural potential of plants to remove heavy metals from the soil, phytoremediation is yet to become a commercially available technology. For this reason, it seems interesting to investigate beneficial plant-microorganism associations that improve the efficiency of the phytoremediation process. The *Pseudomonas* genus is varied and has great versatility. Many of its species can colonize different niches, due to their metabolic capacity and their ease of adaptation to different conditions (Bravakos et al., 2021). The association with plants is enhanced by the secretion of phytohormones (auxins, gibberellins, etc.), secondary metabolites (flavonoids) and enzymes (aminocyclopropane-1-carboxylate, phenylalanine ammonia-lyase) as well as siderophores, nitrogen fixation, sulfate solubilization, antibiotic production, induced systemic resistance (Sah et al., 2021; Consentino et al., 2022; Mellidou and Karamanoli, 2022), and phytopathogens control (Zhang et al., 2022). SAICEUPSM^T was isolated from the rhizosphere of a *Medicago sativa* plant native to the Almadén mining district. Specifically, it was isolated from a dump slope, where the mercury concentration was 1,071 ppm (Millán et al., 2007). Due to its ability to promote plant growth under conditions of abiotic stress, the strain was identified, and it was verified that both bioinformatics and phenotypic analysis suggest that it may be a new strain, reason why *Pseudomonas mercuritolerans* is proposed as its new taxonomic classification.

The aim of this work is the evaluation of the *in vivo* mercury phytostabilization potential under greenhouse conditions of *Lupinus albus* var., Orden Dorado plants in association with mercury resistant SAICEUPSM^T strain in soil substrates from the Almadén mining district (Ciudad Real, Spain).

Results

Isolation, identification, and phylogenetic analysis

Strain SAICEUPSM^T isolated from the rhizosphere of a *Medicago sativa* plant native to the Almadén mining district was classed as a Gram negative rod-shaped bacterium forming translucent brown colonies that intensify their hue after 72 h, ≈ 1 mm Ø, with smooth borders, and creamy texture. It grows in nutritive agar at 28°C, with aerobic metabolism, oxidase catalase positive, non-endospore-forming. Its size is 624.1–778.7 μm wide and 1.679–2.489 μm long (**Supplementary Figure 1**). These characteristics identify SAICEUPSM^T as belonging to the *Pseudomonas* genus.

The similarity analysis using the *16S rRNA* gene showed a value of 96.60% against *P. iranensis* SWRI54. All other strains analyzed showed lower similarity indices (**Table 1**).

Whole genome sequence (WGS) analysis revealed that average nucleotides identity value (ANI) value against *P. iranensis* SWRI54 was 95.34%. In the same way, the average amino acids identity (AAI) offered a value of 97.04%. For this reason, it cannot be concluded that the SAICEUPSM^T strain belongs to *P. iranensis* SWRI54. According to the

TABLE 1 Similarity of 16S rRNA with the 13 closest taxa and the SAICEUPSM^T strain.

Subject	Type strain (T)	Adhesion	Identity (%)
Pseudomonas iranensis	SWRI54	CP077092.1	96.60%
Pseudomonas allokribbensis	IzPS23	CP062252.1	90.65%
Pseudomonas hamedanensis	SWRI65	CP077091.1	90.08%
Pseudomonas kribbensis	46-2	CP029608.1	89.52%
Pseudomonas tensinigenes	ZA 5.3	CP077089.1	89.39%
Pseudomonas gozinkensis	IzPS32d	CP062253.1	88.81%
Pseudomonas glycinae	MS589	CP014205.2	87.96%
Pseudomonas zeae	OE 48.2	CP077090.1	87.82%
Pseudomonas monsensis	PGSB 8459	CP077087.1	87.27%
Pseudomonas azerbaijanoriens	SWRI123	CP077078.1	85.19%
Pseudomonas eucalypticola	NP-1	CP056030.1	84.60%
Pseudomonas mandelii	LMG26867	LT629796.1	84.74%
Pseudomonas prosekii	LMG26867	LT629762.1	84.62%

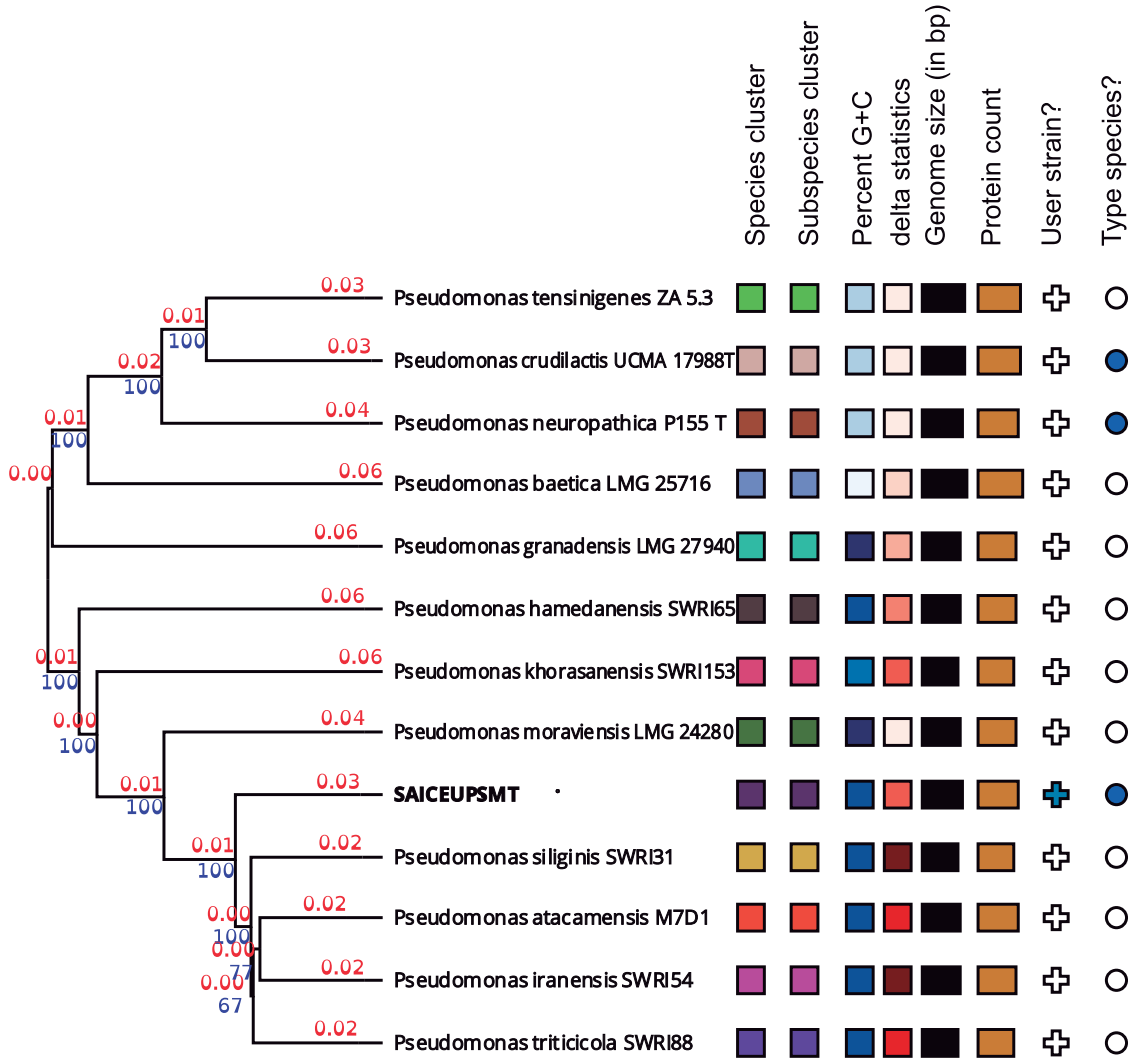


FIGURE 1
Phylogenetic tree from the complete genome of the species of the genus *Pseudomonas*. Numerical values indicate the relative distance between the analyzed species. The branch lengths are scaled in terms of Genome Blast Distance Phylogeny (GBDP) d5. The blue numbers are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 55.2%. The branch length values (in red) represent the evolutionary time between two nodes. Unit: substitutions per sequence site. The tree was rooted at the midpoint.

of the CLSI identification criteria (Clinical and Laboratory Standards Institute) strain SAICEUPSM^T could be a new species within the *Pseudomonas* genus. The phylogenetic activity of Genome Blast Distance Phylogeny (GBDP) (**Figure 1**) positioned SAICEUPSM^T within the cluster of *P. siliginis* SWRI31, *P. atacamensis* M7D1, *P. iranensis* SWRI54 and *P. triticicola* SWRI88. SAICEUPSM^T is segregated from the species in its cluster. The threshold used to discriminate nearby species is between 95–96% for ANI and 70% for DNA-DNA hybridization *in silico* (dDDH) (Richter and Rosselló-Móra, 2009). The analysis of dDDH shows that the highest value for SAICEUPSM^T is below this threshold (**Table 2**). The ANI analysis was 95.42%. These data confirm that the SAICEUPSM^T strain does not belong to any of these species (**Supplementary Table 1**: Calculated ANIb values, **Supplementary Table 2**: Calculated Tetra values, and **Supplementary Table 3**: Intergenomic distance).

Phenotypic description and plant growth promotion activities characterization

This strain is a siderophores producer and presents the ability to degrade ethylene precursor 1-aminocyclopropane-1-carboxylate deaminase (ACC) *via* enzyme ACC deaminase (ACCd). Additionally, produces the auxin class phytohormone

TABLE 2 DNA-DNA hybridization *in silico* (dDDH), confidence interval (CI), and GC percentage difference between SAICEUPSM^T and *Pseudomonas* spp. closely related.

Subject	Assembly accession	dDDH	CI	Diff GC mol %
Pseudomonas atacamensis M7D1	GCA_004801935	61.7	[58.9–64.5]	0.01
Pseudomonas iranensis SWRI54	GCA_014268585	61.5	[58.6–64.3]	0.01
Pseudomonas triticicola SWRI88	GCA_019145375	60.8	[57.9–63.6]	0.10
Pseudomonas siliginis SWRI31	GCF_019145195	59.7	[56.8–62.4]	0.09
Pseudomonas moraviensis LMG 24280	GCF_900105805	46.8	[44.2–49.4]	0.28
Pseudomonas khorasanensis SWRI153	GCA_014268505	37.6	[35.2–40.1]	0.17
Pseudomonas hamedanensis SWRI65	GCA_014268595	36.8	[34.3–39.3]	0.11
Pseudomonas granadensis LMG 27940	GCA_900105485	34.0	[31.5–36.5]	0.27
Pseudomonas tensinigenes ZA 5.3	GCA_014268445	33.6	[31.2–36.1]	0.71
Pseudomonas crudilactis UCMA 17988T	GCA_017973755	33.5	[31.1–36.0]	0.75
Pseudomonas neuropathica P155 T	GCF_015461835	33.5	[31.1–36.0]	0.66
Pseudomonas baetica LMG 25716	GCA_002813455	33.2	[30.8–35.8]	1.13

Formula d4 (a.k.a. GGDC formula 2): sum of all identities found in HSPs divided by overall HSP length.

TABLE 3 Comparison of the phenotype of SAICEUPSM^T with its closest species according to its DNA-DNA hybridization *in silico* (dDDH).

	1	2	3	4	5	6	7	8	9	10	11	12
Fluorescent pigments in King B medium (fluorescein)	+	+	–	+	–	+	+	–	+	+	+	+
Growth at 37°C	+	NA	+	–	–	–	NA	+	NA		+	+
Growth in nutritive agar + NaCl 6%	–	NA	–	/	+	+	NA	–	NA	+	+	+
Nitrate reduction	–	–	–	–	–	+	NA	–	NA	NA	+	–
Citrate utilization	+	+	+	+	NA	+	NA	+	NA	NA	NA	+
Gelatin hydrolysis	–	+	+	+	–	+	+	+	+	+	–	
D-Glucose (oxidation)	+	+	+	+	+	+	+	+	+	+	+	+
L-arabinose (oxidation)	+	+	+	–	+	+	+	+	+	+	+	–
D-galactose (oxidation)	–	+	+	+	+	+	+	+	/	/	+	–
Trehalose (oxidation)	+	+	+	–	–	NA	NA	+	NA	NA	NA	–

Taxons: 1: *Pseudomonas atacamensis* M7D1; 2: *P. moraviensis* LMG24280; 3: *P. granadensis* LMG 27940; 4: *P. baetica* LMG 25716; 5: *P. crudilactis* UCMA 17988T; 6: *P. koreensis* LMG21318; 7: *P. atagonensis* PS14; 8: *P. iridis* P42; 9: *P. allokribbensis* LMG31525T; 10: *P. gozinkensis* LMG31526; 11: *P. laurylsulfativorans* AP3_22; 12: SAICEUPSM^T; +: positive; –: negative; /: weak; NA: data is not available.

Indole-3-acetic acid (IAA, 7.72 $\mu g.ml^{-1}$). Metabolizes citrate, L-Proline aryl amidase and hydrolyzes gelatin, beta hemolytic, and urea transformer. It was negative for the motility, production of acetoin, fermentation of dulcitol, deamination of phenylalanine, production of indole, hydrogen sulfide, decarboxylation of ornithine, lysine, arginine, sucrose, xylose, maltose, mannitol, citrate, and lactose. Table 3 shows different phenotypic characteristics compared to the species with greater phylogenetic proximity.

The bacterium can grow at concentrations between 0 and 6% (p/v) of NaCl, the optimal pH is between 5.5 and 8.0 and within the temperature range of 4 to 37°C, being 28°C its optimal growth temperature. The minimum inhibitory concentrations (MIC) against different antibiotics are shown in Supplementary Table 4.

Regarding the resistance of the strain against heavy metals, quantified by calculating the minimum bactericidal concentration (MBC), SAICEUPSM^T strain was highly resistant to mercury (140 ppm), cupper (400 ppm), chrome (800 ppm), and nickel (400 ppm). In contrast, the cadmium tolerance capacity was 12.5 ppm.

Chemotaxonomic analysis

The analysis by mass spectrophotometry measured by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) generated a list of 10 peaks based on their intensity, which allowed a peptide fingerprint and its comparison with the database (Supplementary Figure 2). The analysis of this profile showed a homologation between SAICEUPSM^T with *P. fluorescens*.

The analysis of fatty acids showed the best represented molecules: C16:0 (35.59%), Sum in Feature 3 (18.21%), C17:0 cyclo (16.73%), and Sum in Feature 8 (10.78%). The profile described by SAICEUPSM^T does not allow the identification of the strain with any nearby species (Table 4).

Genome features

The SAICEUPSM^T genome was formed from 192 contigs, with a genome length of 6,312,264 bp. The GC content was 60.07% mol (Supplementary Figure 3A). A total of 5,522 CDS were identified and assigned to 27 subsystems through the Rapid using Subsystem Technology (RAST) SEED viewer (Supplementary Table 5 and Supplementary Figure 3B), achieving the score of 42% (2337) and through the KEGG analysis 44%. The most represented subsystems were amino acids and derivatives (481), carbohydrates (262), protein metabolism (220), cofactors, vitamins, protein, and pigments (206). The genome sequence project was deposited in NCBI (Bioproject PRJNA847155; Biosample SAMN28920853).

KofamKOALA analyses show that almost all the bacteria's major metabolic pathways were found in SAICEUPSM^T genome (Supplementary Figure 4A and Supplementary Table 6). An in-depth analysis of the mechanisms that could support the phenotypic result of resistance to mercury revealed that SAICEUPSM^T had genes from the mercury resistance operon, finding transport genes (mercuric transport protein, *merT* and mercuric transport protein, *merC*) and reduction genes (Mercuric ion reductase, *merA* and Organomercurial lyase, *merB*) (Supplementary Figure 4B). Likewise, the existence of genes that confer resistance to other heavy metals (cadmium, cupper, chrome, nickel), was analyzed to reinforce the phenotypic observations. The results of the whole gene collection can be consulted extensively in Supplementary Table 7.

The list of genes that code for mechanisms of antibiotic resistance are shown in Supplementary Table 8. Likewise, Supplementary Tables 9, 10 show the genes involved in the main virulence factors: biosynthesis of flagella proteins, adhesion motility, endotoxin, type IV pili, adherence, contraction rate, ion uptake, antiphagocytosis, and secretion systems Type I, Type II, Type III, and Type VI. These operons are incomplete, which is why the strain is not able to express them functionally. The software of the Center for Genomic Epidemiology predicted that the organism does not present a risk of pathogenesis.

In Supplementary Table 11, genes associated with direct and indirect mechanisms of plant growth promoters in SAICEUPSM^T are collected.

Plant growth promotion

The ANOVA analysis of the biometric variables of those plants inoculated with SAICEUPSM^T, revealed the existence of significant differences (p-value < 0.05) in the parameters total weight (Weigth_T, Figure 2A), root weight (Weight_R, Figure 2B), and number of leaves (Leaves, Figure 2C) compared to the controls. The SAICEUPSM^T strain significantly contributed to a higher plant weight, as well as a greater development of the root with respect to the control.

TABLE 4 Fatty acid improve titration.

	1	2	3	4	5	6	7	8
Saturated fatty acid								
$C_{10:0}$	–	–	0.1	–	–	–	–	–
$C_{12:0}$	2.40	+	1.4	1.6	2.26	1.80	1.99	4.09
$C_{14:0}$	0.53	+	0.6	–	–	+	+	–
$C_{16:0}$	35.59	24.67	33.7	32.8	36.09	31.93	32.04	–
$C_{17:0}$	0.56	–	–	–	–	–	–	–
$C_{18:0}$	0.76	–	0.4	+	–	+	+	–
Branched fatty acid								
$C_{17:0}$ cyclo	16.73	+	2.8	11.5	7.48	16.13	17.69	1.31
$C_{18:1}$ ω 7c	–	9.53	–	–	–	–	–	11.22
$C_{19:0}$ cyclo 8cω	0.89	–	–	–	–	+	+	–
Hydroxy fatty acid								
$C_{12:0}$ 2-OH	5.24	10.27	5.2	5.3	5.36	5.14	4.90	3.17
$C_{10:0}$ 3-OH	3.23	+	4.0	3.2	4.16	4.08	4.24	3.89
$C_{12:0}$ 3-OH	4.65	+	–	4.5	4.28	5.40	5.03	4.22
$C_{12:1}$ 3-OH	–	–	–	–	–	–	–	1.86
Summed features								
2	–	–	–	–	–	–	–	10.9
3	18.21		36.2	27.2	30.66	19.82	18.27	38.81
8	10.78		11.3	10.7	10.53	10.92	10.69	

Taxons: 1: *Pseudomonas atacamensis* M7D1; 2: *P. moraviensis* LMG24280; 3: *P. granadensis* LMG 27940; 4: *P. baetica* LMG 25716; 5: *P. crudilactis* UCMA 17988T; 6: *P. koreensis* LMG21318; 7: *P. atagonensis* PS14; 8: *P. iridis* P42; 9: *P. allokribbensis* LMG31525^T; 10: *P. gozinkensis* LMG31526; 11: *P. laurylsulfativorans* AP3_22; 12: SAICEU98^T; –: not detected; +: detected in small unspecified quantities.

Phytoprotection

To verify the potential oxidative stress protection effect SAICEUPSM^T strain under mercury stress conditions, the enzymatic response of *Lupinus albus* grown in soil with a high concentration of the heavy metal was evaluated. Figure 3 shows quantification of catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) enzymes. Incorporating the strain into the root promotes a significant reduction in enzymatic response.

Mercury accumulation in plant

To evaluate the potential mercury phytoprotective capacity of SAICEUPSM^T, the physiological content of this heavy metal in the plant was analyzed (Table 5). It is observed that *Lupinus albus* tends to bioaccumulate mercury mainly at the root level. This accumulation is not statistically different in inoculated plants. The incorporation of SAICEUPSM^T,

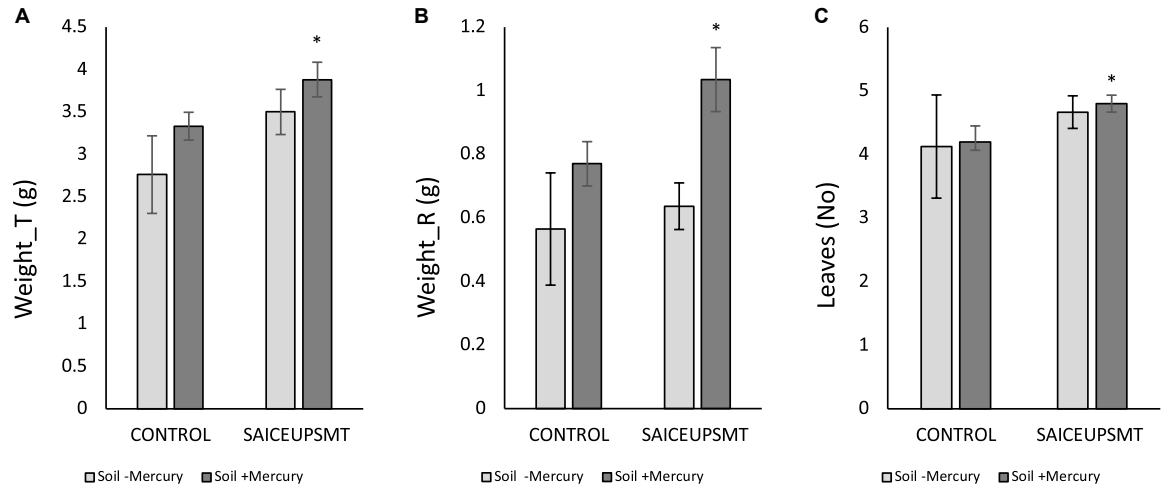


FIGURE 2
Results of the least significance difference (LSD) analysis of the biometric parameters of plants grown in soils with mercury. **(A)** Total weight, **(B)** root weight, and **(C)** total number of leaves. The bars indicate a standard error. *p-value $\leq$ 0.05. s/u, no units.

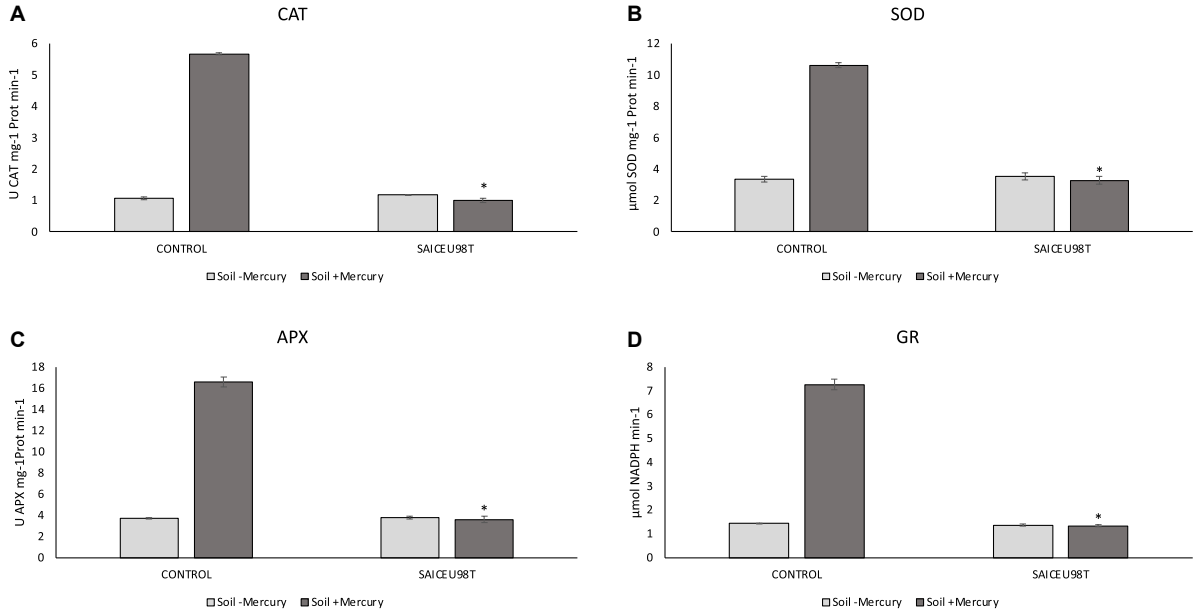


FIGURE 3
Results of Kruskal–Wallis for enzymatic content: **(A)** catalase (CAT), **(B)** superoxide dismutase (SOD), **(C)** ascorbate peroxidase (APX), and **(D)** glutathione reductase (GR). The bars indicate the standard error. *p-value $\leq$ 0.001.

promoted a significant decrease in the accumulation of mercury in the shoot of the plant, respect the control.

Discussion

Lupinus albus model has shown to be a plant with high tolerance to abiotic stress (Fumagalli et al., 2014) as well as having an interesting phytoextraction capacity (Zornoza et al., 2010; Godínez-Méndez et al., 2021; Quiñones et al., 2021). However, mercury is also known to induce physiological and metabolic alterations in plants, as the enzymatic response against oxidative stress (Zelles, 1999; Christakis et al., 2021; Çavuşoğlu et al., 2022) and decreased plant growth. Conversely, the effect of PGPB inoculation may minimize these effects on plants (Heidari and Golpayegani, 2012; Morcillo and Manzanera, 2021) in mercury-contaminated substrates (Pirzadah et al., 2018). The *Pseudomonas* genus,

precisely, has shown in previous works its important role as PGPB in stress conditions (Sandhya et al., 2010). In those plants inoculated with SAICEUPSM^T, the biometric parameters total weight, root weight and number of leaves were significantly higher in soils with high mercury concentration, compared to the same growth conditions, in the absence of PGPB inoculum. The identification of genes coding for plant growth promotion (PGP) activities explains the phenotype of the bacterium. This fact has already been described in other *Pseudomonas* species (Chellaiah, 2018; Kang et al., 2020).

From the results of this work, we confirm that, inoculation with SAICEUPSM^T strain under mercury stress produces a significant reduction in the enzymatic response to oxidative stress (CAT, APX, and SOD). SOD enzyme catalyzes the transformation of singlet oxygen to H_2O_2, with CAT and APX enzymes being responsible for eliminating H_2O_2 by transforming it into H_2O and O_2 (Gill and Tuteja, 2010). Similarly, GR is involved in reducing glutathione disulfide (GSSG) to glutathione (GSH) with NADPH expenditure. GSH plays a very important role in the redox regulation of the cell cycle and in the defense mechanisms against oxidative stress (Sánchez-Fernández et al., 1997). Plants grown in mercury contaminated soils and inoculated with SAICEUPSM^T have also significantly lower GR production. Root colonization by SAICEUPSM^T produces a phytoprotection effect against mercury stress by inducing a better antioxidant enzymatic response, compared to the non-inoculated controls.

Plants of different species, like *Lupinus albus*, can accumulate mercury in different tissues, but the absorption mechanism is still unknown (Zornoza et al., 2010; Quiñones et al., 2021; Robas et al., 2021). This fact can induce a reduction in the production of plant biomass. On the contrary, plants inoculated with SAICEUPSM^T can increase the weight and number of leaves of plants, even in substrates with high concentration of mercury. The incorporation of SAICEUPSM^T significantly reduces the absorption of mercury by the plant, while favoring its development in terms of biomass. That is why we can speak of a phytoprotective effect, which can be associated with the transformation of mercury carried out by the bacteria in the root environment, due to the expression of the genes involved in the metabolism of this heavy metal *mer* (operon). This fact is key for future agronomic applications of the strain, since the improvement in production can be significant, even under conditions of high concentration of mercury, and bioremediation processes. All of this, under safe (environmental and health) conditions since the innocuousness of the SAICEUPSM^T strain has been demonstrated as it lacks functional virulence factors and transmissible or clinically relevant resistance genes. For all the above, the SAICEUPSM^T strain is postulated as a new taxon not described to date, called *P. mercuritolerans*, with potential use in improving plant yield under abiotic stress conditions and in bioremediation processes of heavy metals, such as mercury.

TABLE 5 Mercury accumulation in plant, tested in soils with high mercury concentration.

Substrate	Inoculum	Shoot ($\mu g.g^{-1}$)	Root ($\mu g.g^{-1}$)
Soil + [mercury]	–	0.21 ± 0.02	10.06 ± 0.15
Soil + [mercury]	SAICEUPSM^T	0.13 ± 0.01*	10.04 ± 0.14

*p-value ≤ 0.001.

The growing soil heavy metal pollution in natural environments has increased scientific interest in finding alternatives for the decontamination of these ecosystems. To this end, the participation of PGPB can contribute effectively, both directly on the soil and indirectly, by contributing to the phytoprotection of plants (Singh et al., 2019). The *Pseudomonas* genus can grow and develop under unfavorable conditions, metabolize a wide variety of organic and inorganic compounds, as well as reduce and volatilize heavy metals (Beltrán-Pineda and Gómez-Rodríguez, 2016). SAICEUPSM^T strain has functional genes related to the reduction and volatilization of mercury, resistance to copper, cobalt, zinc, cadmium, as well as siderophores production capacity that help improve tolerance or transform ions of toxic metals, minimizing their toxic effects (Singh and Hiranmai, 2021), which could justify the high resistance values obtained in laboratory tests. There are few bibliographical references that review the tolerance of different species of the *Pseudomonas* genus to mercury. However, the MBC values found in this work highlight the unique resistance of SAICEUPSM^T to said heavy metal. Specifically, phenotypically, the strain ceased to be viable at a concentration of 140 ppm (516.6 μm $HgCl_2$). Sahu et al. (2016) isolated a strain that they considered to be highly tolerant to mercury isolated from an industrial effluent, capable of withstanding up to 200 μm $HgCl_2$ (i.e., almost 2.5 times less than SAICEUPSM^T). In this same sense, Zhang et al. (2012) isolated from a marine sample, a strain of *P. putida* with a tolerance to mercury like the previous authors, of 280 μm, also a much lower value than SAICEUPSM^T. This makes it an excellent candidate for the design of subsequent bioremediation processes for soils contaminated by mercury. The capacity of the strain to resist other heavy metals remains uniquely high, well above values found by other authors (Matyar et al., 2010; Singh et al., 2010) in the *Pseudomonas* genus.

Phenotypic and genotypic characterization techniques allow a multiphase approach of the strains with biotechnological interest. SAICEUPSM^T activity and the set of metabolic tests allowed it to be differentiated from nearby species, such as *P. atacamensis* M7D1. Fatty acid analysis has also been commonly used to identify species and describe new taxa (Cody et al., 2015; Heir et al., 2021). Unfortunately, this method does not always allow accurate identification. Despite the existence of undescribed profiles, the lack of homology makes us think of the possibility of the discovery of a new taxon.

Initially, the analysis of *16S rRNA*, a basic tool for classifying bacterial species, showed a similarity of 96.60% against *P. iranensis* strain SWRI54. This value is below the limits for the demarcation of species, commonly accepted (98.65%). Although the comparison of housekeeping genes is very useful for the identification of bacterial species usually isolated in the clinical field, this marker does not allow discrimination in many isolated environmental strains (Saha et al., 2019). Fortunately, the use of bioinformatics tools for the analysis of the whole genome sequence and its content has been revealed as a useful tool for discrimination and taxonomic ordering (Gutierrez-Albanchez et al., 2021).

The analysis of the total genome size of SAICEUPSM^T was comparable with that of its closest relatives, all of them belonging to the *Pseudomonas* genus. Overall genome sequence comparisons revealed an ANI value between *P. atacamensis* M7D1 and SAICEUPSM^T strain of 95.46% and a dDDH hybridization value of 61.7%. Both values are significantly below the recommended species demarcation values (8.7 and 70%, respectively) (Chun et al., 2018). This suggests that SAICEUPSM^T does not belong to any described species.

The most common mechanism of resistance to mercury in Gram negative bacteria consists of the expression and orderly participation of *merA*, *merB*, *merT*, and *merC* genes included in the *mer* operon. *merA*, has the ability to reduce Hg(II) to Hg(0), being this last one volatile. When the substrate is an organomercurial compound, such as methyl-Hg, it is *merB* that catalyzes the protonolysis cleavage of the C-Hg bond, reducing the Me-Hg to methane (CH_4), and Hg(II) (Christakis et al., 2021). The presence in SAICEUPSM^T genome of all these genes explains their mercury resilience and tolerance against. The fact that this operon does not have a repressor could also explain its high value of MCB which allows to postulate its bioremediatory application.

Antibiotics resistance genes found in SAICEUPSM^T are not transmissible and are common in *Pseudomonas* genus (Heir et al., 2021). On the other hand, genes involved in virulence mechanisms seem to lack functional capacity.

For all the above, we confirm that, inoculation with SAICEUPSM^T strain produces under mercury stress, a significant reduction in the enzymatic response to oxidative stress, significantly promotes plant fitness (increasing total weight, root weight, and number of leaves) and prevents the plant from absorbing mercury from its environment, significantly decreasing its translocation into the shoot. Therefore, SAICEUPSM^T strain is postulated as a new taxon not described to date, called *P. mercuritolerans*, with potential use in improving plant yield under abiotic stress conditions and in heavy metals bioremediation processes, such as mercury.

Description of *Pseudomonas mercuritolerans* sp. nov.

Pseudomonas mercuritolerans (adj.) refers to the strain's tolerance to mercury. It was isolated in 2014 from an autochthonous *Medicago sativa* rhizosphere grown in soils highly contaminated by mercury in the mining district of Almadén (Ciudad Real, Spain). Cells are Gram negative, aerobic, rod-shaped, ranging from 624.1−778.7 μm width to 1,679−2, 489 μm length, non-endospore forming and non-motile. The colonies grow in nutritive agar at an OGT of 28°C forming translucent, round, white-beige colonies. Growth occurs from 4 to 37°C in 24 h. It grows in a pH range of 5.5–8.0 and with a NaCl concentration of 0 to 6%. The optimal temperature, pH, and growth salinity for SAICEUPSM^T are 28°C, pH 7 and 0% salinity. It can't ferment carbohydrates, but it can use urea as a nitrogen source and citrate as the only carbon source; it is catalase and oxidase positive. It produces siderophores (pyoverdine) and can hydrolyze gelatin. The main fatty acids (>81.31% of total fatty acids) were C16:0 (35.59%), sum in feature 3 (18.21%), C17:0 cyclo (16.73%), and sum in Feature 8 (10.78%). The GC genomic DNA content of the type strain is 61.10 mole%. The whole genome sequence has been deposited in the NCBI Bioproject (PRJNA access number 847155), BioSample (SAMN 28920853).

Materials and methods

Legume plant for phytoremediation

Lupinus albus var., Orden Dorado plants were used for the *in vivo* phytoremediation experiment. Seeds were provided by the seed bank of the Center for Scientific and Technological Research of Extremadura (Spain).

Bacteria for phytoremediation

SAICEUPSM^T was selected from a larger set of isolates for its special promotion of plant growth under mercury stress conditions.

Plant growth promotion activities

To determine the *in vitro* production capacity of auxins (3-indoleacetic acid, IAA), a colorimetric technique was used with Van Urk Salkowski's reagent (Ehmann, 1977). The bacterium was grown in LB medium (Texas, USA) at 28°C for 4 days, in shaking conditions. The liquid medium was then centrifuged. In total, 1 ml of the supernatant was mixed with 2 ml of Van Urk Salkowski reagent (2% $FeCl_3$ in 35% $HClO_4$ solution) and kept in darkness. Optical density (OD) was measured at 530 nm after 30 min and 120 min. Results were quantified in ppm ($\mu g.ml^{-1}$). Glick protocol (Glick, 1995) was followed

to differentiate the degradation of ACC by the action of the enzyme ACCd of bacteria that could fix nitrogen. The culture medium contained 1.8% Bacto-Agar (Difco Laboratories, Detroit, MI, USA), low in nitrogen content, supplemented with ACC (30 mmol). The plaques were then inoculated and cultured for 3 days at 28°C, monitoring growth daily. The results were qualitatively evaluated (presence/absence of ACCd enzyme). The production of siderophores was quantified using chrome Azurol S (CAS) agar, described by Alexander and Zuberer (1991) the interpretation was based on the quantitative analysis of the production of siderophores, manifested by the appearance of a halo around the bacteria colonies after 72 h of incubation at 28°C. The ability to solubilize phosphates was tested following the protocol described by De Freitas et al. (1997). Tricalcium phosphate agar (TPM) medium was used (Nautiyal, 1999), with a final pH set to 7 with 1 mole.l^{-1} with HCl. After inoculation, the plaques were incubated at 28°C for 72 h. The inorganic phosphates solubilizer colonies showed clarification halos that were evaluated qualitatively (presence/absence). All PGPB activities were analyzed in triplicate.

Determination of mercury and other heavy metals minimum bactericidal concentration

Mercury, cadmium, copper, chrome, and nickel MBC was evaluated using Müller Hinton agar (Pronadisa®, Madrid, Spain), supplemented with different concentrations of heavy metal salts [$HgCl_2$, $CdSO_4$, $CuSO_4$, $Cr_2(SO_4)_3$, $NiSO_4$]: 800, 400, 350, 200, 175, 150, 100, 87.5, 75, 50, 43.75, and 25 μg.ml^{-1}. MBC was determined to be the lowest concentration of heavy metals salts capable of visually inhibiting >99.9% of bacterial growth after 24 h incubation. All assays were carried out in triplicate.

Substrates and setup of the experiment trays

Two types of substrates were used for the tests. The first of them was a soil with a high concentration of mercury, coming from a mining product dump (called Cerro de los Buitrones) in the Almadén mining district (38° 77′ 35″ N; 4° 85′ 07″ O, Ciudad Real, Spain). The total mercury concentration in this first substrate is 1,710 ppm (Millán et al., 2007).

The second substrate, used as a control, was a soil with a minimum natural concentration of mercury, from the area furthest, called Fuente del Jardinillo (38° 76′ 01″ N; 4° 76′ 79″ O, Ciudad Real, Spain). The use of this substrate instead of one devoid of mercury was done to guarantee the physical-chemical homogeneity of the sample. In both cases, rhizosphere-free soil samples were taken (≈50 kg per soil) from the surface down to 30–35 cm. They were transported under refrigerated conditions (4°C) to the laboratory, where they were stored (4°C) until the assay (<24 h after sampling). Both soils were sieved to guarantee the elimination of the most voluminous fractions and to homogenize the granulometry in each test and replicas.

Four sterile forest trays were used (Plásticos Solanas S.L., Zaragoza, Spain), each composed of twelve alveoli, with a capacity of 300 cm^3, with a light of 5.3 × 5.3 cm. Four pre-germinated *Lupinus albus* seeds (2.0 ± 0.5 cm emerged radicle) were sown in each alveolus.

Inoculant preparation

SAICEUPSM^T strain was incubated in nutritive agar (Pronadisa®, Madrid, Spain) supplemented with 50 ppm of $HgCl_2$ (24 h, 25°C). A bacterial suspension was prepared in 0.45% saline solution and adjusted to 0.5 McFarland (OD 10^8 cfu.ml^{-1}). It was intended to avoid an increase in salinity, which could be aggravated by the incorporation of mercury salts, and which could compromise the correct development of seedlings. Each seed was inoculated with 1 ml of suspension.

Growth under greenhouse conditions

Plants were grown for 6 weeks in a phytotron equipped with white and yellow light (photoperiod of 11 h of light, light intensity 505 μmol.m^{-2}·s^{-1}, stable room temperature 25 ± 3°C). Irrigation was performed every 48 h by capillarity with sterile tap water, with an experimental volume of 350 ml/tray.

Evaluation of the effects of mercury on plant development

Plant biometry

For biometric parameter determination, after 6-week experiment, whole plants were harvested. Roots and shoots were washed with distilled water. With the recently harvested plants, the following parameters were measured: total weight (g), shoot weight (g), root weight (g), shoot length (cm), root length (cm), total number of leaves (N_o), and total number of secondary roots (N_o).

Plant antioxidant response

Enzymes were extracted at 4°C from 1 g of fresh sample, with a mortar and using 50 mg of polyvinylpolypyrrolidone (PVPP) and 10 ml of the following medium: 50 mm of K-phosphate buffer (pH 7.8) with 0.1 mm EDTA (for SOD, CAT, and APX). The same medium, supplemented with 10 mm of β-mercaptoethanol was used for GR. Superoxide dismutase (SOD) enzyme was measured based on SOD'S ability to inhibit the reduction of tetrazoyl nitro-blue (NBT) by photochemically

generated superoxide radicals. A unit of turf is defined as the amount of enzyme needed to inhibit the rate of NBT reduction by 50% at 25°C (Burd et al., 2000). Catalase production was quantified using Aebi method (Aebi, 1984). H_2O_2 consumption was monitored for 1 min at 240 nm. This was carried out by mixing 50 mm of potassium phosphate buffer with 10 mm of H_2O_2 and 100 μl of the extract. APX content was measured in a 1 ml reaction containing 80 nm of potassium phosphate buffer, 2.5 mm of H_2O_2 and 1M sodium ascorbate. H_2O_2 was added to begin the reaction and absorbance reduction was measured for 1 min at 290 nm, to determine the oxidation ratio of ascorbate (Amako et al., 1994). Glutathione reductase (GR) enzyme was estimated spectrophotometrically, according to the Carlberg and Mannervik (1985) method at 25°C and 340 nm. To do this, the reaction mixture contained 50 mm of Tris-$MgCl_2$ buffer, 3 mm, 1 mm of GSSG, 50 μl of enzyme, and 0.3 mm NADPH, added to initiate the reaction. Enzyme concentration was calculated with the initial rate of the reaction and the molar extinction coefficient of NADPH ($\varepsilon 340 = 6.22\ mm^{-1}\ cm^{-1}$).

Mercury accumulation in plant

The root and shoot fraction of each replica were dried in dry heat furnaces at 60°C for 24 h. Each fraction was crushed and digested separately in acid medium (HNO_3/HCl 2%/0.5% weight/volume) under pressure for the determination of trace elements according to the UNE-EN 13805 standard. The product of the digestion was then analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Using a calibration curve, a correlation was established between the concentration of the standard ($\mu g\ l^{-1}$) and the signal (ICP-MS) of mercury element. The element signal value in the 12 samples was interpolated on the calibration curve, resulting in the total concentration of the mercury in the sample. The mercury standard values for establishing the calibration line were as follows, expressed in $\mu g.l^{-1}$: 0.00; 0.05; 0.10; 0.50; 1.00; 5.00; 10.00. Final units' mg kg^{-1}.

$$Cf\left(\frac{\mu g}{Kg}\right) = X\left(\frac{\mu g}{L}\right) \cdot D \cdot \frac{V\,(mL)}{W\,(g)} \cdot 10^{-3}$$

Cf (mg kg^{-1}) is the metal content of the sample, X ($\mu g\ l^{-1}$) corresponds to the interpolated experimental value or the extrapolated experimental value of the standard addition; D is the dilution factor; V (mL) is the volume of the flask, and W(g) to the weight of the sample.

SAICEUPSM^T strain identification

Transmission electron microscopy (TEM)

To determine the size and shape of the analyzed strain, the Prism E scanning electron microscope (SEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used. The culture was observed in suspension. A drop of Formvar was used in transmission electron microscopy (TEM) Cu grids of mesh 200 as a sample support in the grid for TEM microscopy. The measurement conditions were working distance of 10 mm; 24 pA electron current; electron acceleration of 30 kV and size of 2 points, pressure of 375 Pa, at 4°C and a humidity of 50%. The electron microscopy images were obtained by the research support service (SAI) of "X-ray diffraction and scanning electron microscopy" (SAI-DRX-MEB) of the San Pablo CEU University (Madrid, Spain).

Biochemical tests

Oxi/Ferm Pluri Test® (Liofilchem, Italy) was used. Next, the automatic characterization was carried out with the VITEK® 2 equipment and with the VITEK®2 GN identification cards (bioMérieux, Marcy-l'Étoile, France). The motility of the bacterium was tested in Motility Test Agar, (Liofilchem, Italy). Antimicrobial sensitivity was determined using *E*-test in Müller Hinton agar (Pronadisa®, Madrid, Spain) using the following antibiotics: piperacillin and piperacillin with tazobactam, cefepime (bioMérieux, Marcy-l'Étoile, France); ceftazidime, imipenem, imipenem with EDTA, amikacin, gentamicin, and ciprofloxacin (Liofilchem, Italy).

Fatty acids

The analysis of cellular fatty acids was carried out in the Spanish Collection of Type Crops (CECT) at the University of Valencia, Spain. Cells were grown in M2 medium for 48 h, 30°C; extractions and determinations were carried out according to the standard protocol of the MIDI Microbial Identification System (Sasser, 1990) using a chromatograph Agilent 6850 (Agilent Technologies) following the method TSBA6 (MIDI, 2008, version 6.1. Newark, DE: MIDI Inc.).

Phylogenetic analysis

Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) was used. It was carried out in the Vitek MS IND system (BioMérieux, Marcy-l'Étoile, France) at the Carlos III Health Institute (Majadahonda, Madrid). Slides were inoculated with a sterile handle. A total of 1 μl of the matrix solution (VITEK MS-CHCA: mixture of 3.10 g of 2.5-dihydroxy 36 benzoic acid dissolved in 100 ml of water-ethanol-acetonitrile in 1/1/1 ratio) was added to each well and allowed to dry at room temperature. the temperature. Mass spectra were generated with the Axima Assurance system (Shimadzu Corporation, Kyoto, Japan), using the Shimadzu Launchpad software program and the SARAMIS MS-ID v1 database application (AnagnosTee GmbH) for automatic measurement and identification. All strains were analyzed in duplicate. No pre-treatment was used before inoculation on the slide. High confidence identification was considered when the assessment was equal to or greater than 97%.

SAICEUPSM^T genomic DNA was extracted from fresh cells. Amplification of the *16S rRNA* gene was done and BLAST algorithm was used to search for similar sequences. An *in silico* genome analysis was carried out among the most closely related species, for this purpose the Type (Strain) Genome Server (TYGS) service was used using blast+ software. The resulting intergenic distances were used to infer a balanced evolution with branch support through FASTME 2.1.6.1. Compatibility was inferred from 100 pseudo-bootstrap replicas. Trees were rooted in the midpoint and visualized with PhyD3. For the calculation of ANI and AAI the tools available in JSpeciesWS and the ANI calculator[1] were used.

Genome sequencing and bioinformatics analysis

For the extraction of genomic DNA, the QIAamp DNA Kit (QIAGEN®, Hilden, Germany) was used. SAICEUPSM^T genome was obtained by whole genome sequencing using an Illumina Miseq platform. A total of 200 ng of genomic DNA were used, measured by fluorimetry (Quant-iT Picogreen, Thermo Fisher). To make libraries, the NEB Next ultra II FS DNA preparation kit was used, according to the manufacturer's protocol (New England Biolabs). The initial fragmentation time was 7.5 min, and the final PCR amplification was done with six cycles. The resulting DNA fragments were evaluated and quantified by bioanalyzer using a 7500 DNA (Agilent) chip. The libraries were quantified by qPCR using the master mix "Kapa-SYBR FAST qPCR kit for LightCycler480." Libraries were sequenced in Illumina's Miseq equipment following manufacturer's instructions, in a pair end 2 × 300 type race using "Miseq reagent kit v3 600 cycles."

The quality of the sequenced reads was processed using FastQC v.0.11.3 (Babraham Bioinformatics) (Andrews, 2010). They were filtered for low quality reads using Prinseq (Schmieder and Edwards, 2011) and adapter regions using CUTADAPT (Martin, 2011). To eliminate duplicate reads or those that were only present in forward or reverse, FASTQCollapser and FASTQIntersect were used. The *de novo* assembly of the genome was performed with SPAdes v.3.13 (Center for Algorithmic Biotechnology) (Bankevich et al., 2012), the metrics of the assemblies were obtained through SeqEditor (Hafez et al., 2021) and annotated using the Prokka software, version 1.13[2] (Seemann, 2014) and Rapid using Subsystem Technology (RAST) version 2.0[3] with predetermined parameters (Aziz et al., 2012). The genomes of the related strains were obtained from the Genbank database.

Additional bioinformatic analyses were performed. tRNAscan-SE v.2.0[4] was used to predict tRNAs. Several clinically important antimicrobial resistance genes and virulence determinants were searched through functional annotation data generated from Rast, Prokka, and ResFinder 4.1 annotation lines. Several mercury resistance and plant growth promotion genes were searched through functional annotation data generated from Rast and Prokka annotation lines. KEGG software was used to examine the metabolic pathways of the strain, and PathogenFinder v.1.1., Rast and Prokka software were used to estimate pathogenicity.

Statistical analysis

The Kolmogorov–Smirnov test was performed to check the normality of all variables. After that, for the analysis of the biometric data, an ANOVA test was carried out to analyze the plant response in the presence of SAICEUPSM^T in substrates under high and low mercury concentration. For those parameters that showed statistical significance (p-value ≤ 0.05), the multiple comparison test least significant difference (LSD) was performed, to identify if SAICEUPSM^T produced any significant variation in plant biometry, compared to controls. For the analysis of the phytoprotective capacity, a Kruskal–Wallis analysis was performed. For the evaluation of mercury accumulation in plants, the normality of the sample data was verified using the Shapiro–Wilk test. An ANOVA analysis was then performed to determine the existence of significant differences (p-value ≤ 0.05) between treatments. All statistical differences refer to the comparison of the variables when the plant has been inoculated, compared to their respective non-inoculated controls. SPSS v.27.0 software was used (Version 27.0 IBM Corp., Armonk, NY, USA).

Data availability statement

The data presented in this study are deposited in the Genbank repository, accession number: JAMSHA000000000.

Author contributions

PJ, VF, and MR: conceptualization and methodology. VF: software. PJ and MR: validation and supervision. DG, VF, and LG: formal analysis. DG, VF, LG, and MR: investigation. VF and LG: resources. PJ, VF, and LG: data curation. LG and MR: writing—original draft preparation and visualization.

1 http://enve-omics.ce.gatech.edu/ani/, accessed 5 July 2022.
2 https://github.com/tseemann/prokka
3 https://rast.nmpdr.org/
4 http://lowelab.ucsc.edu/tRNAscan-SE/

PJ, AP, and MR: writing—review and editing. AP and PJ: project administration and funding acquisition. All authors read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1032901/full#supplementary-material

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EDITED BY
Nirmalendu Basak,
Central Soil Salinity Research Institute
(ICAR), India

REVIEWED BY
Rishikesh Singh,
Panjab University, India
Megha Kaviraj,
National Rice Research Institute (ICAR), India

*CORRESPONDENCE
Sandeep Kumar
✉ sandeepsihag1992@gmail.com;
✉ sandeep.kumar5@icar.gov.in
Ram Swaroop Meena
✉ meenars@bhu.ac.in;
✉ rsmeenaagro@gmail.com

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Pulse-based cropping systems for soil health restoration, resources conservation, and nutritional and environmental security in rainfed agroecosystems

Sandeep Kumar[1]*, K. A. Gopinath[2], Seema Sheoran[1], Ram Swaroop Meena[3]*, Ch. Srinivasarao[4], Sandeep Bedwal[5], Chetan Kumar Jangir[6], Kancheti Mrunalini[7], Ramdhan Jat[8] and C. S. Praharaj[9]

[1]ICAR-Indian Agricultural Research Institute, Regional Station, Karnal, India, [2]ICAR-Central Research Institute for Dryland Agriculture, Hyderabad, India, [3]Department of Agronomy, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India, [4]ICAR-National Academy of Agricultural Research Management, Hyderabad, India, [5]Department of Soil Science, Chaudhary Charan Singh Haryana Agricultural University, Hisar, India, [6]National Research Centre on Seed Spices, Ajmer, India, [7]ICAR-Indian Institute of Pulses Research, Kanpur, India, [8]Department of Agronomy, Chaudhary Charan Singh Haryana Agricultural University, Hisar, India, [9]ICAR - Directorate of Groundnut Research, Junagadh, Gujarat, India

Pulses are an important source of energy and protein, essential amino acids, dietary fibers, minerals, and vitamins, and play a significant role in addressing global nutritional security. The global pulse area, production, and average productivity increased from 1961 to 2020 (60 years). Pulses are usually grown under rainfed, highly unstable, and complex production environments, with substantial variability in soil and environmental factors, high year-to-year output variability, and variation in soil moisture. Since the last six decades, there is not much satisfactory improvement in the yield of pulses because of their cultivation in harsh environments, coupled with their continuous ignorance of the farmers and governments in policy planning. As a result, the global food supplies through pulses remained negligible and amounted to merely ∼1.0% of the total food supply and 1.2% of the vegan food system. In this situation, protein-rich food is still a question raised at the global level to make a malnutrition-free world. Pulses are a vital component of agricultural biological diversity, essential for tackling climate change, and serve as an energy diet for vegetarians. Pulses can mitigate climate change by reducing the dependence on synthetic fertilizers that artificially introduce nitrogen (N) into the soil. The high demand and manufacture of chemical fertilizers emit greenhouse gases (GHGs), and their overuse can harm the environment. In addition, the increasing demand for the vegetal protein under most global agroecosystems has to be met with under a stressed rainfed situation. The rainfed agroecosystem is a shelter for poor people from a significant part of the globe, such as Africa, South Asia, and Latin America. Nearly, 83% [over 1,260 million hectares (ha)] of cultivated land comes under rainfed agriculture, contributing significantly to global food security by supplying over 60% of the food. In rainfed areas, the limitation of natural resources with the shrinking land, continuous nutrient mining, soil fertility depletion, declining productivity factor, constantly depleting water availability, decreasing soil carbon (C) stock, augmented weed menace, ecological instability, and reduced system productivity are creating a more challenging situation. Pulses, being crops of marginal and semi-marginal soils of arid and semi-arid climates, require less input for cultivation, such as water, nutrients, tillage, labor, and energy. Furthermore, accommodation of the area for the cultivation of pulses reduces the groundwater exploitation, C and N footprints, agrochemical

application in the cropping systems, and ill effects of climate change due to their inherent capacity to withstand harsh soil to exhibit phytoremediation properties and to stand well under stressed environmental condition. This article focuses on the role of pulses in ecological services, human wellbeing, soil, environmental health, and economic security for advanced sustainability. Therefore, this study will enhance the understanding of productivity improvement in a system-based approach in a rainfed agroecosystem through the involvement of pulses. Furthermore, the present study highlighted significant research findings and policy support in the direction of exploring the real yield potential of pulses. It will provide a road map to producers, researchers, policymakers, and government planners working on pulses to promote them in rainfed agroecosystems to achieve the United Nations (UN's) Sustainable Development Goals (SDGs).

KEYWORDS

climate change, energy and nutrition, pulse-based cropping system, rainfed agroecosystem, soil-human health, sustainable food systems, system productivity and profitability

1. Introduction

Approximately 3.1 billion people in the world (~40% of the global population) had experienced hunger or did not have a healthy diet in 2020, and up to 112 million people experienced hunger from 2019. In addition, ~200 million people are also suffering from malnutrition, in particular, due to a lack of dietary protein, vitamin A, and micronutrients such as zinc (Zn), iron (Fe), selenium (Se), and iodine (I) (Lal, 2016a; Sheoran et al., 2022a). Beyond hunger, a growing population had to reduce their food quantity. Pulses, a rich source of these elements, are potential crops for the food and nutritional security of the global population. Pulses are required in a daily diet of 52 g per capita day^{-1} (Barik, 2021). However, their production and consumption are continuously being ignored; the net result is that the average per capita consumption has stagnated at ~19.5 g over the last three decades (FAOSTAT, 2022). This figure dramatically varies across geographical locations, countries, and socioeconomic classes, being maximum in Eastern Africa (44.2 g per capita day^{-1}), Southern Asia and the Caribbean (34.0 g per capita day^{-1}), and Western Africa and Central America (~30.0 g per capita day^{-1}) (FAOSTAT, 2022). Globally, the average daily consumption of pulses has remained almost stagnant for the last three decades except in South Africa, where its consumption had steadily increased from 21 g per capita day^{-1} in 1985 to 29.5 g per capita day^{-1} in 2019 (Rawal and Navarro, 2019; FAOSTAT, 2022). Along with this development, the global average supply of protein from pulses is also low (4.2 g per capita day^{-1}), accounting for only 8.4 and 5.0% of the average protein being supplied from vegetal (50.02 g per capita day^{-1}) and vegetal plus animal sources (83.2 g per capita day^{-1}), respectively (FAOSTAT, 2022).

As regards the current situation, global food security has become one of concern due to a rapid increase in population, climate change, and limited lands available for cultivation; at the same time, interest in proteins from plant sources is also increasing, as opposed to animal protein, for a healthy living. Pulses are a significant component of agricultural systems for their vital contribution to sustaining agricultural growth due to their resource-conserving nature and environment-friendly attribute; the increase in pulse production acts as a panacea to manage the changing climate (Kumar et al., 2022a). Pulses are treasured by smallholder farmers in rainfed agroecosystems who depend on the food they store between harvests. Even though pulses have several nutritional advantages, their area under cultivation accounted for only 12.7% (93.18 Mha) of cereals' (736 Mha) area and 3.0% [89.82 Mt (million tons)] of their production in 2020 (FAOSTAT, 2022). This stat shows the complete ignorance of pulse cultivation by the farmers and governments on the global platform. Pulses are cultivated primarily in rainfed ecosystems and on marginal soils (depleted and degraded soils) and harsh environments that result in a low, highly variable, and unpredictable production and productivity of pulses, compared to cereals (Shukla et al., 2022). Although, throughout the globe, pulses are essential key players in the rainfed agroecosystem, owing to their ability to withstand and perform well under harsh environments, including water, temperature, fertility, and nutritional stress, and their lesser demands on resources (Lal, 2017). Cultivating pulses in rotation with other crops restores soil health and adaptation and mitigates climate changes in the rainfed agroecosystem. They positively impact soil properties, resource utilization, biological nitrogen fixation (BNF), N economy, and production sustainability. These crops also deliver the effect on soil C and N sequestration and increase the productivity of the companion crops growing in rotational sequence. Overall, pulses positively impact dietary energy, soil and human health, and nutritional, environmental, and economic security.

The present study explicitly attempts to analyze the change in pulse production and consumption patterns across the globe and their impact on nutrient intake in terms of energy and protein. Most data (1961–2022) used in this study have been obtained from the Food and Agriculture Organization of the United Nations (FAOSTAT) assessed in 2022 and analyzed to present a valid conclusion. Overall, this review has made an effort to analyze the significance of pulses in food consumption, nutritional security, and soil health restoration *vis-à-vis* environmental sustainability. This article provides a road map to the producers, researchers, policymakers, and government planners to promote pulses in rainfed agroecosystems under the United Nations (UN) umbrella to achieve the Sustainable Development Goals (SDGs). The SDGs have been employed to increase public awareness about the importance of

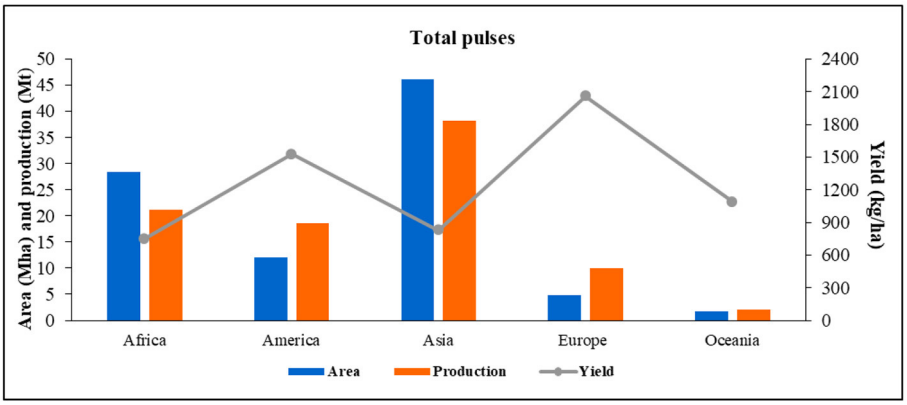

FIGURE 1
Area, production, and yield of total pulses in different regions of the world in 2020 (Data Sources: FAOSTAT, 2022).

pulses to achieve advanced sustainability (Diet Health Club, 2014; Lal, 2016a).

2. Global trends and status of pulse cultivation

Increasing global pulse production is challenging so as to meet the global demand for pulses. Globally important pulses include dry beans such as *Phaseolus* spp. (kidney bean, pinto bean, black turtle bean, navy bean), mung bean (*Vigna radiata*), urd bean (*Vigna mungo*), rice bean (*Vigna umbellata*), moth bean (*Vigna aconitifolia*), lima bean (*Phaseolus lunatus*), adzuki bean (*Vigna angularis*), scarlet runner bean (*Phaseolus coccineus*), and Tepary bean (*Phaseolus acutifolius*); chickpea (*Cicer arietinum*); dry pea including garden pea (*Pisum sativum* var. *sativum*) and protein pea (*Pisum sativum* var. *arvense*); cowpea (*Vigna unguiculata*); lentil (*Lens culinaris*); pigeon pea (*Cajanus cajan*); broad bean including horse bean (*Vicia faba equina*), broad bean (*Vicia faba*), and field bean (*Vicia faba*); lupin (*Lupinus* spp.); vetches/common bean (*Vicia sativa*); bambara bean/earth pea (*Vigna subterranea*); and pulses such as lablab (*Dolichos lablab* or *Lablab purpureus*), jack bean (*Canavalia ensiformis*), sword bean (*Canavalia gladiata*), winged bean (*Psophocarpus tetragonolobus*), velvet bean (*Mucuna pruriens* var. *utilis*), and yam bean (*Pachyrhizus erosus*) (https://en.wikipedia.org/wiki/Legume). The average global pulse production increased from 40.8 Mt in 1961 to 89.8 Mt in 2020, with an overall increment of 120.2% (0.82 Mt year^{-1}) over the base year. The increase in production was augmented primarily due to the expansion of the area under pulse cultivation from 64.0 Mha in 1961 to 93.2 M ha in 2020 (0.49 M ha year^{-1}, 45.6%). Furthermore, with the implementation of improved varieties and agricultural practices, productivity had also increased steadily from 637 kg ha^{-1} in 1961 to 964 kg ha^{-1} in 2020 at an annual increase rate of 5.4 kg ha^{-1} (51.3%), contributing to an increase in total pulse production (Figure 1).

The global area and total production of pulses decreased between the 1960s and the 1980s, but both these parameters increased steadily during the 1990s and the 2000s and increased speedily during the 2010s. In contrast, the average yield of pulses increased consistently from the 1960s to the 2010s (644–964 kg ha^{-1}). In comparison to the 1960s, there was a 120.2% increase in production, a 51.3% increase in the average seed yield, and a 45.6% increase in the pulse area until the end of the 2010s (Figure 2).

In the long journey of ~60 years (1961–2020), all the continents significantly contributed to improving areas, production, and productivity of pulses (Figure 3).

The analysis of the global pulse data from 1961 to 2020 shows a large temporal and spatial variability in total area, production, and average yields (Figure 4).

As per the data obtained from FAOSTAT (2022), Asia contributed the largest area under pulse cultivation (46.1 Mha), with a maximum production of 38.2 Mt in 2020. Although Asia is the major producer, importer, and consumer of pulses, it contributed 61.0% of the total world's pulse production in 1961; since then, its contribution decreased to 42.6% in 2020 due to a significant increment in areas under pulses in both African (4.0 times) and American (1.8 times) continents. In 2020, Africa, America, and Europe produced 21.2 (23.6%), 18.5 (20.6%), and 9.9 Mt (11.0%) of pulses from 28.4, 12.1, and 4.8 Mha areas, respectively. While Oceania had the least contribution, accounting for only 2.2% of the total production. Productivitywise, the trends in different continents were that Europe had the highest productivity with 2,058 kg ha^{-1}, followed by America (1,525 kg ha^{-1}), Oceania (1,089 kg ha^{-1}), Asia (830 kg ha^{-1}), and Africa (749 kg ha^{-1}). In 2020, the top pulse-producing countries were India (26.0%), Canada (9.1%), China (5.3%), Myanmar (4.5%), Nigeria (4.1%), Russia (3.8%), and Brazil (3.4%). There are significant interregional and intercountry yield variations. The average yield of developed countries is >1,200 kg ha^{-1} (e.g., Canada and the USA >20 q ha^{-1}), while the average yield of developing countries is <1,000 q ha^{-1} (except Ethiopia at 1,880 kg ha^{-1}); for instance, most African and South Asian countries' yields are <500 kg ha^{-1} (FAOSTAT, 2022). Still, considering the need to produce more nutritious food, the areas under pulses must increase to 296 Mha by 2030 and 238 Mha by 2100, in addition to the productivity of 1,800 and 2,150 kg ha^{-1}, respectively (Lal, 2016a). India is the largest producer (one-fourth), consumer (27%), and importer (14%) of pulses in the world (Dixit et al., 2015; Kaur et al., 2018). It accounts for one-fifth of the total food grain-producing areas of the world and contributes to nearly 7–10% of the total food grain production

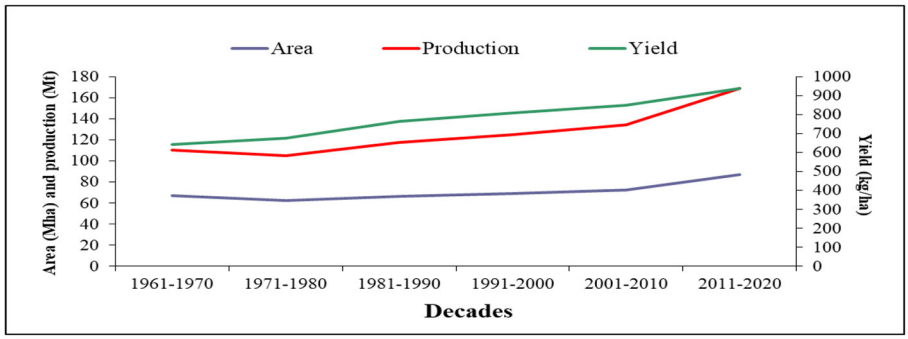

FIGURE 2
Decadal change in the area, production, and average yield of pulses over the globe (Data sources: FAOSTAT, 2022).

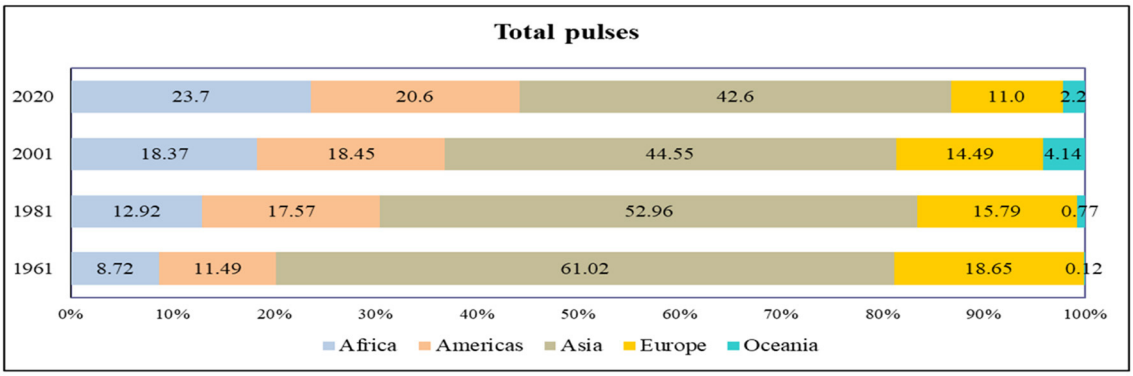

FIGURE 3
Continent-wise share in the global pulse production, ending 1961, 1981, 2001, and 2020 (in percentage) (Data Sources: FAOSTAT, 2022).

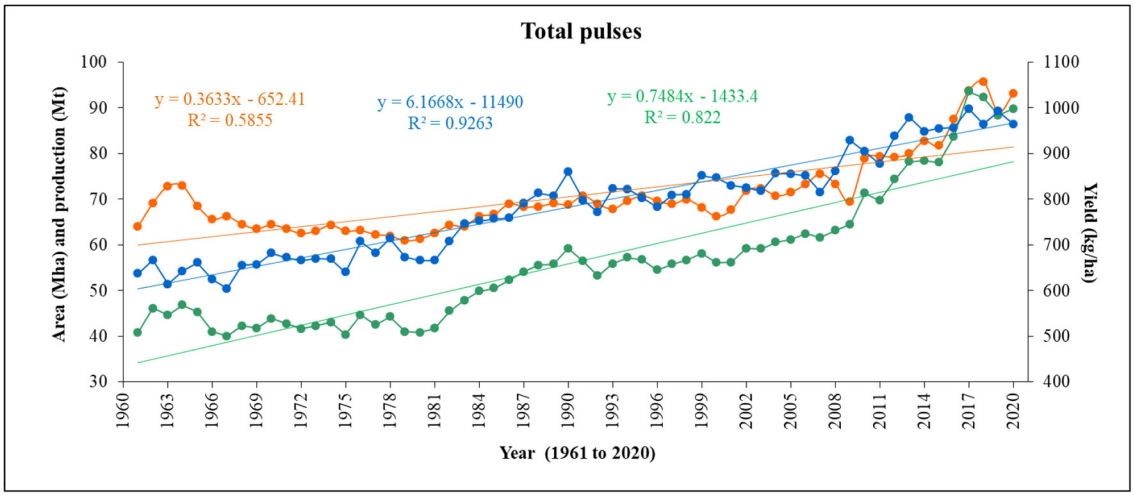

FIGURE 4
Temporal variability in total area, production, and yield of pulses in the world over the last 60 years (1961–2020) (Data sources: FAOSTAT, 2022).

(Kaur et al., 2018). However, to meet the growing population's domestic demands for food and protein, pulse production needs to increase by 39 Mt by 2050 (Dixit et al., 2015). The major pulses cultivated and consumed in India are chickpea, dry bean (mung bean, urd bean, moth bean, and red kidney bean), pigeon pea, lentils, dry peas, etc., in the order of their decreased total production in 2020.

The data obtained from FAOSTAT (2022) clearly indicate that pulses contribute to the total and vegetal food supplies (Table 1). Yet, globally, the food supply through pulses is negligible, ∼1.0% of the

TABLE 1 Dietary habits of the world and the top ten countries with a major share in each item with the minimum total food supply of 1 Mt (FAOSTAT, 2022).

Vegetal % of total food supply		Non-vegetal % of total food supply		% share of pulses of total food supply		% share of pulses of total vegetal food supply	
Niger	97.2	CHS	35.1	Niger	8.6	Niger	8.9
Afghanistan	96.6	Albania	28.1	Ethiopia	5.8	Ethiopia	6.3
Nepal	95.3	Austria	24.5	Rwanda	5.3	Burkina Faso	5.8
Benin	94.5	Mongolia	20.1	URT	3.6	Rwanda	5.7
Rwanda	94.3	France	17.0	Burkina Faso	3.5	Guatemala	4.3
Guinea	94.3	Lithuania	16.7	Guatemala	3.5	URT	4.2
Sudan	94.3	Finland	13.8	Haiti	3.4	Haiti	3.9
Tajikistan	93.5	Slovenia	13.4	Kenya	3.2	Cameroon	3.6
Ethiopia	91.9	Indonesia	12.8	Cameroon	3.1	Kenya	3.5
Kenya	91.6	Guatemala	12.6	Togo	2.4	Togo	2.6
World	83.7	World	11.0	World	1.0	World	1.2

CHS, China, Hong Kong SAR; URT, United Republic of Tanzania.

total global food supply and 1.2% of the total global vegetal food supply. Of the total food consumption, people in Africa consume the highest pulses, which are 1.9% of the total food consumption and 2.1% of the entire vegetal food system. Whereas the pulse consumption in Oceania is the lowest, it is 0.2 and 0.3% of the continent's total food supply and vegetal food supply, respectively (FAOSTAT, 2022). Of the global vegetal and non-vegetal food supplies, the Asian continent accounts for the maximum shares (61.7 and 54.4%), while Oceania accounts for the minimum shares (0.4 and 0.8%). Asia (57.1%) ranks first in the context of the percentage of the global food supply through pulses, followed by Africa (25.2%), America (14.0%), Europe (3.6%), and Oceania (0.1%) (FAOSTAT, 2022). Among countries with a minimum total food supply of 1.0 Mt, Niger (97.2%) and Afghanistan (96.6%) have the highest share of vegetal food. In contrast, the consumption of non-vegetal food as a part of whole food is the highest in China, Hong Kong SAR (35.1%) (Table 1). Similarly, the contribution of pulses to the total food supply and total vegetal food supply of the country was the highest in Niger (8.6 and 8.9%), closely followed by Ethiopia (5.8 and 6.3%), respectively, which was much higher than the global average (1.0 and 1.2%). The protein supply from pulses, vegetal and non-vegetal sources of the top 10 countries is highlighted in Table 1.

3. Pulses for energy intensification and nutritional security

The latest estimates from the United Nations (UN) state that over 3 billion people cannot afford energy food in their daily diet, out of which 57% are residing in Sub-Saharan Africa and southern Asia (FAO, IFAD, UNICEF, WFP, WHO, 2020). According to the data for 2019 assessed from FAOSTAT (August 2022), the vegetal sources that include cereals, starchy roots, sugar crops, sugar and sweeteners, pulses, tree nuts, oil crops, vegetable oils, vegetables, fruits, spices, milk, etc., contribute 83.7% of the total global food supply, being the highest in Africa (89.9%) and the lowest in Oceania (72.2%). Similarly, non-vegetal sources comprising meat, offal, animal fats, eggs, and fish seafood share 11.0% of global food supplies, whereas the supply was the maximum in Oceania (17.1%), followed by the Americas (16.0%) and the least in Africa (5.5%). The remaining food was supplied through stimulants, alcoholic beverages, and other miscellaneous sources.

Globally, the average energy supply through pulses is 67 kcal per capita day^{-1}, which in the case of pulses is the highest in Africa (100 kcal per capita day^{-1}), is the lowest in Oceania (16 kcal per capita day^{-1}) (Table 2), and has an average of 146 kcal per capita day^{-1} in India (Table 2). The same trends follow as regards the quantity of food supply through pulses on a gram (g) capita^{-1} day^{-1} and kilogram (kg) capita^{-1} year^{-1} basis. However, the supply of food, energy, protein, and fat through pulses is much less than that supplied by rice (*Oryza sativa*), wheat (*Triticum aestivum*), and their products globally due to their much higher production and consumption. Nonetheless, pulses are an excellent source of beneficial micronutrients for humans compared to cereals such as rice, wheat, maize (*Zea mays*), and sorghum (*Sorghum bicolor*). In 2019, the global supply of protein through pulses was 4.2 g per capita day^{-1}, which was the highest in Africa (6.6 g per capita day^{-1}) and the lowest in Oceania (1.04 g per capita day^{-1}) (Table 2).

The average daily intake of protein from pulses and the share of pulses in total protein sources differ among countries. As per FAOSTAT (2022), the average customary per capita protein supply amount through pulses in four countries is more than 20% of the average protein supply amount from all protein sources. These countries include Burundi (45.6%), Rwanda (36.3%), Niger (31.4%), and the United Republic of Tanzania (20.9%) (Table 3).

The pulse seeds contain a good amount of carbohydrates (40.4–64.2 g) and encompass excellent dietary fiber (10.6–25.6 g) (Table 4). The United States of America's dietary guidelines advocated the daily intake of 33.6 g of dietary fiber for young men and 28.0 g of dietary fiber for young women in the 19–30 age group. The dietary fiber requirement can be fulfilled by consuming 110–320 g of daily pulses, the intake depending on the gender in the same age group and the type of pulse being consumed. A half-cup of pulses (224 g) daily in the diet provides 7–17% dietary fiber, which is 26–68% of the daily recommended fiber intake in women and 18–45% for men (ICRISAT, 2016). One cup (448 g) of cooked chickpea provides 125 g

TABLE 2 Food supply through pulses in different regions of the globe, 2019 (FAOSTAT, 2022).

Element	Africa	Americas	Asia	Europe	Oceania
Food supply quantity (000' tons)	13,738	7,646	31,123	1,923	70
Food supply (kcal capita^{-1} day^{-1})	100.2	70.7	64.5	24.1	15.8
Food supply quantity (g capita^{-1} day^{-1})	29.5	20.7	18.6	7.1	4.6
Protein supply quantity (g capita^{-1} day^{-1})	6.6	4.6	3.9	1.6	1.0
Fat supply quantity (g capita^{-1} day^{-1})	0.53	0.36	0.48	0.13	0.09

TABLE 3 Top ten per capita daily protein supplier countries through all sources, vegetal, and pulses (FAOSTAT, 2022).

Area	Value (g)	Area	Value (g)	Area	Value (g)
All protein sources		Vegetal protein		Pulse protein	
Iceland	143.9	Niger	74.4	Niger	26.7
CHS	129.5	Tunisia	72.3	Rwanda	21.4
Israel	126.1	Egypt	71.8	Burundi	19.5
Portugal	117.7	Turkey	71.3	Cameroon	13.8
Finland	117.5	Morocco	71.2	Ethiopia	13.7
Norway	116.3	Burkina Faso	69.3	URT	12.6
Lithuania	116.0	Algeria	66.8	Burkina Faso	11.0
Albania	116.0	Ethiopia	64.2	Comoros	10.8
Denmark	115.4	China	63.5	Cuba	10.5
USA	115.0	Bosnia and Herzegovina	62.7	El Salvador	10.2

CHS, China, Hong Kong SAR; URT, United Republic of Tanzania; USA, United States of America.

of dietary fiber, accounting for 50% of the daily fiber requirement. In addition, pulses are a beneficial source of protein because they contain amino acids, such as lysine (130 mg g^{-1} in peas to 250 mg g^{-1} in pigeon peas), which complement the amino acid found in cereals (methionine). The pulse protein is cheaper [₹ 100 kg^{-1} protein (US$ 1.0 = ₹ 79.8)] than proteins available in eggs (₹ 200), milk (₹ 300), and fish/meat (₹ 450). It is also easily digestible and ranges from 18.8% in french beans to 36.2% in lupines, with an average value of 20–25% in most pulses (Table 4), and the average is two times that of wheat (∼12%) and three times that of rice (6–7%). However, the protein content varies across the varieties of a particular crop, for example, common bean (20.9–29.2%), pea (15.8–32.1%), lentil (19–32%), faba bean (22–36%), chickpea (16–28%), mung bean (21–31%), cowpea (16–31%), and pigeon pea (16–24%) (Burstin et al., 2011).

Pulse seeds are a rich source of plant-based protein, dietary fiber, vitamin B, and mineral nutrients such as Fe, Zn, potassium (K), phosphorus (P), calcium (Ca), and magnesium (Mg) (Jangir et al., 2017b). Pulses, contributing ∼10% to the daily protein intake and 5% to energy intake, are important for nutritional security. A dietician recommends a daily intake of 0.8 g of protein for every kilogram of bodyweight. Of the total calories required for a vegetarian, ∼10% should come from protein. For instance, the daily energy needs for a male having a bodyweight of 80 kg should be 2,640 calories; therefore, his protein requirement is 72 g (80 × 0.90 g kg^{-1}). The calories gained from protein can be calculated by multiplying 72 g of protein by four calories g^{-1}, which is 288 calories. Thus, ∼10.9% of calories are received from protein (288/2,640) (Mangels et al., 2011). So, out of the total daily calorific needs, typically, 10–12% of calories should come from protein, depending on the vegetarian diet, which is 14–18% for non-vegetarians. The pulse seeds are energy-dense with a calorie range of 329 (Adzuki bean) to 372 (chickpea) in 100 g of raw mature seeds. Based on the data in Table 4, a daily intake of 100 g of the raw, mature pulse would provide, on average, 350 calories of energy and 25 g of protein. However, for the daily intake of protein of 30 g per capita, there is a need for 125 g of pulses per day (Lal, 2017).

Pulse seeds are rich in iron (Fe) content, which is 2–16 times those supplied by rice, barley (*Hordeum vulgare*), and maize. For instance, its concentration in pulse seeds varies from 3.40 (french bean) to 10.85 mg (moth bean), higher than those of rice (0.2 mg), wheat (3.52 mg), and maize (0.52 mg) in 100 g of raw, mature product (https://foodstruct.com/). That makes the pulses a potent food for alleviating iron (Fe) deficiency, ranging from 50 to 70% in children and women, particularly in pregnant women. Similarly, the pulses are high in Zn content, varying from 0.95 mg in lima bean to 9.30 mg in hyacinth bean with a typical range of 2–4 mg per 100 g of raw, mature seeds. The zinc (Zn) content in pulses is two times higher than that in maize and three times higher than that in rice grains. Pulses also contain a good amount of manganese (Mn) in their seeds, ranging from 0.52 mg (lima bean) to as high as 21.31 mg in 100 g raw, mature seeds of chickpea. Although, the typical range of Mn content is 1.0 to 2.0 mg 100 g^{-1} seed. In all, a 100 g of cooked chickpea and pigeon pea supplies 52.2 and 25.4% of the daily recommended intake of Mn (ICRISAT, 2016). The potassium (K) content in most of the pulses varies between 1,000 and 1,500 mg per 100 g of raw, mature seeds with a maximum content in white bean (1,795 mg), lima bean (1,724 mg), black turtle bean (1,500 mg), and pink bean (1,464 mg). The K content in seeds of pulses is 2–11 times more than those of rice (250 mg), wheat (435 mg), maize (287 mg), sorghum (363 mg), and barley (280 mg) in 100 g of raw, mature product. Potassium has the opposite effect of sodium (Na) on the body, increasing Na excretion in the kidney, and thus drawing water molecules with it. It is required for bones, muscle function, heart diseases, lowering blood pressure, and allowing blood vessels to relax (Rodan, 2017). Similarly, magnesium (Mg) is important for maintaining bodyweight, in addition to various metabolic syndromes associated with cardiovascular disease. In this line, pulse seeds are rich in Mg content, ranging from 47 mg in lentils to 381 mg in moth beans in 100 g raw, mature seeds. A 100 g of cooked chickpea and pigeon pea seeds provides 14 and 13.2% of the daily recommended intake of Mg, respectively (ICRISAT, 2016). Pulses are also considerably richer in calcium (Ca) than most cereals and contain ∼100–200 mg of Ca per 100 g of seeds, being the maximum in white beans (240 mg).

TABLE 4 Energy profile of common pulses (https://www.nutritionvalue.org).

Name	Carbohydrate (g)	Energy (calories)	Protein (g)	Dietary fiber (g)	Fat (g)
Adzuki bean	62.90	329	19.87	12.7	0.53
Urd bean	58.99	341	25.21	18.3	1.64
Black turtle bean	63.25	339	21.25	15.5	0.90
Borlotti bean/cranberry bean/Roman bean	60.65	335	23.03	24.7	1.23
Broad bean	58.29	341	26.10	25.0	1.53
Chickpea	62.95	378	20.47	12.2	6.04
Cowpea common	60.03	336	23.52	10.6	1.26
French bean	64.11	343	18.81	25.2	2.02
Mung bean	62.62	347	23.86	16.3	1.15
Hyacinth bean	61.52	344	22.94	25.6	1.69
Kidney bean, red	61.29	337	22.53	15.2	1.06
Lentil	63.35	352	24.63	10.7	1.06
Lima bean	63.38	338	21.46	19.0	0.69
Lupine	40.37	371	36.17	18.9	9.74
Moth bean	61.52	343	22.94	0.00	1.61
Navy bean	60.75	337	22.33	15.3	1.50
Pea	61.63	364	23.12	22.2	3.89
Pigeon pea	62.78	343	21.70	15.0	1.49
Pink bean	64.19	343	20.96	12.7	1.13
Pinto bean	62.55	347	21.42	15.5	1.23
White bean	60.27	333	23.36	15.2	0.85

Value for 100 g, raw, mature seeds.

Pulses are a perfect and balanced diet, as they are low in Na (5–38 mg), glycemic index, and free from cholesterol and gluten. The low Na concentration in a pulse-based diet helps to stabilize blood pressure, stroke, and heart diseases. These crops are an important source of vitamins, particularly vitamin B_9, known as folate. Our body needs this vitamin to form the DNA, other genetic materials, and cell division (West et al., 2020). The aforementioned information is presented in Table 5.

The pulse seeds are abundant in vitamins containing a significant amount of folate (15–649 μg), thiamine (0.273–1.130 mg), riboflavin (0.091–0.333 mg), niacin (0.479–2.630 mg), pantothenic acid (0.732–2.140 mg), vitamin B_6 (0.140–0.774 mg), vitamin C (0–6.3 mg), vitamin E (0–0.82 mg), and vitamin K (0–15.9 μg) in 100 g of raw, mature seeds (Table 6). The seeds of pinto bean (6.3 mg), mung bean (4.8 mg), lupins (4.8 mg), french bean (4.6 mg), red kidney bean (4.5 mg), lentil (4.5 mg), moth bean (4.0 mg), and chickpea (4.0 mg) contain a good amount of vitamin C, in which most of the cereals (e.g., rice, wheat, and barley) are lacking. It helps the body to absorb Fe in a better way. For instance, in the absence of vitamin C in chickpea, the chickpea seeds absorbed Fe slightly until vitamin C was added. The concentration of vitamin B_{12} is present more in germinated pulse seeds that start to increase after 48 h of germination and are at their peak after 96 h (Rohatgi et al., 1955). A 100 g of cooked chickpea provides 44.5% folate, 24.5% vitamin E, 10.5% thiamine, 10.3% vitamin B_6, and 4.3% vitamin K in the daily intake. At the same time, 100 g of cooked pigeon pea seed contributes to the daily recommended intake with 28.7% folate, 13.3% thiamine, 5.4% riboflavin, and 3.7% vitamin B_7. On average, folic acid (folate) present in pulses is nearly six times that in maize and up to 42 times in non-fortified rice. A good amount of folate in pulse seeds has been found to reduce inflammation by reducing inflammatory factors in our bodies.

In addition to the richness of pulse seeds in protein, mineral nutrition, vitamins, carbohydrates, and dietary energy, they also have several other health benefits. A high fiber-based pulse diet (60–70%) helps in weight loss, maintains bowel health, and lowers the risk of colon diseases, apart from reducing heart-related diseases by stabilizing blood pressure and heart inflammation (Jimenes-Cruz et al., 2003). The pulse seeds also enrich the blood and cure skin diseases and ear inflammation, which acts as a tonic and appetizer (Khan et al., 2021). Thus, the consumption of whole pulse seeds protects us against obesity and the development of diabetes and helps to manage people who have already developed type II diabetes mellitus (T2DM). A reduction of 20–30% in the occurrence of diabetes is found if a person takes 2–3 servings of legumes a day. The dietary fiber present in pulse seeds is slowly digestible and aids in alleviating the blood sugar from spiking and the level of insulin. This dietary fiber lowers bad cholesterol in the body [low-density lipoprotein cholesterol (LDL-C)] by preventing bile salts from reabsorbing in the small intestine. For instance, supplementation with seeds of chickpea, navy bean, and pinto bean lowers the LDL-C and high-density lipoprotein cholesterol (HDL-C)

TABLE 5 Mineral profile of common pulses (https://www.nutritionvalue.org).

Name	P (mg)	K (mg)	Ca (mg)	Mg (mg)	Zn (mg)	Fe (mg)	Cu (mg)	Mn (mg)	Na (mg)
Adzuki bean	381	1,254	66	127	5.04	4.98	1.09	1.73	5
Urd bean	379	983	138	267	3.35	7.57	0.98	1.52	38
Black turtle bean	440	1,500	160	160	2.20	8.70	1.00	1.00	9
Borlotti bean/cranberry bean/Roman bean	372	1,332	127	156	3.63	5.00	0.79	0.92	6
Broad bean	421	1,062	103	190	3.14	6.70	0.82	1.63	13
Chickpea	252	718	57	79	2.76	4.31	0.66	21.31	24
Cowpea common	824	1,112	110	184	3.37	8.27	0.84	1.53	16
French bean	304	1,316	186	188	1.90	3.40	0.44	1.20	18
Mung bean	367	1,246	132	189	2.68	6.74	0.94	1.04	15
Hyacinth bean	372	1,235	130	283	9.30	5.10	1.34	1.57	21
Kidney bean, red	406	1,359	83	138	2.79	6.69	0.70	1.11	12
Lentil	281	677	35	47	3.27	6.51	0.75	1.39	6
Lima bean	385	1,724	81	224	0.95	7.51	0.74	0.52	18
Lupine	440	1,013	176	198	4.75	4.36	1.02	2.38	15
Moth bean	489	1,191	150	381	1.92	10.85	0.67	1.82	30
Navy bean	407	1,185	147	175	3.65	5.49	0.83	1.42	5
Pea	334	852	46	63	3.49	4.73	0.81	1.19	5
Pigeon pea	367	1,392	130	183	2.76	5.23	1.06	1.79	17
Pink bean	415	1,464	130	182	3.60	6.77	0.81	1.38	8
Pinto bean	411	1,393	113	176	2.28	5.07	0.89	1.15	12
White bean	301	1,795	240	190	3.67	10.44	0.98	1.80	16

Value for 100 g, raw, mature seeds.

in adult women and adult men, and thus the transportation of all fat molecules in extracellular water around the body (Shutler et al., 2007). Therefore, pulses are a good choice for people having diabetes while enhancing insulin resistance. According to a WHO (World Health Organization) (2008) report, up to 80% of heart attacks, diabetes, and strokes can be prevented by adopting a healthy diet with essential pulses. The presence of soluble fiber prevents the absorption of lipids, fats, and cholesterol in the body and advances cardiovascular health, whereas insoluble fibers reduce gastrointestinal (GI) issues (Rawal and Navarro, 2019). The protein in pulses plays an essential role in reducing cardiovascular disease, owing to lower saturated fats (0.53–9.74 g per 100 g of dry seeds) and high dietary fibers. Winham and Hutchins (2007) advocated that consuming baked beans considerably lowers the cholesterol level in the body and the associated risks of cardiovascular diseases. A recent control study found an inverse association between legumes with endometrial and colon cancers in women. For instance, the seeds of the navy bean are rich in saponin, exhibiting antifungal and antibacterial properties that prevent cancer cell growth. Its seeds are also an outstanding source of *p*-coumaric acid and ferulic acid among the varieties of common beans (Luthria et al., 2006). White bean (navy bean) is a well-known copious plant-based source of phosphatidylserine (a phospholipid constituent of the cell membrane) (Souci et al., 2008). Scientific reports have noted that the pinto bean contains a considerable amount of phytoestrogen coumestrol, exhibiting several health benefits. Some of the critical health benefits of pulses are as follows:

1. Pulses lower the risk of coronary heart disease and cardiovascular disease by 22 and 11%, respectively (Arora, 2019). Pulses also modulate the concentration of glucose, insulin, and homocysteine, and lipid peroxidation in patients with coronary artery disease (Jang et al., 2001).
2. Adzuki bean seeds reduce the triglyceride concentration by inhibiting pancreatic lipase activity (hypertriglyceridemia) (Maruyama et al., 2008).
3. Pulses reduce the risk of endometrial, breast, and colon (colorectal adenoma) cancers (Velie et al., 2005).
4. The consumption of whole seeds of beans controls the glycemic index and helps in weight loss (Jimenes-Cruz et al., 2003).
5. Pulse seeds lower the average body mass index (BMI), waist circumference, waist-to-hip ratio, and the risk of obesity (Williams et al., 2000).
6. Vegetables, including some pulse seeds, reduce the risk of lymphoblastic leukemia (Petridou et al., 2005).
7. Chickpea seeds have tonic, appetizer, stimulant and, aphrodisiac, anthelmintic properties (Khan et al., 2021), which reduce the risk of skin and ear inflammation (Gill, 2019) and serum total and LDL cholesterols (hypertriglyceridemia) (Pittaway et al., 2006).

TABLE 6 Vitamin profile of common pulses (per 100g) (https://www.nutritionvalue.org).

Name	Thiamine (B_1) (mg)	Riboflavin (B_2) (mg)	Niacin (B_3) (mg)	Pantothenic acid (B_5) (mg)	Vit. B_6 (mg)	Folate (B_9) (μg)	Vit. C (mg)	Vit. E (mg)	Vit. K (μg)
Adzuki bean	0.455	0.220	2.630	1.471	0.351	622	0.0	0.00	0.0
Urd bean	0.273	0.254	1.447	0.906	0.281	216	0.0	0.00	0.0
Black turtle bean	0.900	0.193	1.955	0.899	0.286	444	0.0	0.21	5.6
Borlotti bean/cranberry bean/Roman bean	0.747	0.213	1.455	0.748	0.309	604	0.0	0.00	0.0
Broad bean	0.555	0.333	2.832	0.976	0.366	423	1.4	0.05	9.0
Chickpea	0.477	0.212	1.541	1.588	0.535	557	4.0	0.82	9.0
Cowpea common	0.853	0.226	2.075	1.496	0.357	633	1.5	0.39	5.0
French bean	0.535	0.221	2.083	0.789	0.401	399	4.6	0.00	0.0
Mung bean	0.621	0.233	2.251	1.91	0.382	625	4.8	0.51	9.0
Hyacinth bean	1.130	0.136	1.610	1.237	0.155	23	0.0	0.00	0.0
Kidney bean, red	0.608	0.215	2.110	0.780	0.397	394	4.5	0.21	5.6
Lentil	0.873	0.211	2.605	2.140	0.540	479	4.5	0.49	0.0
Lima bean	0.507	0.202	1.537	1.355	0.512	395	0.0	0.72	0.0
Lupine	0.640	0.220	2.190	0.750	0.357	355	4.8	0.00	0.0
Moth bean	0.562	0.091	2.800	1.535	0.366	649	4.0	0.00	0.0
Navy bean	0.775	0.164	2.188	0.744	0.428	364	0.0	0.02	2.5
Pea	0.719	0.244	3.608	0.962	0.140	15	1.8	0.12	15.9
Pigeon pea	0.643	0.187	2.965	1.266	0.283	456	0.0	0.00	0.0
Pink bean	0.772	0.192	1.892	0.997	0.527	463	0.0	0.21	5.7
Pinto bean	0.713	0.212	1.174	0.785	0.774	525	6.3	0.21	5.6
White bean	0.437	0.146	0.479	0.732	0.318	388	0.0	0.21	5.6

Value for 100 g, raw, mature seeds.

4. Rainfed agroecosystem

The importance of rainfed agroecosystems varies regionally, but these agroecosystems provide the majority of food for poor communities in developing countries. The rainfed agroecosystem is home to the production of most of the millets, pulses, cotton (*Gossypium* spp.), oilseeds, and nearly half of the country's rice, apart from providing support to the production of goat, sheep, and cattle. A rainfed agroecosystem is a shelter for poor people in a significant part of the globe, such as Africa and South Asia. Nearly, 83% (over 1,260 million ha) of the cultivated land is under rainfed agriculture, significantly contributing to global food security by supplying around 60% of the food (FAO, 2008) and generating livelihoods in rural areas while providing food for urban people. In North and East Africa, ∼80% of the seasonal crops come under the rainfed agroecosystem, whereas in the Sahelian countries, this figure is over 95% of the total cultivated areas (FAO, 2018). Rainfed agriculture covers ∼95% of the entire cultivated land of Sub-Saharan Africa, 90% of Latin America, 87% of Southern America, 65% of East Asia, 60% of South Asia, and 75% of Near East and North Africa (Srinivasarao et al., 2013). As per an estimate, ∼61% of Indian farmers depend on rainfed farming for their livelihood, while ∼60% of the country's gross cropped area falls under a rainfed agroecosystem, contributing ∼43% to the national food basket (Venkateswarlu and Prasad, 2012). By taking the example of the Indian subcontinent, even though the full irrigation potential of rainfed areas is realized, 50% of the net cultivated area remains rainfed. In addition, the productivity of the rainfed agroecosystem is still far from the potential yield, especially in developing countries, which have attained only ∼30% of the potential yield. In comparison, countries such as Yemen and Pakistan achieved only ∼10% of the potential yield in rainfed areas (Ramirez-Vallejo, 2011), often due to low soil fertility, low nutrient inputs, and limited water availability. The soils of rainfed regions face severe issues of soil degradation, reduced soil fertility, multinutrient deficiency, low soil organic carbon (SOC) stock, low soil water retention capacity, high temperatures, limited rainfall, and biotic stresses (Lehman et al., 2015). The high temperatures coupled with heat waves in these ecoregions accelerate the process of soil erosion, evaporation, transpiration from vegetative surfaces, and oxidation of SOC stocks (Jangir et al., 2017a). In addition, the rainfall variability characterized by rare and high-intensity rainfall or drought, and frequent and more prolonged dry spells break the constant water supply of rainfed crops and thus have become the reason for crop failure in these regions (Ramirez-Vallejo, 2011; Kumar et al., 2016). Again, the high-temperature regime and limited water availability reduce the length of the growing season and, thus, the choice of crops and productivity (Lehman et al., 2015).

Furthermore, rainfed agroecosystems are more vulnerable to the consequences of climate change, including variability and unpredictability of rainfall events and increasing frequency of extreme weather events and, as a result, worse effects on productivity. It is also expected that climate change and other soil constraints could convert ∼30–60 Mha of cultivated lands in rainfed areas to lands unfit for rainfed agriculture in Sub-Saharan Africa by the 2080s (Fischer et al., 2005). As a result, the extent of soil loss in these regions could range from 5 to 150 Mg ha^{-1} $year^{-1}$ depending upon the soil type, presence of vegetation, and slope gradient (Srinivasarao et al., 2013). This condition can be avoided if the soil is sustainably utilized to reduce the loss of its properties and the impact of climate change. Therefore, there is a lot of scope for sustainable utilization of a potential rainfed ecosystem for enough food production under the scenario of a degrading environment in the future.

A fundamental principle for the sustainability of the rainfed agroecosystem is crop intensification, which can alleviate the existing constraints, adapt well, and produce an optimal yield (Lehman et al., 2015). Pulses can naturally tolerate high temperature and drought stress, owing to their inherently low water requirement. Therefore, pulses, either as monocrop, inter-/mixed-crop, or a sequential crop in rotation in a rainfed agroecosystem, can utilize effectively the available water through their deeper root system (Liu et al., 2019). In addition, these crops conserve the limitedly available soil moisture by providing an efficient ground cover and reducing the risk of water loss through evaporation, leaching, percolation, runoff, soil salinization, nutrient leaching, and soil erosion. In addition, this group of crops produces nutrient-rich biomass that potentially increases SOC levels and contributes to a healthy soil biological community (Lehman et al., 2015). Pulses also fit well in the low-fertile rainfed areas because of their low nutrient requirement coupled with the capacity of BNF for their own use and also leaving some residual N for the use of companion crop in inter-/mixed-crop or subsequent crop in a rotation system (Sheoran et al., 2021). Similarly, these low-fertile rainfed areas are good for restoring soil health, improving micronutrient concentration in the edible parts, and reducing the N and C footprints of the agroecosystems (Lal, 2016b). It is envisaged further that pulse cultivation in a rainfed environment could usher in the second green revolution in the era of emerging health crises such as coronavirus 2019 (COVID-19), climate change, and degrading natural resources for ensuring food and nutritional security, strengthening ecosystem services, and enhancing production sustainability. Therefore, looking toward the miracle of pulses is yet to be harnessed appropriately by focusing more on sustainable pulse production in rainfed conditions by increasing cultivable areas following the best management practices under site-specific soil and environmental conditions.

The area under pulses can be expanded by eliminating fallow periods in rainfed agroecosystems (Ghosh et al., 2019). This step includes the diversification of rice–wheat, maize–wheat, and rice–rice rotation, rice–fallow ecology (22.2 Mha) in India, Bangladesh, and Nepal, and summer fallow (2–3 Mha) in cereal–cereal rotation (rice–wheat in particular) in Indo-Gangetic Plains (IGPs). In addition, these crops can be grown as inter- and/or mixed-crops (e.g., 4–6 Mha in India) in long-duration crops such as banana (*Musa* spp.), sugarcane (*Saccharum officinarum*), newly established orchards, cotton, and millets; relay (*utera/paira*) cultivation in the rice-based system; and relay their inclusion in the agroforestry system. Northeastern hill zones of India under low-input farming could also be a targeted niche for pulse cultivation. Vertically, adequate pulse availability can be ensured by increasing productivity and avoiding postharvest losses. Including pulses in rotation with non-legume crops improves soil health, restores degraded lands, and thus increases the productivity of subsequent crops in rotation and component crops in inter-/mixed-cropping (Meena et al., 2022). Pulses fix atmospheric N at an estimated annual rate of around 3.0 Tg (Teragram = million metric tons). Hence, there is an urgent need to focus more on promoting pulses in the rainfed agroecosystem.

TABLE 7 Possible intercropping systems capable of large-scale advancement and adoption in India.

Intercropping systems	Possible niches
Cotton + pigeon pea	Madhya Pradesh, Gujarat, Maharashtra, Karnataka, Andhra Pradesh, and Telangana
Cotton + mung bean/urd bean/cowpea	Punjab, Haryana, Madhya Pradesh, Gujarat, Maharashtra, Karnataka, Andhra Pradesh, and Telangana
Indian mustard + chickpea/lentil/mung bean/urd bean	Punjab, Haryana, Uttar Pradesh, Madhya Pradesh, and West Bengal
Potato + French bean	Punjab, Uttar Pradesh, Madhya Pradesh, Bihar, Jharkhand, and West Bengal
Sugarcane + mung bean/urd bean/cowpea	East Uttar Pradesh, Bihar, West Bengal, Maharashtra, Karnataka, Andhra Pradesh, Telangana, and Tamil Nadu
Chickpea/lentil + autumn planted/ratoon sugarcane	Uttar Pradesh, Bihar, and Maharashtra
Soybean + pigeon pea/urd bean	Madhya Pradesh and Maharashtra
Pearl millet/sorghum + pigeon pea	Uttar Pradesh, Madhya Pradesh, Gujarat, Maharashtra, Karnataka, Andhra Pradesh, and Telangana
Groundnut + pigeon pea	Gujarat, Madhya Pradesh, Maharashtra and Uttar Pradesh
Groundnut/sorghum/pearl millet + mung bean/urd bean/cowpea	Rajasthan, Gujarat, Uttar Pradesh, Bihar, Madhya Pradesh, Maharashtra, Karnataka, and Telangana

5. Pulse-based agroecosystems for rainfed areas

Pulses are an integral part of a profitable agroecosystem because of their energy-rich nutritious food, feed, and forage (Singh et al., 2009). Crops and cropping sequences are chosen for sustainable intensification (SI), economic profitability, energy-rich diversified nutritious food, climate resilience, and ecological services in a system-based approach. This group of food crops can be included in rainfed agroecosystems, owing to their inherent hardy nature, low input requirements, and immense capacity to grow well under prevailing fragile and harsh climatic stresses. Pulses can be grown as green manuring crops, catch crops, and ratoon crops under sequential cropping or monocropping in diverse agroecological regions. In the intercropping system, pulses are taken as a mixed intercrop (e.g., homesteads), row intercrop (e.g., pearl millet + mung bean), and relay intercrop (e.g., mung bean–maize–potato–wheat). The dominant agroecosystem in which pulses are encompassed is double and triple cropping. Relay cropping (*paira/utera*) is practiced in

humid regions of northeastern India and drier areas of central and coastal southern India for better use of residual soil moisture. Pulses are the best crop in relay croppings, such as mung bean, urd bean, and lentil, along with rice which facilitates double cropping and system sustainability. In northern India, the development of early maturing cultivars of mung bean, urd bean, and pigeon pea made crop diversification possible and the SI of existing non-pulse crops. In addition to providing excellent nutritious food on hilly slopes, fodder pulses, such as mung bean, urd bean, french bean, and rice bean, also act as tremendous cover crops. Many traditional rainfed agroecosystems have been either modified or replaced by several new pulse-based agroecosystems following sustainability concern and marketing opportunities in rainfed areas. For example, rice–chickpea/lentil, rice–mung bean/urd bean, groundnut (*Arachis hypogaea*)/soybean (*Glycine max*) + pigeon pea, and potato + french bean. In the existing scenario of natural resource degradation in agriculture coupled with increasing demands for nutritious food, the importance of pulses in the agroecosystem is enhanced. Hence, pulses must be incorporated into the predominant rainfed cropping systems. However, there is hardly any scope for expansion of the area under pulses because of the intense competition with cereals and cash crops. Nonetheless, almost 4–6 million ha can be easily devoted to pulses by intercropping them with other crops (Praharaj and Blaise, 2016). This strategy is also in accord with SI and other innovative systems. Some of the possible intercropping systems recommended for the Indian subcontinent are presented in Table 7 (Praharaj and Blaise, 2016; Singh, 2018).

5.1. Significance of pulses in cropping systems in a rainfed agroecosystem

Pulses are vital to food and nutritional security and occupy a unique niche, particularly in dryland or rainfed farming, short growing seasons, summer fallowing, short monsoon season, marginal and/or degraded soils, and impoverished ecoregions (Lal, 2017). In 1961, pulses were grown in 150 countries, and the global number increased to 171 in 2019 (FAOSTAT, 2022). In South Asia, pulses are grown as inter-/mixed crop, sequential crop, monocrop, relay crop, etc., fulfilling the local demands of nutritional food and cash (Adarsh et al., 2019). In Europe, pulses such as faba bean are intercropped with wheat, and the bean supply N to companion cereals. In some regions such as India's Indo-Gangetic plains (IGPs) and the Great Plains of North America, the traditional summer following is being replaced with short-duration pulses (Gan et al., 2015). In Australia, pulses have become an important cash crop since the 1980s, wherein faba beans are grown from 20 to 40°S in the summer season, and chickpeas from 10 to 40°S (south) in the winter season (Lal, 2017). In the African continent, pulses have been grown since ancient times and will continue to be a part of their rainfed agricultural systems that are prone to water scarcity.

Pulses in cereal-based cropping systems have an essential role in improving system productivity, soil fertility status, and soil physical properties by reducing continuous submergence and breaking the chain of insects and pests. Pulses in rotation account for the sustainability of the cropping system by fixing atmospheric N and supplying fixed N to the succeeding cereal. This helps in scavenging mineral N, promoting nutrient cycling deep in the soil, improving the SOC stock, reducing soil compaction and erosion, enhancing weed suppression, and improving mycorrhizal colonization (Jakhar et al., 2017). This crop group enhances employment generation, provides nutrient-rich balanced food, and improves the socioeconomic conditions of small and marginal farmers. Pulse–cereal production system has a low C and water footprint, high soil C accretion capacity, nitrogen fertilization, and improved soil biodiversity (Adarsh et al., 2019). In sequential cropping, component crops compete for residual moisture, nutrient, soil, and light. In the cereal–pulse sequence, the previous pulse crop efficiently utilizes light due to the quick land-covering capacity of the dense canopy (Singh et al., 2009), while the subsequent cereal crop effectively utilizes the residual nutrients and moisture from the previous pulse crop (Adarsh et al., 2019). The improved yield of succeeding cereal crops after pulses results from integrated effects of residual N, soil health improvement, soil water conservation, and pest control in a rainfed agroecosystem.

Pulses aid in economizing the N use in successive non-legume crops, owing to their residual effect. Pulse residues encompass ∼20–80 kg N ha^{-1} accounting for nearly 70% of biologically fixed N depending on crop and environmental conditions (Giller, 2001). On average, in sequential cropping, the previous pulse crop supplies 18 to 70 kg N ha^{-1} to the soil (Kaur et al., 2018). This N use remains available for following non-legume crops and, thus, reduces fertilizer requirement up to the extent of 25–30% (Garg and Geetanjali, 2007)—for instance, growing cowpea and mung bean before pearl millet in the preceding season supplies ∼60 kg N to the pearl millet crop (Ghosh et al., 2007). While in pulse–wheat sequential cropping, pulses contribute to between 20 and 40% of the N requirement of wheat. However, it is not easy to quantify the amount of fixed N transferred to the subsequent cereal crop due to higher variability in N fixation. The BNF also reduces chemical N inputs in the rotation, which enhances fertilizer use and consequently reduces the environmental consequences of chemical fertilization.

Pulses in the cropping system also improve physical soil conditions, such as aggregate stability and soil structure, while reducing bulk density. Aggregate stability is an important index of long-term soil quality improvement in a pulse-based cropping system due to the presence of fungi. The fungi secrete glomalin (a glycoprotein) that entraps solid-oxide-oxygen-ion conducting membrane (SOM), soil minerals, and plant debris to form a stable soil aggregate and improve soil structure further (Singh et al., 2009). In addition, soil bulk density also responds positively to changes in management over the long-term pulse-based cropping system (Figure 5) (Wang et al., 2020). Legumes also enhance earthworm activity, which along with the root channel of pulses, increases soil porosity, promotes aeration, increases water-holding capacity (WHC), and percolates deeper into the subsoil (Kumar et al., 2020) (Figure 5). Pulses influence strongly the exploitation of groundwater. For example, replacing lowland rice with pigeon peas in the rice–wheat system reduces the depletion rate of the groundwater aquifer, improves water use efficiency (WUE), and thus leads to efficient water utilization in the era of climate change accelerated water crises.

Growing pulses also impacts the chemical properties of the soil and improves soil health status in the cereal-based system (Wang et al., 2020). Pulses fix atmospheric N and reduce the dependency on soil N and thus lower soil pH (Singh et al., 2009). Even a slight decrease in soil pH increases the availability of micronutrients and microbiota activity in the rhizosphere. The capacity of pulses to reduce soil pH is in the decreasing order of chickpeas, peas,

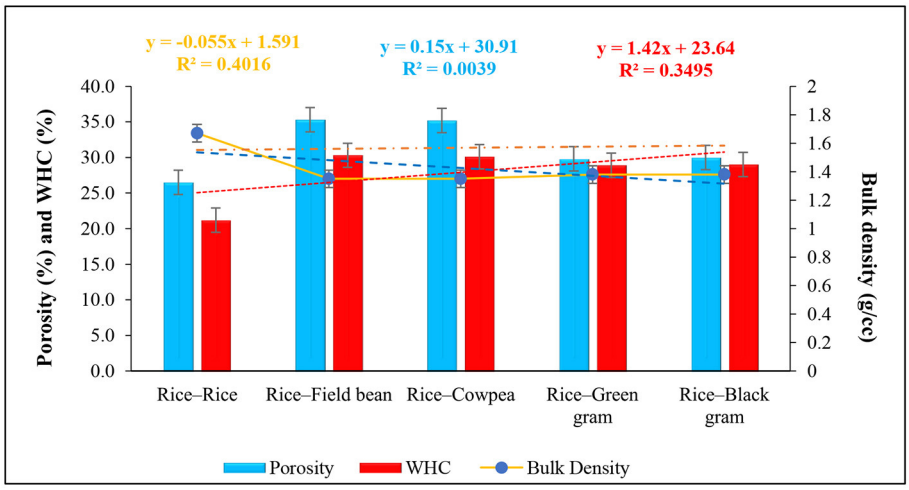

FIGURE 5
Inclusion of pulses in rotation significantly improved soil porosity ($y = 0.15x + 30.91$; $R^2 = 0.0039$), water holding capacity ($y = 1.42x + 23.64$; $R^2 = 0.3495$), and reduced bulk density ($y = -0.055x + 1.591$; $R^2 = 0.4016$). The rice–field bean (33.21, 42.92, and −19.16%), rice–cowpea (32.83, 41.98, and −19.16%), rice–mung bean (12.45, 36.32, and −17.37%), and rice–urd bean (13.21, 36.79, and −17.37%) improved porosity, WHC and bulk density, respectively over the mono-cropping of rice–rice (*Date source*: Prakash et al., 2008).

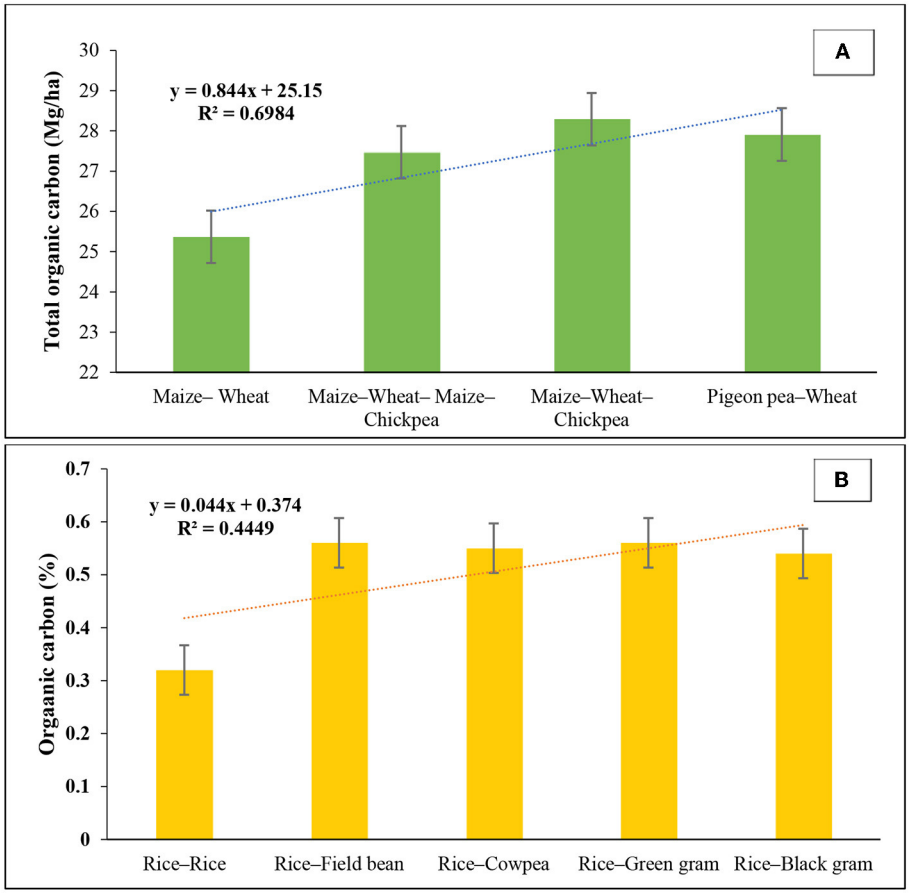

FIGURE 6
Effect of pulse-based cropping systems on SOC content. The pulse-based cropping systems have a higher soil C sequestration capacity than mono-cropping systems, **(A)** Cropping system of maize–wheat–maize–chickpea (27.5 Mg ha^{-1}), maize–wheat–chickpea (28.3 Mg ha^{-1}), and pigeon pea–wheat (27.9 Mg ha^{-1}) improved SOC by 8.3, 11.5, and 10.0% over the maize–wheat system (25.4 Mg ha^{-1}) ($y = 0.844x + 25.15$; $R^2 = 0.6984$) (*Date source*: Venkatesh et al., 2013); **(B)** The rice–field bean, rice–cowpea, rice–mung bean, and rice–urd bean added 75.0, 71.9, 75.0, and 68.8% more SOC to the soil over rice–rice monoculture ($y = 0.044x + 0.374$; $R^2 = 0.4449$) (*Data source*: Prakash et al., 2008).

and pigeon peas. The decline in soil pH is more beneficial to the following non-pulse crops that are grown under a neutral or saline soil environment. Pulses also add considerable biomass-C to the soil as leaf litter, root biomass, and release of other C compounds (Figures 6A, B). Thus, soil C stock is improved because of the combined effect of eliminating the fallow through the cultivation of a pulse crop and input of extra C to the soil through a pulse-inclusive system (Ghosh et al., 2019). The biomass-N added through these residues becomes available to the successive cereal crops upon decomposition.

Along with N in leaf, shoot, and seed, a large amount of fixed N remains stored in roots and nodules after the crop harvest, which accounts for ∼50% of cumulative N in chickpeas and ∼30% of cumulative N in lentils and peas (Herridge et al., 2008). In rotation, pulses produce lesser but higher-quality biomass than cereals. They also aid in more biomass production of subsequent non-pulse crops through N benefits that positively impact soil productivity (Liu et al., 2019). Along with N economization in the agroecosystem, the green manuring of pulse-based residues (e.g., mung bean) improves available soil P as the root exudates help to mobilize the soluble P in soil. Roots of pulses are known to release certain acidic substances and H^+ (hydrogen) ions into the rhizosphere, which solubilize the native insoluble P and thus ensure its availability to the plants. For instance, the secretion of citric acid from roots acidifies the rhizosphere zone that solubilizes P from Ca–P complexes.

Pulse-based rotations are also known to improve the soil's biological environment by releasing low-molecular-weight organic substances *via* root exudates. Soil microbes use these compounds as a source of energy and multiply quickly. The results of a field experiment revealed a more significant soil microbial population and more biomass in the rice–wheat–mung bean and maize–wheat–mung bean systems, compared to those in the rice–wheat and maize–wheat rotation systems, respectively (Kushwaha et al., 2007-2008). The increased microbial population and their functionaries speed up further the process of mineralization–mobilization following the soil environment. The microbial, enzymatic activities and biochemical processes also increase upon soil incorporation of residues of pulses, which over the long term improve soil health and overall system productivity.

Cereal + pulse intercropping is identified as the best intercropping system in the rainfed agroecosystem that helps improve soil health, eliminates soil sickness resulting from monocropping, breaks insect–pest and disease cycles, and has a smothering effect on weeds (Mobasser et al., 2014). Pulses while intercropping also minimize or reduce environmental issues through effective utilization of available resources (i.e., water, nutrient, land, and energy) and fulfill the needs of diverse, balanced food and nutrition needs for poor and marginal farmers. The cereal + pulse intercropping systems under rainfed conditions provide the cereal component's full yield, in addition to the bonus yield of up to 60% of the pulse crop (Ghosh et al., 2007). From a competition point of view in the intercropping system, component crops strive for different resources due to their distinct growth and rooting pattern. For instance, in maize + mung bean intercropping, there is minimal competition for resources because of their differences in peak resource requirement (Adarsh et al., 2019). Similarly, maize + pigeon pea is one of the best examples of minimum competition among component crops. Maize would have already completed its life cycle by the time pigeon pea needs the maximum nutrients at flowering. Thus, the entire system is benefitted through a higher weed smothering efficiency and reduction in the infestation of insect pests and diseases, apart from improving most yield traits due to synergetic interaction. Pulses significantly reduce the N requirement of the component crop and thus decrease external chemical fertilization and associated soil toxification due to chemical leaching in deeper soil strata. In an experiment (Layek et al., 2018) on the intercropping of maize with legume crops, the maize crop responded to up to 60 kg N ha^{-1} application. In contrast, the sole maize responded to a dose of 120 kg N ha^{-1} because the component legume significantly contributed to the N needs of maize. Therefore, pulse-based rotations are needed to sustainably intensify the existing cereal-based production system toward a pulse-based production system in rainfed environments.

5.2. Sustainable intensification through a pulse-based rainfed agroecosystem

A continuous increase in food demands for the escalating global population under climate change puts significant pressure on the availability and quality of natural resources (Kumar et al., 2016). Resource constraints over the soil, water, and biodiversity will also directly affect agricultural production in a rainfed agroecosystem in the coming time (Pretty and Bharucha, 2014). At the same time, the rice–wheat system—a significant food production system in South Asia is a supplier of nearly 40% of India's total food production (Gupta and Seth, 2007), causing higher exploitation of natural resources, including groundwater and soil fertility. The yield of the cereal-based cropping system is also either declining or stagnant, which may create problems in achieving the goal of 60% more food production to feed the projected population of 9.1 billion by 2050 (FAO, 2017). The perils of severe water shortage and its deteriorating quality, soil sickness, dwindling economic profitability, and deteriorating available natural resources are some of the barriers to the sustainability of the agroecosystem in South Asia (Chaudhury et al., 2016; Gupta and Kumar, 2019). Therefore, global farmers are challenged to produce more with limited natural resources under adverse conditions.

Food security and production sustainability can be achieved by more efficient input use through desirable modifications to the existing agroecosystem and input management, seeking to capitalize on a more efficient natural resource base (Kumar et al., 2017c; Rani et al., 2020). The SI is an agroecosystem production process wherein crop production is improved without adversely impacting the environment, degradation of natural resources, and cultivation of more land (Pretty and Bharucha, 2014). This implies the use of SI in attaining more food production, advanced environmental goals, and services by various means. The SI seeks to shift toward natural services and social capital without trading off crop productivity and environmental safety. A better concept of SI focuses on the intensification of available resources, making their better use, *for example,* water, land, biodiversity, soil fertility, and technologies (Pretty and Bharucha, 2014). Along with achieving adequate crop production, SI also takes care of ecosystem services and natural resources to maintain productivity factors and mitigate the consequences of the green revolution (Hazra and Bohra, 2020).

For the sake of the sustainability of the agroecosystem, there is a need to formulate and implement some alternative strategies that produce enough diversified and balanced food for the global population and feed to soil with safeguarding the production environment. Pulses are the most realistic and potential crops for SI as these are more eco-friendly and efficient resource utilizers that may become a means to improve system productivity and higher economic returns for the small and marginal farmers in the rainfed ecosystem (Jangir et al., 2016). As these are low-input-requiring crops, they can be grown widely on marginal and wastelands of rainfed areas with fewer management practices and limited water, labor, and other inputs (Adarsh et al., 2019). Recently, the concern about expanding acreage under pulses is growing to progressive niches to achieve self-sufficiency in pulse production.

The sustainability and production economics of a cereal-based system can be significantly enhanced by the horizontal expansion of areas by bringing the fallow periods under cultivation of short-season pulse crops in a rainfed agroecosystem. In addition, to improve the grain and protein yield of the subsequent non-pulse crop, diversified crop rotation also ensures the sustainability of land use and production system. Three types of the fallow period are there in rainfed agroecosystems, *viz*., summer fallow or pre-*Kharif* fallow (April–June), rice fallow (November–February), and fallow period in drylands (*Kharif* fallow in the absence of adequate rainfall or *rabi* fallow due to lack of residual moisture). After harvesting the *rabi* crops and planting *Kharif* crops, there is a summer fallow of ∼80–90 days. Summer fallowing leaves land unprotected and bare, deprived of any crop planted for one growing season, generating a chance to lower production in a rainfed agroecosystem (Gan et al., 2015). In addition, summer fallowing has heavy soil and environmental consequences. Therefore, an alternative production system needs to be identified that can overcome the negative impacts of summer fallowing and redeem positive benefits apart from advancing system productivity (Ghosh et al., 2019). Shifting this summer fallow toward pulses in the cereal-based system is a win–win situation in terms of system productivity, net profitability, water productivity, efficient energy utilization, and reduced global warming potential (GWP) (Figure 7) (Kumar et al., 2018).

This short period could be diversified and effectively utilized by taking short-duration pulse crops such as mung bean, cowpea, and urd bean, under the rainfall, irrigation facilities, and production cost (Gan et al., 2015). In this context, Ghosh et al. (2019) noted 10–14 and 5–11% improvement in grain yield of rice and wheat, along with 70 and 79% higher system productivity in lowland rice–wheat and maize–wheat systems, respectively, with the inclusion of pulses (mung bean) in the summer fallow period (April–May) at Kanpur, India. The replacement of wheat with chickpea in the lowland rice–wheat system also improved rice grain yield by 5–8%. Long-term experiments are an effective tool for identifying system productivity, compatibility of component crops, stability, and economic efficiency. Considering this, after analyzing for 30 years, St. Luce et al. (2020) reported that the wheat–canola–wheat–field pea system gained 14–38% higher economic yield concerning continuous wheat and summer fallow–wheat–wheat system in semi-arid regions of Saskatchewan, Canada. This shows that diversifying existing summer fallow and monoculture with pulses has better fallow utilization, better soil health, improved protein-rich yield of the subsequent crop, and bonus yield from pulses that provide nutritious and health-conscious food and economic advantages (St. Luce et al., 2020; Wang et al., 2020). Although, for promoting pulses in summer fallow, adequate availability of quality seed, reducing production costs, managing labor costs, and optimizing production techniques must be accomplished. Farmers accept and adopt any technology only when it is economically flexible. Furthermore, it is also vital to select the pulse crop to optimize resource utilization by considering the suitability of the agroecosystem. Although, changing the existing agroecosystem may face other challenges in addition to the inadequacy of irrigation facilities which is the major challenge obstructing the cultivation of summer pulses in cereal-based rotation in the rainfed ecosystem. Growing mung bean in summer fallow demands frequent irrigation due to high evaporative demands that are not sustainable, resulting in a rapid decline in groundwater. Although, black beans could be a better option during March–May for SI, specifically in rice–maize, rice–potato, etc., in eastern India (Hazra and Bohra, 2020). Therefore, this new system needs an assessment of resource use efficiency of water, N, and ecological footprint in each unit of land and production system. Still, the widespread adoption of summer pulses, instead of leaving fallow, will require finding more reasons for the non-adoption of this system and focusing on solutions through adaptive research in different agro-ecological regions.

India alone has ∼11.695 Mha fallow area out of 22.2 Mha of entire fallow regions of south Asia after harvesting rice. Of this, ∼82% falls in the Eastern States, including West Bengal, Odisha, Jharkhand, Assam, Bihar, Chhattisgarh, and Eastern Uttar Pradesh, and the remaining lies in Peninsular India, *viz*., Karnataka, Tamil Nadu, and Andhra Pradesh (IIPR, 2016). These areas are from where rice is taken in the monsoon season (June to October), while the period between November and February remains uncultivated. This is due to the lack of irrigation and good rainfall, low residual moisture, excessive moisture in December, soil compaction and cracking in vertisols, growing long-duration rice varieties, lack of appropriate pulse varieties, and the problem of stray animals. Short-duration and high adaptive capacity with fewer inputs under climate change fit pulses best in these fallows to increase the overall system productivity (Table 8).

Therefore, diversifying existing central cereal-based systems with the inclusion of pulses during the fallow period could prove a trump card for sustainable yield, food security, and restoration of soil health in a rainfed agroecosystem (Sravan and Murthy, 2018). Taking another crop in rainfed rice fallow has a lot of challenges, including unfavorable soil properties, due to puddling, rapid residual soil moisture depletion, unavailability of appropriate cultivars, socioeconomic status of farmers, and lack of awareness among them (Kumar et al., 2017d; Hazra and Bohra, 2020). Pulses help eradicate rice production's existing constraints, such as depleting natural resources, yield stagnation, and other soil and environmental issues, which may facilitate economic development and alleviate hunger and poverty. The traditional relay cropping can be followed in eastern India, where long-duration rice cultivars are grown, wherein pulse seeds are broadcasted in standing rice 10–12 days before rice harvesting, permitting efficient utilization of the residual soil moisture. The rotation of pulse crops in rice-fallow needs locally adapted short-duration rice cultivars, water-efficient and early maturing pulse cultivars, and farm mechanization (zero tillage machinery). The rice fallow areas are not much

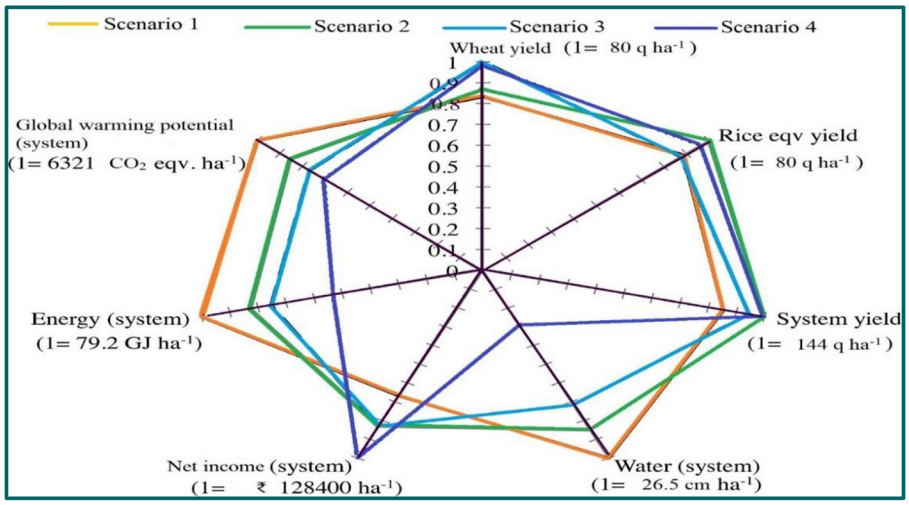

FIGURE 7
Several indicators of assessing the long-term performance of diverse scenarios in IGP at Karnal, India. The reduced–till rice–wheat–mung bean system (scenario 2) produced considerably greater system yield (17%), net return (24%) with 17, 15, and 15% fewer energy inputs, irrigation water, and global warming potential (GWP) over conventional rice–wheat–fallow system (scenario 1). By shifting from scenario 2 to CA-based rice–wheat–mung bean system (scenario 3) there was further saving of 9, 15, and 10% energy inputs, irrigation water, and GWP with similar profitability but yield reduction of 8 q ha^{-1}. The shifting from scenario 3 to scenario 4 (CA-based maize–wheat–mung bean system) gave 21% more net income than scenarios 2, 3, and 5% more system yield than scenario 3 with 29–35% fewer energy inputs, 59–66% less irrigation water, and 8–18% less GWP than scenario 2 and 3 (Modified; Kumar et al., 2018). Variable means are normalized on a 0–1 scale, where 1 represents the greatest absolute value of that variable. The highest absolute value is also shown for each parameter.

TABLE 8 Potential pulse crops and potential varieties for rice fallows in different states (NAAS, 2016; Singh et al., 2017).

Crop	States	Suitable varieties
Lentil	Assam, West Bengal, Bihar, Odisha, Eastern Uttar Pradesh, Chhattisgarh, and Jharkhand	Narandra Masoor 1, WBL 58 KLS 218, HUL 57
Pea	Jharkhand, Chhattisgarh, Eastern Uttar Pradesh, and Northern Madhya Pradesh	IPFD 10–12, IPFD 11–15, KPMR 400, Prakash, DMR 57 and 11, Malviya Matar 15, Rachana
Chickpea	Chhattisgarh, Bihar, Uttar Pradesh, Jharkhand, West Bengal, and Madhya Pradesh	Pusa 372, Pusa 547, JG 14, JG 16, Rajas, Pant G 186, GCP 105
Mung bean	Odisha, Andhra Pradesh, Tamil Nadu, and Karnataka	IPM 2–14, Virat, Shikha, HUM 16, TU 40, VBG 04-008, LBG 787, TM 2000-2
Urd bean	Coastal Andhra Pradesh, Tamil Nadu, Karnataka and Odisha	LBG 402, LBG 752, LBG 709, Pant U 31, IPU 2-43
Grass pea (Lathyrus)	Tal area of Bihar, Chhattisgarh, and West Bengal	Prateek, Ratan, Mahateora
Lablab bean	Andhra Pradesh, Tamil Nadu, Karnataka, Jharkhand and Bihar	Arka Vijay, Pusa Sem 2 and 3, CO 12, 13, and 14, KDB 405

explored; therefore, breeding and identifying new varieties suited to the soil and agroecological conditions, improved management practices, and technological intervention are primarily essential. For this, there is a strong need for a robust database and associated human, organizational, and governmental support in the agronomic management of pulses, which are highly exposed to climate change as pulses are crops of a rainfed agroecosystem (Lal, 2017).

The formulation and implementation of an appropriate scheme have a great role in exploring these fallow lands for cultivating pulses using improved management technologies. Recently, the Indian government has taken outstanding initiatives to screen and identify pulse crops and their cultivars, agronomic management, and other promotional technologies. To promote pulse cultivation in these fallow areas, recently in 2016–2017, a scheme Targeting Rice Fallow Areas (TRFA) was implemented under Rashtriya Krishi Vikas Yojana (RKVY) in eastern India by the Department of Agriculture Cooperation and Farmers Welfare (DAC&FW) targeting 4.5 M ha rice fallow to bring under winter pulses in the next 3 years by releasing a fund of ₹ 75 crores (US$ ~10.3 million) (NAAS, 2016). In 2016–2017, the TRFA was initiated in 15 districts of 6 states (West Bengal, Odisha, Jharkhand, Chhattisgarh, Bihar, and Assam) to cover 19.14 lakh ha areas. In 2017–2018, the government extended the areas of the scheme to 40 districts and 4,000 villages to cover 15 lakh ha with the support of Minikit distribution, cluster demonstration, and farmers' training (MoA&FW, 2018). Most rice fallows were covered with lentils, peas, mung beans, chickpeas, urd beans, pigeon peas, and lathyrus. The government is further planning to extend the TRFA program in the rice fallows of southern, northeastern, and Himalayan states by the year 2022 with new norms of assistance (MoA&FW, 2019). Several institutional and organizational projects are ongoing in different regions of the country to strengthen pulse cultivation in fallow soils. The government is also promoting areas

under pulse cultivation to enhance their production, specifically lentils, chickpeas, and lathyrus in rice fallow through another plan, National Food Security Mission-Pulses (NFSM-Pulses). The NFSM-funded projects-CG institutes are International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)—Enhancing chickpea production in rainfed rice fallow land of Chhattisgarh and Madhya Pradesh (2008), International Center for Agricultural Research in the Dry Areas (ICARDA)—Expanding lentil production in Eastern and Northeastern states under rice-based production system in India (2010), and ICARDA—Enhancing grass pea production in Eastern and Northeastern states under the rice-based production system (2010). The farmers are encouraged to grow pulses under the NFSM through cluster demonstration, quality seed distribution, arranging for improved machinery/tools/technologies, water application tools, and other facilities. To support this scheme, other governmental plans like Mahatma Gandhi National Rural Employment Guarantee Act (MGNREGA), RKVY, and Pradhan Mantri Krishi Sinchayee Yojana (PMKSY) are also contributing by developing rainwater harvesting structures like farm ponds, which can be used further as supplemental irrigation to enhance the survival rates and productivity of pulses in rice fallow. In addition, other research and development activities undertaken for promoting pulse cultivation in rice fallow are the All India Coordinated Pulse Improvement Project (AICRP) (1967) on Mung bean, Urd bean, Lentil, Lathyrus, Rajmash and Pea (MULLaRP) (1995), mitigating abiotic stresses and enhancing resource-use efficiency in pulses in rice fallows (2010), and brainstorming meeting on rice fallows (2013). Pulses are climate smart as they simultaneously adapt to climate change and contribute toward mitigating its effects. Therefore, introducing them to farming systems can be vital to increasing resilience to climate change through diversification of the income source, increased stability to climate extremes, and increased productivity.

6. Adaptation potential of pulses to climate change

Climate change, food production, and food security are interconnected; therefore, it is impossible to evaluate them independently. Climate change gives rise to several abiotic stresses, including drought, cold, heat waves, flood, submergence, high temperature, and increased attacks of insects, pests, and diseases (Kumar et al., 2019c; Sheoran et al., 2022b). This climate change is putting an extra burden on already water-stressed systems and will intensify the competition for water with other sectors. Heat stress and water scarcity are probably the most acute stresses faced by the agricultural production system (Pereira, 2016), which will be aggravated more with the predicted increase in temperature by 2–4°C over the next 100 years in a rainfed agroecosystem. Pulses have an inherent capacity to adapt and perform well under climate change because of their hardy nature to fight abiotic stresses (Table 9).

Pulses can withstand harsh conditions and survive well under water scarcity since they require fewer inputs than input-intensive cereals. Therefore, they play an essential role in climate change adaptation as water becomes scarce and the temperature rises. On a global scale, many neglected pulse species can be used for specific niches because of their adaptation to marginal environmental conditions. Therefore, pulses will be a game-changer in the impending climate change era, owing to their capacity to supply nutritious food under punitive soil and ecological requirements. Globally, pulses, beans, and cowpea demands are expected to increase by 155% from 2015 to 2050 (Kissinger, 2016). Despite India being the world's largest producer of pulses in the world, the country will need to increase pulse production by 32 and 39 million tons by 2030 and 2050 to fulfill the fast-growing population's domestic needs (Dixit et al., 2015). Therefore, to attain the targeted food production under the growing stresses of climate change, pulses are the first choice of farmers and scientists for enhancing climate change resilience as they adapt better to changes in climatic stimuli (FAO, 2016a).

Pulses differ in morphophysiological traits and have wider adaptability than cereals; naturally, they are drought-tolerant crops and can sustain and produce an economic yield in the rainfed agroecosystem. Pigeon pea is one of the most drought-tolerant pulse crops that can also be produced under drought spells. The ability of pigeon peas to perform even under drought conditions is attributed to a higher osmotic adjustment in leaves (0.1–1.6 MPa; mega pascal) (Basu et al., 2018) and a deeper root system. Osmotic adjustments help the pigeon pea plant to moderate stomatal conductance and photosynthetic functions, even at low water potential, diminish floral dropping, delay leaf senescence, and improve the rooting system and water extraction from deeper soil strata (Basu et al., 2018). Some of the identified pigeon pea varieties having higher osmatic adjustments and adaptive capacity to drought conditions are AL 201, AL 1855, TGT 501, BSMR 853, BDN 708, BDN 2008-12, ICP 13673, ICP 84031, VKS11/24-1, VKS11/24-2, GRG 815, GRG 2009-1, JKM 7, MAL 13, RVK 275, Bahar, and Bennur Local (Basu et al., 2018). Naturally, lentils can grow well under fewer water conditions, and even supplying irrigation/rainfall before the reproductive stage of the crop causes significant yield losses. It can withstand water-stressed conditions through drought avoidance and tolerance mechanisms (Gupta et al., 2019). Its drought avoidance mechanisms involve the availability of short-duration cultivars and associated early flowering, faster early crop growth, extensive rooting network, and higher yield potential. In addition, various morphological features, such as leaf surface, length, orientation, stomatal behaviors, and canopy structure, pay to drought avoidance mechanisms for climate change adaptation (Biju et al., 2017). Early maturity of lentil cultivars helps to mature and set seeds before the commencement of terminal heat, water stress, and resultant enforced maturity that causes yield loss. Recently, efforts have been made by ICARDA to develop short-duration lentil cultivars to escape from terminal heat stress, such as BARI M 5, BARI M 4, BARI M 6, Idleb 3, Bakaria, and Precoz, without any yield reduction (Basu et al., 2018). While the drought tolerance mechanisms show dense leaf pubescence, a high osmotic adjustment of the leaf (0.6 MPa), controlled stomata closure, and increased response of antioxidants (Gupta et al., 2019).

Furthermore, the wild landraces of lentils are more resilient to climate change and can be included in the breeding program of lentils to enhance their adaptive capacity to climate change. In chickpeas, a higher osmotic adjustment (0–1.6 MPa) and the production of glandular hairs are important adaptive traits for delaying the effects of drought, either by reducing stomatal density and/or transpiration or by increasing leaf reflectance that helps in more interception of non-photosynthetic radiations (Basu et al., 2018). Even these winter pulses (e.g., chickpeas and lentils) require physiological stress (i.e., drought) to terminate flowering and induce seed set. Summer pulses, such as mung bean and moth bean, do not perform well under

TABLE 9 Adaptive traits of pulse crops to climate extremes.

Pulse crop	Niche	Adaptive traits	References
Chickpea	Semiarid tropics and dry areas	Heat and water-stress tolerant; resistance to pod borer, Fusarium wilt, A*schochyta* blight, *Botrytis* gray mold, and root rot	Whish et al. (2007)
Pigeon pea	Tropical climate, semiarid and less humid regions	Short stature; resistance to Fusarium wilt, *Phytophthora* stem blight, sterility mosaic virus, pod borer, and pod fly	Kumar et al. (2019a)
Mung bean	Arid and semiarid regions, warm-season	Very short duration, photo-and thermal insensitive; heat and water-stress tolerant; favors stressed conditions; resistance to powdery mildew and mung bean yellow mosaic virus	Tickoo et al. (2006), Gupta et al. (2019)
Moth bean	Arid regions, warm climate	Early and synchronous maturity, erect plant habit; tolerant to heat and water stress; grown in low soil fertility; tolerant to yellow mosaic virus	Tiwari et al. (2018), Gupta et al. (2019)
Urd bean	Semiarid regions, hot humid season	Very short duration, photo-and thermal insensitive; tolerant to excessive moisture stress; resistant to powdery mildew and mung bean yellow mosaic virus	Gupta et al. (2019)
Cowpea	Wider adaptation, arid and semiarid regions	Early maturity, fast initial growth, and better source/relationship	Kumar and Dixit (2003), Hall (2012)
Pea	Semiarid climate, cool areas	Dwarfness, tendril, leaflessness, and earliness; tolerant to terminal heat and water stress; resistance to powdery mildew and rust	Kumar and Dixit (2003)
Lentil	Semiarid tropics and dry areas	Heat and water-stress tolerant; resistance wilt, A*schochyta* blight, Stemphylium blight, rust, and black aphid	Sarker and Erskine (2002)
Lathyrus	Indian and Mediterranean region	Tolerant to both excessive water and drought conditions	Kumar and Dixit (2003), Kumar et al. (2019a)
Horse gram	Arid and semiarid regions	Early maturity, green foliage up to maturity; thermo-insensitive, tolerant to water stress and soil acidity	Kumar and Dixit (2003), Kumar et al. (2019a)
Common bean	Tropical climate	Short height, early maturity; tolerant to heat, water stress and cold; resistant to common bacterial blight	Kumar and Dixit (2003), Kumar et al. (2019a)
Lima bean	Climate of Mexico, Argentina, and Georgia	Bushy compact plant, climbing type; well suited under limited water and marginal soils	Kumar and Dixit (2003), Kumar et al. (2019a)
Rice bean	Dry regions of arid and semiarid climates	Determinate growth habits, early maturity; tolerant to water stress and soil acidity	Isemura et al. (2010), Kumar et al. (2019a)
Tepary bean	Tropical climate, dry season	Short duration, deep-rooted crop; resistance to water stress and common bacterial blight	Kumar and Dixit (2003), Kumar et al. (2019a)
Adzuki bean	Temperate and sub-tropical climate	Tolerant to water stress; resistance to bacterial blight	Kumar and Dixit (2003), Kumar et al. (2019a)
Hyacinth bean	Sub-humid and semiarid regions	Early maturity; tolerant to water stress and soil salinity	Kumar and Dixit (2003), Kumar et al. (2019a)

continuous water supply and reduced temperature. They require stressed conditions (i.e., water stress and temperature stress) for their outstanding performance, which will arise because of climate change. Hence, mung bean and moth bean, being the warm-season crops, are expected to be less affected by high temperatures resulting from climate change. In urd bean, the presence of a waxy cuticle above the epidermis coupled with dense hairs of the pod wall reduces moisture loss. Thus, the pod continues photosynthetic activities under stressed conditions. The anatomical difference in urd bean imparts drought tolerance to climatic extremes like high temperature and drought.

Pulses provide opportunities to sustainably intensify the existing agroecosystem and help to moderate the risk associated with unpredictable weather and other market issues. Food legumes, such as pigeon peas and mung bean, are a better option for inclusion in the agroforestry system for sustaining soil health, soil C sequestration, food security, and the economics of farms by diversifying sources of income (Roy et al., 2021). This system can stand more strongly against climatic extremes because of the hardier nature of pulses, along with benefitting the soil system (FAO, 2016b). Including pulses in mixed and intercropping systems conserves soil moisture and improves water availability, and thus is an excellent example of high-priority adaptation strategies to conditions of rainfall variability in a rainfed agroecosystem. Similarly, including at least one pulse crop in a cereal-based cropping system, either as an intercrop, sequential crop, or relay crop, enhances the adapting capacity of the system to climactic adversities. In addition to their excellent adaptation capacity, these crops also greatly aid in reducing greenhouse gas (GHGs) emissions, owing to their superb mitigation potential.

7. Climate change mitigation potential of pulses

Pulses in the agroecosystem contribute to climate change mitigation through GHGs emission reduction and SOC sequestration (Lal, 2017). Production and application of synthetic fertilizers in the agricultural production system are the major contributors to GHGs

emissions, whose share in emissions is increasing at the rate of 4% per year from 1961 to 2010 (Smith et al., 2014). In field crops, N fertilization contributes 36–52% of total emissions, which can be significantly reduced by diversifying the existing agroecosystem with pulses to enhance fertilizer use efficiency. Diversification cuts the C footprint of the system by 32–315% compared to continuous monoculture (Liu et al., 2016). Pulses not only have a lower need for nitrogenous fertilizers, but they also cater to the N needs of the subsequent non-legume crop in rotation, and thus, the emission of GHGs is reduced. In addition, less tillage requirement and associated fuel-saving, low irrigation requirement and related water and fuel/energy saving by pumping, and reduced herbicide application due to rapid initial ground cover through faster foliage growth (e.g., cowpea, mung bean) also reduce the emission of GHGs. To confirm the assumption, in western Canada, MacWilliam et al. (2018) tested a pulse-based cropping system under two treatments, *that is,* thoroughly recommended application of synthetic N and reduced dose of synthetic N fertilization to subsequent cereal or oilseed. They observed that the emission of GHGs from cereal and oilseed crops grown after lentil and dry peas was much higher (286–598; 888–987 kg CO_2 eq. t^{-1}) with complete synthetic N application than that from reduced N application (116–598; 311–978 kg CO_2 eq. t^{-1}) without compromising yield. This suggests that emission can be significantly reduced by growing pulses in rotation as a preceding crop as these crops offset the N fertilizer requirements of following non-legume crops and thus an overall reduction in synthetic N application. In the silt loam of Saskatchewan, Canada, Gan et al. (2011b) assessed the C footprint of durum wheat by diversifying the cropping system with pulses (e.g., chickpea, lentil, and dry peas). They found 37% reduced emissions (162 kg CO_2 eq. ha^{-1}) in a pulse–durum wheat system compared to cereal/oilseed–durum systems (251 kg CO_2 eq. ha^{-1}) due to reduced fertilizer application. Therefore, the C footprint of the pulse–durum wheat system was 28% (0.30 CO_2 eq. kg^{-1} of grain) lower than cereal–durum rotation (0.42 CO_2 eq. kg^{-1} of grain). At Swift Current, Saskatchewan, Campbell et al. (2000), based on a long-term study of 17 years, reached the result that CO_2 eq. emissions from N_2O (nitrous oxide) emissions and energy inputs were 180 and 170 kg ha^{-1} lower for wheat–lentil and wheat–field pea rotation, respectively, over the spring wheat monoculture. By making the same assumptions, Zentner et al. (2002) found a reduction of CO_2 eq. emissions by 160 or 40 kg ha^{-1} net SOC reduction upon inclusion of chickpea over the one that did not. The result of another experiment (Gan et al., 2011b) suggested that durum wheat grown after pulse crops (e.g., chickpea, lent, and dry peas) released total GHGs of 673 kg CO_2 eq. that was 20% less when durum wheat was grown after cereal crop. The C footprint of durum wheat preceded by pulses was 0.20–0.30 (averagely 0.25) (kg CO_2 eq. kg^{-1} of grain) were 32% lower than durum wheat grown after the cereal crop. Pulses in rotation also reduced the C footprint (kg CO_2 kg^{-1} of grain) of canola (0.80), mustard (0.59), spring wheat (0.46), and chickpea, lentils, and dry peas (0.20–0.30) (Gan et al., 2011a).

The N_2O emission under pulse cultivation may be more than that released under the cereal field, which must be adequately managed. Jain et al. (2016) quantified the GHGs emission under pulses, oilseeds, millets, and cereals in northwestern India. The analyzed data showed that the total amount of N_2O emissions in pulses, oilseeds, millets, and cereals was 67, 55, 43, and 40% of applied N, respectively. Sah and Devakumar (2018) analyzed the data for five decades (1960–2010) and found the maximum emission of 23.75 Tg C eq. ha^{-1} was from rice fields while it was the lowest from pulses, *for example,* pigeon pea with a value of 2.98 Tg C eq. ha^{-1}. Similarly, Jain et al. (2016) measured the lowest C equivalent emission in pigeon pea (833 kg C ha^{-1}), while it was the highest in wheat (1,042 kg C ha^{-1}). However, the GWP for pigeon peas and wheat was 3.05 and 3.97 tons CO_2 eq. ha^{-1}, respectively, but the GWP per unit of seed yield was the highest in pulses and the lowest in cereals might be due to a higher emission of N_2O in pulses.

Pulses promote soil C sequestration by improving soil organic stock contributed by fallen leaves, straw, roots, rhizodeposition, *etc.*, and by improving microbial biomass carbon (MBC) (Kumar et al., 2018). A significant fall of 3.0 and 1.67 Mg ha^{-1} leaves (dry weight) was found in pigeon pea crops sown during rainy and postrainy seasons, respectively. In addition, chickpeas and lentils also have a high leaf fall during senescence. The way of the addition of leaf C to the soil can significantly contribute to the soil C stock. In IGPs of India, Venkatesh et al. (2013) observed that the diversification of the existing maize–wheat system toward a more remunerative pulse-based system, *that is,* maize–wheat–mung bean or pigeon pea–wheat significantly improved the SMBC by 10–15%. In fine sandy soils of the south-central portion of Washington, Wang et al. (2012) found that the growing of faba bean added a maximum amount of soil C (597 g C m^{-2}) into the soil compared to other winter crops, purple vetch (378 g C m^{-2}), triticale (369 g C m^{-2}), ryegrass (342 g C m^{-2}), mustard (247 g C m^{-2}), and white bean (149) g C m^{-2}. In IGPs, Venkatesh et al. (2013) revealed that pulses (e.g., mung bean and pigeon pea) in rotation had nearly 10% more organic C level in soil and C management index than those obtained without pulses, *that is,* maize–wheat system. In IGPs, the inclusion of pulses in the traditional puddled rice–wheat system improved the soil C flux and MBC by 6 and 85% as regards the conventional practice of a continuous rice–wheat system (Ghosh et al., 2012). In Asia, also the growing of mung beans in the summer fallow of the existing rice–wheat system produced an additional 4.5 Mg ha^{-1} dry matter that directly contributed to the soil C pool (Yaqub et al., 2010). In the black soils of semi-arid tropical India, Chaudhury et al. (2016) observed that monocropping of cotton had no C addition to the soil. Still, the organic and inorganic C increased by 0.75 and 1.12% upon rotation with pigeon peas. Pulses have a positive impact on the emission reduction of GHGs, but still, it is small. The improved N availability and associated soil quality under pulses in rotation improve the soil's organic status, which helps to reduce GHGs emissions. Although, the buildup of the C pool in soil dramatically depends upon the pattern of C translocation among different species. For instance, pasture crops translocate 30–50% of biomass belowground, equivalent to around 2.2 Mg C ha^{-1}; in cereals, this figure is 20–30%, corresponding to ~1.5 Mg C ha^{-1} (Kuzyakov and Domanski, 2000). The C:N (CN ratio) of pulse crop root, shoot, and seed are also considered crucial determinants for enhancing soil C stock. Usually, the CN ratio of pulses stover (e.g., lentil, chickpea, and peas) is ~17 compared to wheat (~32) and oilseeds (e.g., mustard, canola, and linseed) (~41). Likewise, root C mass in the top 20 cm significantly differed with crop species as 41% for chickpeas, 55% for lentils, peas, linseed, *etc.*, while in mustard, wheat, and canola, it was 66% (Gan et al., 2011b). In search of water and nutrients, chickpea roots got more profound in the soil profile, which after harvesting, leave root residues at a greater depth and thus are less prone to microbial decomposition. Ghosh

et al. (2012) found that in the rice—wheat system of IGPs, taking chickpeas, instead of wheat, totally or as a minimum in alternate years added more organic C to the soil over the rice–wheat system. So, the extensive root system of pulses is the critical factor in conserving available natural resources, including water, along with an increased nutrient uptake and associated energy conservation.

8. Pulses for enhancing resource use efficiency

Soil, water, energy, nutrients, air, biodiversity, forest, watershed, and vegetation are significant gifts of nature whose conservation is a must to sustain crop production. The degradation of these resources is an important environmental issue that drastically affects the biological system and sustainability of agroecosystems throughout the globe (Kumar et al., 2018). The misuse of soil and water resources makes the production system sick, leading to a reduced response of applied inputs such as fertilizer, irrigation, agrochemicals, tillage, *etc*. At the same time, climate change threatens crop production, causing significant challenges to food security and efficient resource management (Bajiya et al., 2017; Lakhran et al., 2017). Fulfilling the requirements of growing food demand and efficient resource management will not be accessible without significant changes to the existing production systems for long-term ecological sustainability. Pulses have become the brand ambassador of resource-efficient agriculture in rainfed agroecosystems, owing to their limited demands of water, energy, and nutrients along with C-efficient and ability to grow well in poor and marginal soils. These crops also get a high net return to the farmers with the reduced cost of cultivation and higher marketing price.

Diversifying the existing agroecosystem with pulses is more productive and profitable and bridges the gap between food and nutrition, while being resilient to extreme climatic events. Pulses are water-efficient crops as they consume less water and have the capacity to grow on residual soil moisture, such as chickpeas and lentils, in northern and central India, and mung bean, urd bean, cowpea, chickpea, lathyrus, and lentil in rice fallows of southern, eastern, and northeastern India during November to February. At the same time, winter pulses (i.e., chickpeas and lentils) can be grown with only one irrigation in IGPs, whereas at the same time, the wheat crop requires 5–6 irrigations (Kumar and Yadav, 2018). Pulses have lesser water demand and higher WUE than cereal, oilseeds, and other commercial crops because of their morphological and physiological characteristics, as described in section 7 and Table 9. The water requirement for rice is 100–200, 30–40 cm for wheat, and 150–250 cm for sugarcane, while pulses need only 15–25 cm of water (Kumar and Yadav, 2018). Therefore, with the same quantity of water, ∼4.8 times more areas can be occupied under pulses than rice. Cereals consume ∼60% of global water, while pulses consume only 4% of global water (Kumar et al., 2018). Pulses on average require only 2,500 gallons of water to produce one ton of seeds, while an equal amount of eggs, chicken, pork, and beef is being produced by ∼3,200, 4,500, 5,900, and 20,700 gallons of water, declaring them very water-efficient crops. In this line, the production of one kilogram of *dal* (pulse splits) requires ∼1,250 liters of water, while the output of the same quantity of chicken, mutton, and beef requires 4,325, 5,520, and 13,000 liters of water (FAO, 2016a), hence, pulses are climate-smart crops with few water requirements. The more profound and extensive network of roots enables them to extract moisture from deeper soil sections, strengthening their survival ability in the rainfed system. In silt loam soils of Swift Current and Stewart Valley of Saskatchewan, Canada, Gan et al. (2009) found that the average WUE of pulse crops (*i.e.,* chickpea, lentil, and dry peas) was 4.08 kg ha^{-1} mm^{-1}, significantly more than oilseeds (3.64 kg ha^{-1} mm^{-1}) and spring wheat (5.5–7.0 kg ha^{-1} mm^{-1}). They also critically observed that the pulses removed most of the water from the top 0.6 m of soil and left behind more than wheat and oilseeds. This unused and conserved soil moisture could be used better by a deep-rooted crop to be raised after pulses which may improve the WUE of the entire cropping system in rainfed environments. In another study at Swift Current, Saskatchewan, Canada, Miller et al. (2001) also observed the improved WUE in pulse crop (dry peas) (9.1 kg ha^{-1} mm^{-1}) concerning the wheat (6.4 kg ha^{-1} mm^{-1}) under fallow and stubbled-crop conditions. The WUE of dry peas varied from 59 to 186% (averagely 132%) compared to wheat crops due to the higher productivity of dry peas and reduced water withdrawal below 0.6 m soil section (Wang et al., 2012). The study by Wang et al. (2012) suggested that among pulses, dry peas had the greatest WUE with an average value of 8.3 kg ha^{-1} mm^{-1} while the lowest was in chickpeas (5.62 kg ha^{-1} mm^{-1}). Similarly, in Mediterranean types' conditions of South-eastern Australia, Siddique et al. (2001) found that the maximum WUE for seed yield in peas was up to 16 kg ha^{-1} mm^{-1}, while the WUE for dry matter production in faba bean and narbon bean was maximum with a value of 30 kg ha^{-1} mm^{-1}.

Integration of pulses into cropping systems enables the fixation of free atmospheric N into a plant-available form for their own use and then hands over the leftover N to the companion crop. This ensures a better utilization of nutrients under intercropping systems through complementary interaction and niche facilitation in the rooting zones of companion crops, reducing competition among component crops for nutrients, and thus enhancing efficiencies of nutrient use (Gitari et al., 2018; Punia et al., 2020). Likewise, the roots of pulse crops exude particular types of chemical substances that can solubilize native P in soil by replacing the P from the exchange site, thus increasing its availability in soil solution, which the intercropping system can efficiently utilize. The higher N use efficiency (NUE) of pulse crops is associated with the gathering and accumulation of N in their plant system rather than the release in the atmospheric system (Hocking and Reynolds, 2012; Kumar et al., 2022b). The improved NUE reduces their application through fertilizers and minimizes groundwater pollution and the circulation of chemical ions in the food system, ensuring healthy food production. Gan et al. (2015) in Saskatchewan, Canada, in a 3-year pulse-based cropping cycle, found that diversifying summer fallow with pulse crops enhanced the fertilizer-N use efficiency (NUE_f) by 33% over the summer fallow in rainfed dry areas. The pulse cropping system improved the NUE_f by 36.6 and 62.6% for seed and protein yield, respectively, over the fallow period. Similarly, compared to the cereal monoculture, the improvement in NUE_f for seed and protein yield was 99 and 186.6% in the pulse system. Deep-rooted pulses such as lablabs have the capacity for a better exploration of water and nutrients from deeper soil horizons, so competition for these resources in the surface profile is reduced, and shallow-rooted crops such as potatoes get the benefit. Using the concept, in Nairobi, Kenya, Gitari et al. (2018) reported a significant improvement in N and phosphorus use efficiency (NUE and PUE) in pulse + potato intercropping over sole potato planting. The NUE of potato + lablab, potato + bean, and potato + peas was

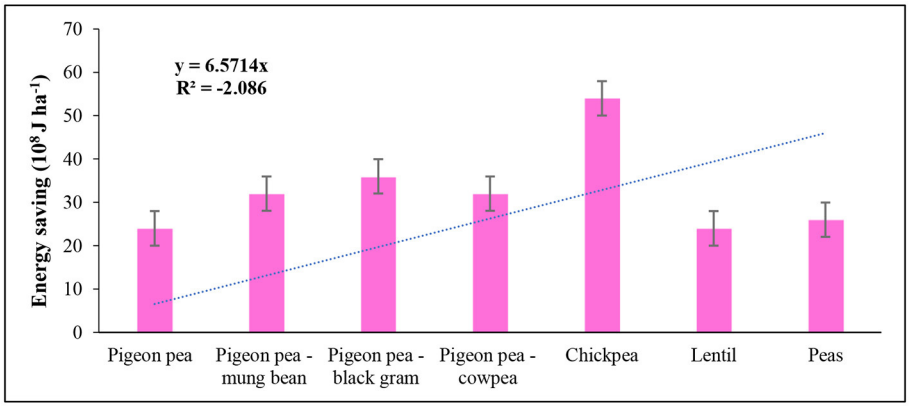

FIGURE 8
Quantum of energy saved by pulse crops through *N* economy ($y = 6.5714x$, $R^2 = 0.7475$). Sole cropping of chickpea saved maximum energy (54×10^8 J ha^{-1}) by saving 68 kg N ha^{-1} followed by pigeon pea–urd bean rotation (36×10^8 J ha^{-1}), while the lowest energy saving was observed from both lentil and pigeon pea (24×10^8 J ha^{-1} each) having N economy of 30 kg N ha^{-1} (*Data source:* Ahlawat and Srivastava, 1997; Kumar and Yadav, 2018).

30, 19, and 9% greater than that of the sole potato, respectively, while in the case of PUE, this improvement was 6, 14, and 21% compared to that of potato monoculture. In the semi-arid regions of Saskatchewan, Canada, St. Luce et al. (2020), in a long-term experiment of 30 years, concluded that the wheat–canola–wheat–field pea system had greater NUE_f (26.4 g kg^{-1}) and NUE (4.1 g kg^{-1}), compared to those attained from green manure–wheat–wheat system (18.1 and 2.7 g kg^{-1}, respectively). Fertilizer is the most significant proportion of total energy inputs whose production, packaging, transportation, and application consume a lot of energy. A third of the total energy input to crop production goes to the production of fertilizers, one-third to mechanization, and one-third to other inputs, including electricity, labor, pesticides, and transportation. According to a study, diesel and fertilizer share 61.94% of the total consumption of energy, followed by electricity (16.01%), chemicals (10.19%), and human power energy (8.64%) (Hatirli et al., 2006). Pulse crops require lesser fertilizers than cereals, specifically nitrogenous fertilizers (~20 kg N ha^{-1}), while cereals (rice and wheat) need 120–150 kg N ha^{-1}. This saving of nitrogenous fertilizer by more than 100 kg ha^{-1} essentially saves energy (Figure 8). Amenumey and Capel (2014), in an experiment, reported that dry bean requires only 3% energy from fertilizers out of the total energy inputs that were significantly lower than those of oilseeds (10–41%), potato (25%), grain crops (19–60%), vegetables (12–30%), and fruit crops (2–23%).

8.1. Pulses for soil health restoration and chemistry in a rainfed agroecosystem

Approximately 30% of agricultural land, 20% of forest land, and 10% of rangelands are affected by land degradation (Ayub et al., 2019). In addition, nearly one-third of the soils on the planet have already been degraded, and over 90% are expected to be degraded by 2050 (IPBES, 2018). Globally, land degradation affects 2,000 million ha of land, where 1,500 million people reside (FAO, 2020a). Every year, 12 million ha of land is degraded throughout the globe, corresponding to 23 ha per min. On average, soil erosion caused a loss of 24 billion tons of fertile soil that threatens the livelihood of 1.5 billion people and is likely to displace 135 million by 2045 (United Nations, 2012). According to the aforementioned estimation, it could lead to up to a 50% loss in crop yield (FAO, 2020b). Several factors are responsible for land degradation; indiscriminate use of agrochemicals/poor quality water, mining, and deforestation are vital to them. During the green revolution in the 1960s, more cultivated areas were brought under a cereal-based cropping system that decreased the areas under pulses from 13.5 to 7.5 Mha in northern India, which drastically deteriorated soil quality, subsequently associated with lower fertility and caused a severe issue of sustainable crop production (Srivastava and Mukhopadhyay, 1997). These factors individually or collectively contribute to poor crop production by surging wind and water erosion, soil salinity, desertification, soil sickness, declining soil fertility, and heavy metal contaminations (Rani et al., 2019). To sustain the production system and ecosystem services to meet the global food demand with a sloping cultivated land, appropriate measures must be taken to stop further degradation and restore the already degraded soils. This helps to improve livelihood, boost food security, and aid people to adapt to the climatic extremes.

Pulse-based crop rotation increases crop yield, improves overall soil quality, and manifests sustainable production (Jangir et al., 2019). Improved fertility status, SOC, nutrient cycling, soil structure, and aggregation, enhanced soil microbial functions, and system productivity are important indicators to restore degraded soils (Figure 9). Pulses improve the fertility status of soil by enhancing SOC, available N, P, K, and other micronutrients of importance to plants. Compared to the crop residues of cereals and others, the residues of pulse crops are N-rich, along with a significant amount of C content. Soil bacteria take energy and N from these residues and facilitate a faster conversion of soil residues to SOC (Kumar et al., 2018). Likewise, short-duration pulses can help reduce C loss from fallow lands and boost C sequestration. Venkatesh et al. (2013) reported that soil under pulse production had an ~10% higher SOC stock for longer than that without pulses. The increment in SOC stock optimizes the sulfur (S) and N cycle in soil and compensates for other negative impacts of the environment on agroecosystems. The improved SOC tightly binds the soil particles to make the aggregates more stable and resistant to breakdown. These groups

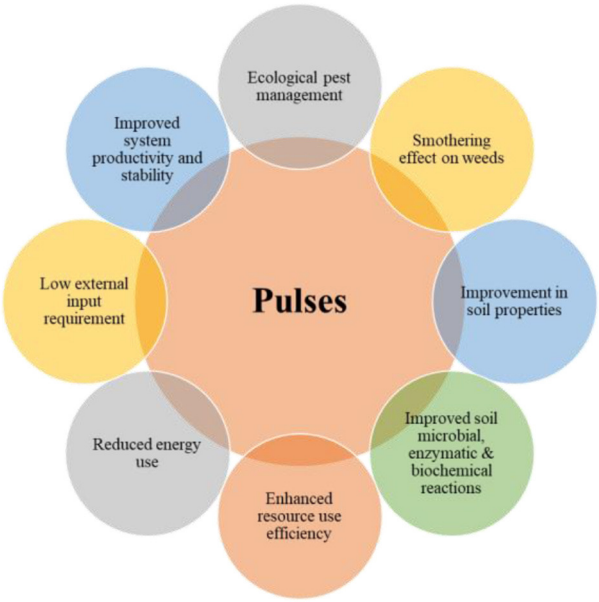

FIGURE 9
Pulses for soil health restoration and ecosystem services (Modified, Rani et al., 2019).

of crops increase the length of fungal hyphae in soil by 1.9 to 2.5 times (19–292 mg g^{-1}) to that of soils without pulses. These fungal hyphae, in turn, prove to be a crucial binder of soil aggregates of >0.25 mm size, called macroaggregates, while the formation of micro-aggregates (<0.25 mm) takes place with the help of humic substances (Kumar et al., 2018). Thus, the enhanced soil aggregation promotes the formation of favorable soil structure and tilth. In Himachal Pradesh, India, Sharma et al. (2000) reported that a pulse-based system increased the percentage of macro-aggregates. Likewise, at Kanpur, India, Hazra et al. (2019) found an improvement in soil macroaggregates of >3.0 mm by 1.59, and 1.25 times in continuous pulse crops (35%) concerning uncultivated fallow (22%), and non-pulse crops (28%), respectively. They also observed a significant reduction in soil compaction cone index in continuous pulse crops (39) than in non-pulse crops (46) and uncultivated fallow (55) at 0.05% of the significance level. In Inceptisol (Typic Ustochrept) soils of Kanpur, Uttar Pradesh (India), Hazra et al. (2019) also reported higher retention of coarse macroaggregates (>0.25 mm) in pigeon pea–wheat and maize–wheat–mung bean rotations by 67.4 and 26.6% ($p < 0.05$), compared to the maize–wheat rotation, respectively. Similarly, the pulse-based cropping system also recorded significantly higher meso-aggregates (0.25–2.0 mm) over the maize–wheat system. Similarly, Kumar et al. (2019a) also found improved macro- and meso-aggregates, and aggregate mean weight diameter in legume-based crop rotations such as rice–chickpea–mung bean and rice–chickpea compared to the rice–wheat rotation in 0–20- and 20–40-cm soil depths. In addition to BNF, the nutrient-rich pulse residues add a considerable amount of N, P, K, and micronutrients to the soil ecosystem upon decomposition and mineralization that can be beneficially used by soil microbes and succeeding crops (Kumar et al., 2018).

The proliferation of soil microbes, enzymatic and associated biochemical processes into the rhizosphere regions due to root exudates adds to nutrient acquirement, soil nutrient reservoir, and retrieval of soil processes (Rani et al., 2019; Kumar et al., 2021). Hence, it reduces external chemical fertilization and advances ecological services and crop yield while minimizing the chemical pollution in soil, thus securing soil health. Pulses also ensure an enhanced growth of P-mobilizers and solubilizers, further advancing P acquisition and reducing external P fertilization. In addition, adaptive morphological and physiological approaches have been developed in pulses at multiple levels, including the continuation of higher P in root nodules, compared to surrounding tissues/organs, enhancement of root exudates' excretion, and root surface area. These approaches also include the articulation of transporters and aquaporins for improved P uptake and an increase in fixed N per unit of the nodule to reimburse for a decrease in the nodule number (Rani et al., 2019). Pulses promote the recycling of nutrients (i.e., C, N, and P), owing to their deeper, healthy, and profuse root network that helps in a more efficient resource utilization and reduces nutrient losses, *that is*, NO_3 and thus enhances the nutrient recovery and NUE (Kumar et al., 2019b). These crops reduce NO_3 leaching by 20–80% over non-legume crops by storing the inorganic soil N in the organic state between two crop seasons (Meisinger et al., 1991). In Denmark, Stagnari et al. (2017) found an increased N uptake by 23–59% in various field crops grown after field pea and lupin in rotation. In addition, the release of organic acids (e.g., piscidic acid and citric acid) into the soil through roots solubilizes the insoluble soil nutrients, including both macro- and -micronutrients, whose P solubility is well known. Long-term pulse cultivation significantly increases the buildup of these nutrients, which improves soil fertility status and, thus, soil health and quality.

8.2. Effect of pulses on nitrogen economy and biological fixation equations

It is well known that pulses leave a considerable amount of N in the soil after harvest. An estimate of the N economy in pulse-based cropping systems depicts the addition of 6,68,000 tons of N into the soil (Singh et al., 2009). Herridge et al. (2008) and Peoples et al. (2009) reported 47 and 87 Mt N $year^{-1}$ through legume-based BNF and fertilizer application, respectively, that collectively cost over US$ 50 billion. The intrinsic N-fixing capability of pulse crops helps in meeting the N demands (N economy) of succeeding cereal crops due to its residual N effect (Figure 10). Mineralizable organic N, MBC, and mineral N bring about improvement in N economy. Furthermore, pulses are reported to have a high NO_3 content in their soil profile, where the maximum increase in NO_3 is observed with chickpea among *rabi* pulses with 20.4 kg ha^{-1}. This extra NO_3 is due to its reduced use during crop growth stages of pulses, which is termed the nitrate sparing effect.

The N-fixing ability takes place at the rate of 1.0 kg ha^{-1} day^{-1} in the growing season, which is considered the potential N-fixing ability of legumes within given environmental conditions (Kumar et al., 2017a). Approximately two-thirds of fixed N are available for the next growing season. On average, legumes can fix N around 30–150 kg N ha^{-1}, which varies with rhizobial population, legume species grown, soil properties, management practices, and prevailing environmental conditions (Kumar et al., 2017a). A study in Australia showed an N economy of 40–90 kg N ha^{-1} in the first year, and the carryover

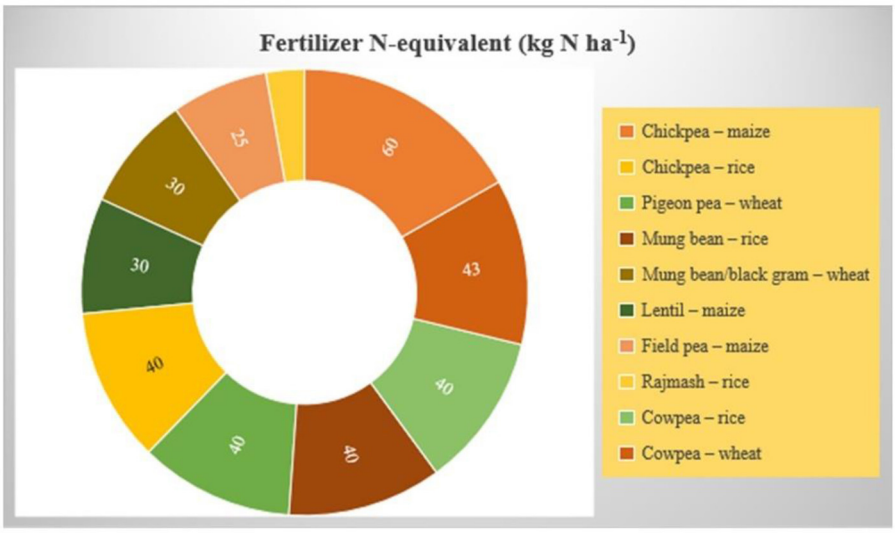

FIGURE 10
Nitrogen (N) economy due to the inclusion of pulses in the cereal-based system (*Data source*: Ali and Venkatesh, 2009).

effect was 20–35 kg N ha^{-1} through the inclusion of legumes in a cereal-based cropping system, in the second year (Peoples et al., 2015).

It is essential to assess BNF to evaluate the sustainability of pulse-based cropping systems. Quantifying BNF in the right way is crucial to achieving economic viability and long-term sustainability (Anglade et al., 2015). Different methodologies are used to estimate the N fixation of a crop, but no single method is universally accepted for all legume species. The most widely used methods are N differences, the Isotope dilution method (^{15}N natural abundance and ^{15}N enrichment method), and Acetylene reduction by nitrogenase enzyme and Xylem (Ureide) solute technique. Several empirical and simulation models are used recently to assess the predefined N fixation rate for different legume species (Liu et al., 2011). The principles and calculations of BNF using diverse methods are given in the following Table 10.

So, this group of pulses has a lot of benefits together with system productivity and overall economic efficiency of the crop production system.

9. Effect of pulses on system productivity and economic efficiency

With the increasing demand for food, declining per capita land area, and escalating human population, the improvement in crop productivity became necessary through sustainable intensification (SI) under a rainfed agroecosystem (Lal, 2017). In coming times, eliminating hunger and poverty and sustainable development will be major goals, and pulses could play a crucial role in achieving them, owing to their nutritious seeds, eco-friendly and less input requiring nature while increasing the carrying capacity of the land (Ali and Gupta, 2012). The SI of existing cropping systems, by involving pulses (e.g., intercropping/mixed cropping, relay cropping, sequential cropping, catch cropping, and ratoon cropping), improves the system productivity as a whole (Singh et al., 2009) and can feed the world.

Pulses benefit the cropping system by enhancing the pulse itself and subsequent crop yield and reducing input application because of improved soil health and fertility. Pulses can arrest the declining productivity of a continuous non-pulse-based system by improving the soil's physical, chemical, and biological environment (Singh et al., 2009). Thus, pulses being a part of an integrated plant nutrient supply chain of the cereal-based system should be promoted. There are several scientific reports on the role of pulses in improved productivity of subsequent cereal and oilseed crops while enhancing climate change resilience (Angus et al., 2015; Gan et al., 2015; Liu et al., 2019). For instance, pulses increase the yield of subsequent wheat crops by up to 35% in Northern America (Gan et al., 2015) and nearly 20% throughout the globe (Kirkegaard et al., 2008). The important indices used for assessing the productivity and economic efficiency of pulse-based cropping systems are Land Equivalent Ratio (LER), Crop Equivalent Yield (CEY), Relative Crowding Coefficient (RCC), Aggressivity Index (AI), Intercropping Advantage (IA), Competition Ratio (CR), Actual Yield Loss (AYL), Area Time Equivalent Ratio (ATER), Cropping Intensity (CI), Multiple Cropping Index (MCI), and Monetary Advantage Index (MAI) (Layek et al., 2018). In Van, Turkey, Ciftci and Ulker (2005) reported that the diversification of wheat (20%) through mixed cropping with lentils (80%) increased the LER to 1.15. The yield advantages of 20 and 15% were also obtained by diversifying mustard (*Brassica* spp.) through mixed cropping (75 + 25%) and intercropping (2 paired + lentil broadcasting), respectively, at Mymensingh, Bangladesh (Sekhon et al., 2007). The higher LER (1.61) was also found with lentil + linseed intercropping (3:2) in Bangladesh (Miah and Rahman, 1993). Kermah et al. (2017) reported higher productivity and economics of different grain–legume-based intercropping systems in the northern and southern Guinea savanna, respectively. The results indicated higher productivity and economic benefits in the intercropping system than sole cropping in terms of LER (1.16–1.81 and 1.07–1.54) in northern and southern Guinea savanna, respectively, due to an efficient and productive use of natural resources by intercropping. In Eastern Himalayan regions of India,

TABLE 10 Different methodologies and principles to assess biological *N*-fixation.

S.No.	Method	Principle	Formula	References
1.	*N* differences	The difference in total *N* accumulation in the shoot of the legume crop and control crop is determined as symbiotic *N* fixation	$Q = [N$ yield (legume) – N yield (control)] + [N soil (legume) – N soil (control)]	Peoples et al. (1989)
2.	Nodule tissue	Total *N* plant α nodule tissue formed (weight)	Regression equation: $NF = b\,X$	Döbereiner (1966)
3.	*N* yields	Linear relationships with *N* yields	BNF (kg N $ha^{-1}year^{-1}$) $= \left[\alpha cult \times \frac{Y}{NHI} + \beta cult\right] \times BGN$	Anglade et al. (2015)
4.	**Isotope dilution method**			
a.	Natural ^{15}N abundance	With increasing *N* fixation, there is a decline in the abundance of ^{15}N in the N_2-fixing plant as *N* assimilation from the soil is diluted by atmospheric *N* of lower ^{15}N abundance fixed in root nodules of plant	$P = 100 \times \frac{\delta^{15}N\ (soil\ N) - \delta^{15}N\ (legume\ N)}{(\delta^{15}N\ (soil\ N) - B)}$	Peoples et al. (1989)
			$\%N = 100 \times \frac{(^{15}N_{ref}) - (^{15}N_{leg})}{(^{15}N_{ref} - B)}$	Chalk and Craswell (2018)
b.	^{15}N enrichment method		$P = 100 \times 1 - \frac{(atoms\ \%^{15}N\ excess\ in\ legume\ N)}{(atoms\ \%^{15}N\ excess\ in\ soil\ derived\ N)}$	Peoples et al. (1989)
5.	Acetylene reduction by the enzyme nitrogenase	Detection of nitrogenase activity (as nitrogenase reduces acetylene to ethylene)	ARA = Nitrogenase activity × dry weight of the nodules	Chalk and Craswell (2018)
6.	Xylem (Ureide) solute technique	Differences in xylem *N* solute concentration between symbiotic plants and non-nodulated plants to assess their dependency on *N* fixation/soil mineral *N*	$Relative\ ureide\ index\ (\%) = \frac{(Ureide\ N)}{(Total\ sap\ N)} \times 100$	Peoples et al. (1989)
7.	Empirical models	Pre-defined *N* fixation rate α to harvested yield, plant *N* concentration and total *N* derived from *N* fixation	$Nfix = \alpha \cdot DM \cdot f_{leg} \cdot N_{con} \cdot \%Ndfa \cdot (1 + R_{root})$	Liu et al. (2011)
8.	Simulation models	Pre-defined *N* fixation rate (*Nfix*) is estimated concerning response functions (soil/plant water status, soil temperature, soil/plant *N* concentration, *C* supply and growth stage of the crop)	$Nfix = Nfixpot\ fT\ fW\ fN\ fC\ fgro$	Liu et al. (2011)

2* NF, N fixed; b, regression slope of total plant N with nodule weight; X, corresponding nodule weight.
3* α_{cult} and β_{cult}, slope and intercept coefficients; Y, yield (kg N ha^{-1} $year^{-1}$); NHI, N harvest index; BG, factor determining below-ground contributions, i.e., nodules, roots and rhizodeposition (factor 1.3 for pulses).
4* P, percentage of pulse N fixed from atmospheric N; $\delta^{15}N$ (soil N), obtained from non-N fixing plant grown in the same soil as a pulse; B, $\delta^{15}N$ of N fixing plant grown with N as the sole source of N (values varies with different legumes); $^{15}N_{ref}$, abundance of the ^{15}N isotope in the reference plant; $^{15}N_{leg}$, abundance of the ^{15}N isotope in the pulse.
7* DM, yield; f_{leg}, legume proportion (if intercropped); N_{con}, N concentration in pulse; %Ndfa, total N proportion derived from N fixation; R_{root}, ratio of fixed N below and above ground.
8* Nfixpot, N fixation rate (g N fixed day^{-1}); fT – Soil temperature function, fW – Soil water status function; fN – Soil mineral N/root substrate N concentration function; fC – Substrate C concentration in plant/root function; fgro – Crop growth stage.

Choudhary et al. (2014) reported a higher LER (1.63, 1.75, and 1.66) upon diversifying maize through intercropping with cowpea (1:2), french bean (1:2), and urd bean (1:2), respectively. By taking french bean as an intercrop in the upland rice field, the french bean increased the rice equivalent yield from 2.03 to 6.0 t ha^{-1} at midhills in Meghalaya and thus improved the total system's productivity per unit of land areas. Similarly, Oseni (2010), in the Bauchi in the northern Guinea savanna of Nigeria, reported higher RCC, CR, and aggressivity index while intercropping sorghum with cowpea in the different ratios over the sole cropping of both component crops. Furthermore, Kumar et al. (2015) highlighted the improved rice equivalent yield and system productivity by different pulse-based cropping systems. This observation shows a strong relationship between the crop yield/ N concentration in seed and the N fixed by pulse crops.

The impact of pulses on the productivity of pearl millet (*Pennisetum glaucum*)-based system is synergistic in the rainfed agroecosystem of northern India. Intercropping of mung bean with pearl millet increased equivalent yield of pearl millet grain by 15.3% over the sole pearl millet (Prasad and Nanwal, 2001). In another experiment, Varia and Sadhu (2011) observed the highest LER and RCC (3.47 and 2.04) in the pearl millet + mung bean intercropping system (1:2 and 1:1, respectively). While strip cropping of pearl millet + mung bean (4:4) produced 26% more yield benefits for the continuous pearl millet monoculture (Singh and Joshi, 1994). Likewise, strip cropping of pearl millet with mung bean (8:4) and cowpea (6:3) had higher LER, ATER, CR, MAI, RCC, and aggressivity, when compared to monocropping (Sharma and Singh, 2008). Aggressivity values showed that intercropping of mung bean and cowpea did not compete with pearl millet. The pearl millet + pigeon pea intercropping system also produced a greater pearl millet equivalent yield (PEY) (43.1 and 48.2 q ha^{-1}) over the sole cropping of pearl millet and pigeon pea, respectively (Ansari et al., 2012). Pearl millet with moth bean (1:7) generated higher PEY, LER, and net return over sole cropping and other intercropping systems (Kuri et al., 2012).

Inclusion of peas in rotation with wheat positively impacts system yield, compared to continuous wheat. Superior performance of the pulse-based system over that of wheat monoculture was observed recently in terms of system yield, stability, and overall productivity

(Liu et al., 2019). Farmers reported improved productivity of successive wheat crops (29.76 q ha^{-1}) after pea, compared to continuous wheat (25.06 q ha^{-1}). Among different crop rotations, the pea–wheat system showed a better performance in protein-based system yields that were 9–26, 22–82, and 26–82% higher than those of lentil–wheat, chickpea–wheat, and wheat monoculture, respectively (Liu et al., 2019). Similarly, Miller et al. (2003) observed that the wheat grain yield increased by 13 and 19% when grown as a subsequent crop after lentil and pea over wheat monoculture, respectively. This observation may be associated with peas' more profuse, shallow, and branched root system than chickpeas and lentils, which improved the nutrient and water uptake under stressed conditions (Liu et al., 2011). Due to the shallow root system, pea and lentil crops take water from top soils only and leave behind deep soil moisture for succeeding cereal crops, thereby improving crop performance in a rainfed agroecosystem (Liu et al., 2019).

The economic benefits of pulses in the agroecosystem are due to an improvement in total/system grain yields over the respective single crop. The saving of fertilizers' input, particularly N-fertilizer, irrigation, and higher returns from the inclusive pulse crop directly reflect the improved production economics. Competition among the component crops is an essential factor in the intercropping system that helps to know the compatibility of component crops; therefore, agroecosystems' economic and biological suitability and spatial arrangements can be determined. For example, intercropping of cowpea with maize had a higher actual intercropping advantage and yield loss over the respective monocultures (Takim, 2012). For instance, the pearl millet + mung bean intercropping system (30/60 cm) recorded the highest profitability in terms of net monetary returns (₹ 12,270 ha^{-1}; US$ ∼170) and benefit–cost (2.75) over sole cropping (Hooda and Khippal, 2000). Hooda et al. (2004), in another experiment, found that pearl millet + mung bean generated greater net monetary returns (₹ 16,043 ha^{-1}; US$ ∼220) and benefit–cost (1.90) over monocropping of pearl millet (₹ 13,120 ha^{-1}; US$ ∼180). Likewise, Singh et al. (2003) observed that mung bean + cluster bean (*Cyamopsis tetragonoloba*) intercropping in paired row planting was more profitable than other intercropping systems in terms of the cluster bean equivalent yield (1.77 t ha^{-1}), net return (₹ 6,846 ha^{-1}; US$ ∼95), and income equivalent ratio (1.9). By looking toward the productivity gain and economic benefits of pulses in various ways, the identification, characterization, landmarking, and implementation of pulse-based agroecosystems in rainfed areas is essential.

10. Constraints, opportunities, and researchable issues for promoting pulses in a rainfed agroecosystem

In the era of the growing challenge of COVID-19, climate change, increasing food and protein demand of the global population, shrinking land and water resources, and rising prevalence of biotic and abiotic pressure, the production sustainability of the system is of prime concern. Existing non-legume-based cropping systems cannot maintain their long-term productivity level and are suffering from soil fertility depletion, extensive nutrient mining of natural nutrient reserves, declining productivity factor, reduced groundwater aquifer, decreasing soil C stock, augmented weed menace, and ecological instability (Kumar et al., 2017b). In this regard, pulses are in front most and have an immense potential for promoting ecosystem services, land restoration, resource conservation, and nutrient cycling while reducing groundwater extraction, chemical farming, and the ill effects of climate change.

There is a vast scope for increasing pulse production through the expansion of the horizontal area under pulses. The horizontal area can be increased by an inter-and/or mixed-cropping of pulses in crops such as sugarcane, potato, maize, cotton, mustard, pearl millet, and many other crops; by *utera/paira* cultivation in the rice-based system; and by the inclusion of pulses in an agroforestry system. In addition, the area can be increased significantly in huge rice fallows of India, Bangladesh, and Nepal by growing pulses after rice to take advantage of the residual soil moisture through agricultural conservation practices such as zero tillage and residue mulching. The production system can be intensified sustainably by replacing summer fallows with pulse crops such as mung bean, urd bean, and cowpea, which could bring additional 2–3 Mha areas under pulses in a more unsuitable rice–wheat system in Indo-Gangetic Plains (IGPs) of India (Kakraliya et al., 2018).

However, significant barriers to the successful adoption of pulses in rice and summer fallow are the timely unavailability of quality seeds of required varieties, lack of irrigation facilities, soil constraints, the poor financial status of farmers, and lack of clear vision of the government for sustainable intensification of these unused lands. In addition, a higher water requirement for summer pulses due to increased evaporative demands and a higher incidence of pests and diseases are also significant issues. Farmers of these regions must be supported with an efficient marketing network, enabling 100% procurement on minimum support price and technology transfer mechanisms, with particular emphasis on climate-resilient production technologies. The technology or management practices should be location-specific and economically flexible to understand better system ecology, obstructions, and possibilities for their higher adoption by the farmers. For the promotion of pulse cultivation, practical efforts have to be made through knowledge sharing, efficient technological intervention, site-specific input management, supplemental irrigation through a precise water applicator (*i.e.,* drip and sprinkler technology) coupled with rainwater storage, and the use of quality seeds of short-duration high-yielding stress-tolerant/resistance cultivars, according to the soil and environmental conditions.

Therefore, research must be initiated on following the problems of farmers by their active participation to advance the location-specific set of practices for easy adoption of technology. Yet, the potential of pulse crops for deep soil C sequestration remains poorly understood. An in-depth investigation is thus warranted to quantify the stabilization of pulse crops belowground root biomass. Increasing the content of the resistant C pool or humus is a vital strategy to enhance the SOC pool. A comprehensive assessment of cereal and legume residues in increasing the resistant C pool would help to develop strategic crop residue management. The potential of pulse genotypes for SOC restoration is another researchable issue. Variations in WUE and nutrient acquisition of some genotypes are also tested; however, there is scope to test these genotypes as to their C sequestration potential, so that the inclusion of pulses achieves SOC enrichment in the cereal-based system without incurring additional costs. Presently, the area under CA in the rice-wheat

cropping system (RWCS) is increasing in South Asian countries, and pulses are vital for crop diversification. All the possible inclusions of pulses in the RWCS under CA practices need to be studied to advocate appropriate pulse-inclusive crop rotation for resource conservation and climate change mitigation. A comprehensive estimation of GHGs emissions in cereal–cereal and cereal–pulse systems provides useful information for the strategic management of GHGs emissions following the cropping system approach. In addition, investing in the shifts in soil microbial activities, abundance, and communities driven by different agricultural practices would be conducive to maintaining and enhancing the fertility and productivity of soils and protecting soil ecosystems against disturbances.

It is necessary to prioritize the formulation and implementation of novel policies by encouraging investment in research, technology transfer, farmers' training, knowledge sharing, capacity building, and application of advanced technologies based on the region-specific available resources. Global investments in pulses' research, development, and extension are too low (US$ 175 million) compared with cereals, *that is,* maize receives billions in investments. Therefore, investments in research and development (R&D) in pulses must be promoted among scientists and farming communities. Research on pulses is needed to increase productivity, improve climate resilience, and understand the health and nutritional benefits and value of ingredients and food processing. The Indian government is already working to promote pulse cultivation in rice fallows under the TRFA, NFSM-Pulses, and other smaller schemes/projects/programs. Its impact on increased pulse production has been seen in the last 3–4 years, but still, to make the program successful, more efforts, mainly centered on small and marginal farmers, are needed in this direction. Farmers can also be encouraged to cultivate pulses by providing financial assistance and subsidies or free accessibility of quality seeds to make programs and schemes more successful. The policy agenda needs to be centralized for improved productivity, risk management capacity, improved marketing facilities, financial assistance for technology adoption, and government subsidies and financial incentives. This calls for a holistic approach to develop an action plan by identifying the ecological and economic constraints and taking advantage of the existing opportunities by utilizing the available technical and scientific information. There is a need to bring together science and policies to generate a common platform that will be useful and applicable to all living entities, including soil. For achieving that purpose, a better coordination should be made possible among the scientific communities, farmers, and democrats for designing an efficient policy framework. With the help of scientists, farmers should also come forward to identify such management practices that are productive, eco-friendly, and have a wider scale adaptability. Research and development activities must be promoted in conservation practices and land-use decisions across the agroecological regions and most soil types. Farmers' feedback on the easiness, incentives, and economics of technology should be collected and accordingly, the gap has to be refined for further modification and improvement in the existing technology. Therefore, increasing the acreage and yield of pulses during the fallow period by adopting an appropriate package of management technologies may give birth to another green revolution and could prove to be a turning point in mitigating the food and nutritional security needs of millions of food-insecure and malnourished people.

In addition, still, some of the specific researchable issues have to be rectified to enhance the agronomic yield of pulses while sustaining the environment:

- Quantifying the rate of soil carbon sequestration and stabilization under a wide range of soils, agroecology, and pulses.
- Assessing GHGs emissions (specifically, N_2O) and calculating the C footprint of diverse pulse-inclusive systems under a wide range of agroecology.
- Quantifying the N fixation capacity of different pulses and finding factors affecting them.
- Developing soil, climate, and system-specific set of practices to reduce N_2O emission, dinitrogen–dinitrogen monoxide (N_3O) leaching, and trade-off of BNF.
- Determining the impact of crop management strategies on the nutritional status of pulses, including the protein and micronutrient (Zn and Fe) levels.
- Adapting pulses to conservation agriculture (CA)-based systems and recognizing site-specific components of CA-based systems.
- Identifying agronomic management of soil and water to narrow the yield gap under resource constraints.
- Finding soil, water, and crop management systems for sustainable intensification of pulse-inclusive systems.
- Improving use efficiencies of different resources, including water, nutrients, energy, and land of the pulse-based system, under diverse soils and climates, and identifying the site-specific loopholes.

11. Conclusions

Pulses are healthy food, rich in dietary fiber, protein-energy, essential amino acids, vitamins, and minerals, and they have several other health benefits. Pulses are a precious source of nutritious food for 828 million insecure, 690 million undernourished, and 200 million malnourished people, mainly when the prediction showed the same hunger level in 2030 as in 2015. In addition to provisioning food, feed, and fuel, pulses have numerous subsidiary benefits for soil restoration, climate-resilient agroecosystems, and improved ecosystem services. Being hardy, they grow well in harsh environmental conditions, including rainfed agroecosystems where most of the food-insecure populations live. Most pulses are of short duration and fit well into an existing niche in most farming/cropping systems. Their natural ability to adapt to a wide range of soils and climates with limited additional resource requirements makes them the best crop for securing the food supply of global hunger under conditions of climate change. Pulses improve the yield of component/subsequent non-legume crops and their bonus yield, thus enhancing the overall system's productivity, economics, and environmental sustainability. Pulses aid in economizing N use in the system by adding 18–70 kg N ha^{-1} to the soil through the BNF process. Hence, they help to reduce the demand for chemical fertilizers by 25–30% and safeguard the soil and environment from the ill effects of chemical fertilizers. By cutting the demand for fertilizers and other inputs, the pulses save energy in the cropping system and thus reduce the C footprint. These crops effectively restore sick and degraded soil by improving the SOC stock and soil microbial functionaries and offsetting GHGs emissions in a rainfed

agroecosystem. Subsequently, pulses in the production system enhance adaptation to climate change by conserving/maintaining soil moisture, SOC, and health. Overall, pulses promote the four pillars of sustainability, *that is*, economic, environmental, social, and institutional, through diverse interactive natural progressions. Pulses also directly contribute to the UN's SDGs for human wellbeing and natural resource conservation. Thus, considering the future plans to cater to the feeding needs of the exploding population and the significance of pulses in health, nutrition, and ecosystem management, the areas covered under the production of pulses must be increased to 296 Mha by 2030 and 238 Mha by 2,100, in addition to a productivity of 1,800 and 2,150 kg ha^{-1}, respectively.

Author contributions

Conceptualization and validation: SK, KG, and RM. Data curation: SK, SS, and RM. Formal analysis: SK and SB. Methodology: SK, RM, SB, and SS. Resources: RM and RJ. Supervision: KG, CS, and RM. Visualization: SK, SS, RM, and SB. Writing—original draft: SK, SS, RM, SB, CJ, and KM. Writing—review and editing: RM, SK, CS, KG, RJ, and CP. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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EDITED BY
Priyanka Chandra,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Nalin Nilusha Wijayawardene,
Qujing Normal University, China
Jadson Diogo Pereira Bezerra,
Universidade Federal de Goiás, Brazil

*CORRESPONDENCE
Rufin Marie Kouipou Toghueo
✉ rufin.toghueo@irnasa.csic.es

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Diaporthe species associated with the maritime grass *Festuca rubra* subsp. *pruinosa*

Rufin Marie Kouipou Toghueo*, Beatriz R. Vázquez de Aldana and Iñigo Zabalgogeazcoa

Plant-Microorganism Interaction Research Group, Institute of Natural Resources and Agrobiology of Salamanca, Consejo Superior de Investigaciones Científicas (IRNASA-CSIC), Salamanca, Spain

Festuca rubra subsp. *pruinosa* is a perennial grass growing in sea cliffs where plants are highly exposed to salinity and marine winds, and often grow in rock fissures where soil is absent. *Diaporthe* species are one of the most abundant components of the root microbiome of this grass and several *Diaporthe* isolates have been found to produce beneficial effects in their host and other plant species of agronomic importance. In this study, 22 strains of *Diaporthe* isolated as endophytes from roots of *Festuca rubra* subsp. *pruinosa* were characterized by molecular, morphological, and biochemical analyses. Sequences of the nuclear ribosomal internal transcribed spacers (ITS), translation elongation factor 1-α (*TEF1*), beta-tubulin (*TUB*), histone-3 (*HIS*), and calmodulin (*CAL*) genes were analyzed to identify the isolates. A multi-locus phylogenetic analysis of the combined five gene regions led to the identification of two new species named *Diaporthe atlantica* and *Diaporthe iberica*. *Diaporthe atlantica* is the most abundant *Diaporthe* species in its host plant, and *Diaporthe iberica* was also isolated from *Celtica gigantea*, another grass species growing in semiarid inland habitats. An *in vitro* biochemical characterization showed that all cultures of *D. atlantica* produced indole-3-acetic acid and ammonium, and the strains of *D. iberica* produced indole 3-acetic acid, ammonium, siderophores, and cellulase. *Diaporthe atlantica is* closely related to *D. sclerotioides*, a pathogen of cucurbits, and caused a growth reduction when inoculated in cucumber, melon, and watermelon.

KEYWORDS

grasses, *Festuca rubra subsp. pruinosa*, endophyte, multi-locus phylogeny, *Diaporthe atlantica*, *Diaporthe iberica*

1. Introduction

The genus *Diaporthe* is highly complex, according to Index Fungorum (2022) comprises nearly 1,000 fungal names without taking into account the 984 names attributed to its asexual state *Phomopsis*. *Diaporthe* is worldwide-distributed and associated with a broad range of host plants. Some members of this genus are important pathogens, responsible for several economically significant plant diseases including stem, root and fruit rots, gummosis, cankers, leaf spots, blights, diebacks, decay, and wilts on hosts such as citrus, grapevines, soybean, peach, or sunflower, to name just a few (Udayanga et al., 2011; Marin-Felix et al., 2019). They can also colonize decaying plant tissues as saprophytes (Thongkantha et al., 2008), or live endophytically inside healthy plant tissues (Gomes et al., 2013).

As endophytes, *Diaporthe* species have been isolated from agricultural, medicinal and ornamental plants growing in a wide variety of locations and habitats (Huang et al., 2015; Dos Santos et al., 2016, 2021; Yang et al., 2018). They have also been reported as dominant taxa in fungal microbiomes of plant species like *Tectona grandis* (Murali et al., 2006), the Brazilian medicinal plants *Vochysia divergens* and *Stryphnodendron adstringens* (Noriler et al., 2018), olive trees (Martins et al., 2016), *Fagus crenata* (Tateno et al., 2015) and the grass *Festuca rubra* subsp. *pruinosa* (Pereira et al., 2019). Non-pathogenic *Diaporthe* species have been recognized as plant growth promoters (Vázquez de Aldana et al., 2021; da Silva Santos et al., 2022; Toghueo et al., 2022), and biocontrol agents (Marak et al., 2002; Dos Santos et al., 2016; Dini-Andreote, 2020; Abramczyk et al., 2022). From an industrial and pharmaceutical point of view, they are well-known producers of enzymes (Lange et al., 2021; Baluyot et al., 2022) and secondary metabolites exhibiting a wide range of activities including antimicrobial, antiviral, antioxidant, anti-inflammatory and anticancer (Chepkirui and Stadler, 2017; Xu et al., 2021). Therefore, studies aiming to analyze and better characterize *Diaporthe* species are of interest, particularly to fully comprehend not only their role as ubiquitous species in nature, but also their potential as metabolite producers, plant growth promoters, biocontrol agents, or for control measures in case of potential pathogenicity.

Previously, *Diaporthe* species were identified on the basis of morphological characteristics (e.g., colony phenotype, size, shape, and type of spores) and host specificity (Rehner and Uecker, 1994; Udayanga et al., 2011). This methodology of *Diaporthe* identification resulted in a proliferation of names in the literature. However, several studies demonstrated that morphological characters are not sufficient for species level diagnoses, and that many species of *Diaporthe* have multihost capability (Rehner and Uecker, 1994; Thompson et al., 2011, 2015; Udayanga et al., 2012). Nowadays, *Diaporthe* species are being redefined based on a polyphasic approach, employing a combination of information including morphological characteristics and multi-locus sequence data (Udayanga et al., 2012, 2014a,b; Gomes et al., 2013; Norphanphoun et al., 2022). Currently, the taxonomy of *Diaporthe* species is being resolved by means of multigene phylogenetic analyses based on sequences of the 5.8S rDNA and internal transcribed spacers (ITS1-5.8S-ITS2), translation elongation factor 1-alpha (*TEF1*), beta-tubulin (*TUB*), histone H3 (*HIS*), and calmodulin (*CAL*) genes (Udayanga et al., 2012; Gomes et al., 2013; Marin-Felix et al., 2019; Norphanphoun et al., 2022). Using this methodological approach, the delimitation of species in the genus has improved. Lately, *Diaporthe* species occurring in several host plant families have been well-characterized and sometimes reclassified, resulting in an actively evolving taxonomy with numerous novel species being described each year (Gomes et al., 2013; Dissanayake, 2017; Guarnaccia et al., 2018; Yang et al., 2018; Guo et al., 2020; Wang et al., 2021; Norphanphoun et al., 2022). Despite the attention given to pathogenic *Diaporthe* species occurring in economically important crops, more effort is needed to understand the ecology and distribution of non-pathogenic species occurring in numerous plant species.

Festuca rubra, commonly known as red fescue, is a perennial grass distributed across a very diverse range of habitats in the Northern Hemisphere. Red fescues are cultivated and used as turfgrasses in ornamental and sports lawns, and some cultivars have been used for phytoremediation and rehabilitation of damaged soils (Braun et al., 2020). Among its several subspecies, *Festuca rubra* subsp. *pruinosa* (*Festuca pruinosa* thereafter) is native to the Atlantic coasts of Europe and North America, where often grows in sea cliffs as a chasmophyte in rock fissures where soil is absent (Castroviejo, 2020). In this highly unhospitable habitat, plants grow with a low nutrient availability and nearly continuous exposure to salinity and desiccating winds. A previous study revealed that the roots of *Festuca pruinosa* plants have a complex endophytic fungal microbiome, and *Diaporthe* is one of its most abundant components, occurring in 54% of the plants, and at all locations analyzed (Pereira et al., 2019). The main goal of this study was to identify the endophytic *Diaporthe* species associated to roots of *Festuca pruinosa* by means of genotypic, morphological, and biochemical analyses.

2. Materials and methods

2.1. Fungal isolates

Twenty-two *Diaporthe* isolates (**Table 1**) obtained from surface-disinfected roots of healthy *Festuca pruinosa* plants were analyzed (Pereira et al., 2019). Plants were collected at four locations in sea cliffs in the North Atlantic coast of Spain: Torre de Hércules (43°23'09" N 8°24'23"W), Cedeira (43°40'46"N 8°01'15"W), Estaca de Bares (43°47'25"N 7°41'16"W), and San Pedro de la Rivera (43°34'43"N 6°13'17"W). In addition, we included a *Diaporthe* isolate (T6) obtained from roots of *Celtica gigantea* (= *Stipa gigantea*), a tall grass growing in dry sandy soils collected in Cuatro Calzadas, Salamanca (40°49'03"N, 5°36'47"W) (Vázquez and Devesa, 1996; Vázquez de Aldana et al., 2021).

2.2. DNA isolation, PCR amplification, and sequencing

Fungal DNA was extracted and amplified from a small amount of mycelium scraped from seven-day-old potato dextrose agar (PDA) cultures using Extract-N-Amp Plant Tissue PCR kits (Merck). The oligonucleotide primers and PCR protocols used for the amplification of the different fungal genes are listed in **Supplementary Table 1**. After amplification, PCR amplicons were sequenced in both directions at the DNA sequencing service of the University of Salamanca (Spain). New sequences generated in this study were deposited at the GenBank nucleotide database (**Table 1**).

2.3. Phylogenetic analysis

To establish the identity of fungal isolates at species level, phylogenetic analyses based on sequences of five genes (ITS, *TUB*, *CAL*, *TEF1*, and *HIS*) were conducted. To determine which *Diaporthe* taxa were closest to our fungal isolates, a concatenated sequence of the five loci was aligned with a similar set of 243 sequences belonging to 95 *Diaporthe* species (TreeBASE, study S13943; Gomes et al., 2013). The alignment was performed using MAFFT (Katoh and Standley, 2013) with default settings, and manually adjusted with MEGA v. 7 (Kumar et al., 2016). Then, a Maximum Likelihood (ML) phylogenetic tree was made using MEGA v.7 to identify the clades to which our isolates belonged. Afterwards, more restricted phylogenetic trees were made using the sequences of the species included in these major clades.

TABLE 1 *Diaporthe* species, host plant, geographic origin, and GenBank accession numbers of strains used in the study. (*Festuca rubra subsp. pruinosa* = *Festuca pruinosa*).

Species	Strain	Isolation source	Origin	GenBank accession number				
				ITS	*CAL*	*HIS*	*TEF1*	*TUB*
Diaporthe ambigua	CBS 114015 (epitype)	*Pyrus communis*	South Africa	KC343010	KC343252	KC343494	KC343736	KC343978
	CBS 117167	*Aspalathus linearis*	South Africa	KC343011	KC343253	KC343495	KC343737	KC343979
Diaporthe arecae	CBS 161.64 (ex-isotype)	*Areca catechu*	India	KC343032	KC343274	KC343516	KC343758	KC344000
	CBS 535.75	*Citrus sp.*	Suriname	KC343033	KC343275	KC343517	KC343759	KC344001
Diaporthe foeniculacea	CBS 123208 (holotype of *D. neotheicola*)	*Foeniculum vulgare*	Portugal	KC343104	KC343346	KC343588	KC343830	KC344072
	CBS 123209; Di-C004/4 (ex-type of *D. neotheicola*)	*Foeniculum vulgare*	Portugal	KC343105	KC343347	KC343589	KC343831	KC344073
Diaporthe ganjae	CBS 180.91 = ILLS 43621 (ex-type)	*Cannabis sativa*	USA	KC343112	KC343354	KC343596	KC343838	KC344080
Diaporthe inconspicua	CBS 133813 (type)	*Maytenus ilicifolia*	Brazil	KC343123	KC343365	KC343607	KC343849	KC344091
	LGMF922 = CPC 20298	*Spondias mombin*	Brazil	KC343124	KC343366	KC343608	KC343850	KC344092
Diaporthe infecunda	CBS 133812 (type)	*Schinus terebinthifolius*	Brazil	KC343126	KC343368	KC343610	KC343852	KC344094
	LGMF918	*Schinus terebinthifolius*	Brazil	KC343132	KC343374	KC343616	KC343858	KC344100
Diaporthe longispora	CBS 194.36 (type of *D. strumella* var. *longispora*	*Ribes sp.*	Canada	KC343135	KC343377	KC343619	KC343861	KC344103
Diaporthe mayteni	CBS 133185 = CPC 20314 (ex-type)	*Maytenus ilicifolia*	Brazil	KC343139	KC343381	KC343623	KC343865	KC344107
Diaporthe musigena	CBS 129519 (holotype)	*Musa sp.*	Australia	KC343143	KC343385	KC343627	KC343869	KC344111
Diaporthe pseudophoenicicola	CBS 462.69 (ex-type)	*Phoenix dactylifera*	Spain	KC343184	KC343426	KC343668	KC343910	KC344152
	CBS 176.77	*Mangifera indica*	Iraq	KC343183	KC343425	KC343667	KC343909	KC344151
Diaporthe sclerotioides	CBS 296.67 (type of *P. sclerotioides*)	*Cucumis sativus*	Netherlands	KC343193	KC343435	KC343677	KC343919	KC344161
	CBS 710.76	*Cucumis sativus*	Netherlands	KC343194	KC343436	KC343678	KC343920	KC344162
Diaporthe atlantica sp. nov	TH2	*Festuca pruinosa*	Spain	ON159897	ON364024	ON398814	ON398836	ON364045
	TH10	*Festuca pruinosa*	Spain	ON159898	ON364025	ON398815	ON398837	ON364046
	TH21	*Festuca pruinosa*	Spain	ON159899	ON364026	ON398816	ON398838	ON364047
	TH56	*Festuca pruinosa*	Spain	ON159900	ON364027	ON398817	ON398839	ON364048
	TH71	*Festuca pruinosa*	Spain	ON159901	ON364023	ON398818	ON398840	ON364044
	TH86	*Festuca pruinosa*	Spain	ON159903	ON364029	ON398820	ON398842	ON364050
	TH91	*Festuca pruinosa*	Spain	ON159904	ON364030	ON398821	ON398843	ON364051
	TH151	*Festuca pruinosa*	Spain	ON159905	ON364031	ON398822	ON398844	ON364052
	TH158	*Festuca pruinosa*	Spain	ON159906	ON364032	ON398823	ON398845	ON364053
	EB4	*Festuca pruinosa*	Spain	OM944045	ON018830	ON018832	ON018831	ON018829
	EB11	*Festuca pruinosa*	Spain	ON159889	ON364015	ON398806	ON398827	ON364036
	EB12	*Festuca pruinosa*	Spain	ON159890	ON364016	ON398807	ON398828	ON364037
	CD39	*Festuca pruinosa*	Spain	ON159887	ON364013	ON398804	ON398825	ON364034
	CD87	*Festuca pruinosa*	Spain	ON159888	ON364014	ON398805	ON398826	ON364035
	SP11 = CECT 21217 (holotype)	*Festuca pruinosa*	Spain	ON159893	ON364019	ON398810	ON398831	ON364040
	SP45	*Festuca pruinosa*	Spain	ON159894	ON364020	ON398811	ON398832	ON364041
	SP130	*Festuca pruinosa*	Spain	ON159895	ON364021	ON398812	ON398833	ON364042
	SP131	*Festuca pruinosa*	Spain	ON159896	ON364022	ON398813	ON398834	ON364043
Diaporthe iberica sp. nov	S32	*Festuca pruinosa*	Spain	ON159892	ON364018	ON398809	ON398830	ON364039
	T6	*Celtica gigantea*	Spain	MT645115	ON364033	ON398824	ON398835	ON364054
	TH77 = CECT 21218 (holotype)	*Festuca pruinosa*	Spain	ON159902	ON364028	ON398819	ON398841	ON364049

(Continued)

TABLE 1 (Continued)

Species	Strain	Isolation source	Origin	GenBank accession number				
				ITS	*CAL*	*HIS*	*TEF1*	*TUB*
Diaporthe sp.1	EB73 = CECT 21219	*Festuca pruinosa*	Spain	ON159891	ON364017	ON398808	ON398829	ON364038
Diaporthella corylina	CBS 121124 = AR 4131	*Corylus sp.*	China	KC343004	KC343246	KC343488	KC343730	KC343972

Type cultures are indicated in bold. CBS, CBS FUNGAL Biodiversity Centre, Utrecht, The Netherlands; CECT, Spanish type culture collection; CPC, Collection Pedro Crous, housed at CBS; ILLS, Illinois Natural History Survey Fungarium; LGMF, Culture collection of Laboratory of Genetics of Microorganisms, Federal University of Parana, Curitiba, Brazil; *TUB*, partial beta-tubulin gene; *CAL*, partial calmodulin gene; *HIS*, partial histone H3 gene; ITS, ITS1-5.8S rDNA-ITS2 internal transcribed spacer region; *TEF1*, partial translation elongation factor 1-alpha gene. Isolates marked with bold are ex-type or ex-epitype strains.

These phylogenetic analyses were based on ML for all the individual loci, and on both ML and Bayesian Inference (BI) for the concatenated sequence of the five loci. The best-fit models for each gene and the concatenated set were determined using MEGA v. 7 and incorporated into the analyses. For the BI, MrBayes v. 3.2.7 (Ronquist et al., 2012) was used to generate the phylogenetic trees under optimal criteria per data partition. The Markov Chain Monte Carlo analysis of four chains ran for 20 000 000 generations and started in parallel from a random tree topology and lasted until the average standard deviation of split frequencies was below 0.01. Trees were saved each 10 000 generations. The first 25% of saved trees were discarded as the burn-in phase and the posterior probabilities were determined from the remaining trees. The resulting phylogenetic tree was printed with Geneious v. 5.5.4 (Drummond et al., 2011). A ML tree for the concatenated sequence of the five loci was based on the Hasegawa-Kishino-Yano model with gamma correction. For other parameters, default settings were used. ML analyses were performed in MEGA v.7 with the tree bisection and reconnection algorithm, where gaps were treated as missing data. The robustness of the topology was evaluated by 1,000 bootstrap replications. *Diaporthella corylina* (CBS 121124) was used as an outgroup. Holotypes were preserved as metabolically inactive cultures at the Spanish Type Culture Collection (CECT). Nomenclatural novelties and descriptions were deposited at the CECT and MycoBank (MB).

2.4. Genetic diversity analysis

The 18 cultures of *D. atlantica* available were used for an analysis of its genetic diversity. For the samples investigated, diversity indices were calculated for each gene and the combined sequence dataset. Parameters such as the Tajima's D (Tajima, 1989), the minimum numbers of recombination events (Rm) (Hudson and Kaplan, 1985), the total number of haplotypes (H), haplotype diversity (Hd), Watterson's θ (θw), the number of segregating sites (S), and the average nucleotide diversity (pi) were calculated using the DnaSP v. 6.12 software (Librado and Rozas, 2009). To overcome the population size effects, Hd, θw, and pi were calculated after 1,000 repetitions, and the median estimate was recorded for each parameter.

2.5. Morphological characters

Agar plugs (6 mm diameter) from the edge of actively growing cultures on PDA were transferred to 9 cm diameter Petri dishes containing one of the following culture media: malt extract agar (MEA), PDA, water agar supplemented with sterile pine needles (PNA) (Smith et al., 1996) or with sterile pieces of *Festuca pruinosa* leaves (FLA). Plates were incubated at 21–22°C under a 12 h/12 h near-ultraviolet light/darkness cycle to induce sporulation as described by Gomes et al. (2013). Cultures were examined periodically for the development of ascomata or conidiomata. Colony diameters were determined on PDA cultures grown at 22–25°C in darkness after 3 days, while colony colors were described after 14 days using the charts of Rayner (1970). For microscopy, fungal structures were mounted in distilled water, and measurements determined for 30 conidia and other structures.

2.6. Biochemical properties of *Diaporthe* strains

The 22 *Diaporthe* strains were investigated for their metabolic activity *in vitro* as follows. Ammonium production was determined both qualitatively and quantitatively, as described by Cappuccino and Sherman (1998). Siderophore activity was determined using the chrome azurol S agar plate assay as described by Schwyn and Neilands (1987). The production of indole-3-acetic acid (IAA) was determined quantitatively using both spectrophotometric (Gordon and Weber, 1951) and chromatographic (Patel et al., 2018) assays. The ability of *Diaporthe* strains to produce amylase, cellulase and protease was analyzed *in vitro* in the supplemented media described by Hankin and Anagnostakis (1975). The phosphate solubilizing ability was determined using Pikovskaya's agar medium, as described by Katznelson and Bose (1959).

2.7. Pathogenicity assays

Because of the close relatedness of the new *Diaporthe* species to *D. sclerotioides*, a well-known pathogen of cucurbits (Shishido et al., 2006, 2014), the pathogenicity of *Diaporthe* strains EB4 (*D. atlantica*) and S32 (*D. iberica*) was tested on cucumber (*Cucumis sativus*) cv. Ashley, melon (*Cucumis melo*) cv. Piñonet, and watermelon (*Citrullus lanatus*) cv. Crimson Sweet.

Mycelial inoculum of both *Diaporthe* strains was produced in 3-week old sugar beet pulp cultures (Vázquez de Aldana et al., 2020), and seedlings of the three cucurbits were obtained after seed germination in sterile vermiculite. Fourteen day old seedlings were transplanted to 200 mL pots containing a substrate consisting of seven volumes of a mixture of peat and perlite (1:1; v:v) previously treated at 80°C for 12 h in a forced air oven, and one volume of *Diaporthe* inoculum. Control seedlings were transplanted to substrate containing only the peat and perlite mixture. One seedling was

transplanted to each pot and each treatment was replicated in six pots. The plants were maintained in a greenhouse, being tap watered on demand for a period of 40 days. After this time, the plants were harvested, the roots were cleaned with tap water and inspected for disease symptoms such as dark lesions. Dry weights of roots and aboveground tissues were measured, and differences among treatments were tested by means of an analysis of variance (ANOVA). Differences among means were tested with the Holm-Sidak method. Statistical calculations were made with SigmaPlot software v. 14.5.

3. Results

3.1. Phylogenetic analyses

A preliminary analysis of the concatenated sequences of the 22 *Diaporthe* strains from *Festuca pruinosa* aligned with 243 sequences belonging to 95 *Diaporthe* species (Gomes et al., 2013) allowed to identify the clades to which the Festuca isolates belonged (**Supplementary Figure 1**). Thereafter, we reviewed other multilocus taxonomic studies (Dissanayake et al., 2017; Gao et al., 2017; Guarnaccia et al., 2018; Guo et al., 2020; Norphanphoun et al., 2022), to search for other *Diaporthe* species closely related to the identified clades that were not included in Gomes et al. (2013). As a result, *Diaporthe columnaris* and *D. cyatheae* (Norphanphoun et al., 2022), were found to belong to the same clade as our endophytic isolates. Due to the lack of all five loci for these two species, they were not included in the multilocus tree. However, available loci were included in the single locus phylogeny to support the classification of our fungal isolates (**Supplementary Figures 2–5**).

Ultimately, a set of 11 *Diaporthe* species closely related to the endophytic *Festuca pruinosa* isolates plus *Diaporthella corylina* (outgroup) were selected for a more restricted multilocus phylogenetic analysis (**Table 1**). A total of 2,786 characters including gaps were included in this phylogenetic analysis, 1,368 of these were conserved and 981 were variable, 649 of which were parsimony informative. The Bayesian analysis of the combined sequences of the five loci was based on the Hasegawa-Kishino-Yano substitution model with gamma distributed rate variation among sites (**Table 2**) and all partitions had Dirichlet base frequencies. The Bayesian analysis lasted 20 000 000 generations resulting in an average standard deviation of split frequencies of 0.001683. The consensus tree was generated and the posterior probabilities were calculated from the 27 443 trees left after discarding the first 25% for burn-in. The topologies resulting from ML and BI analyses of the concatenated dataset were congruent. Bayesian posterior probability (PP $\geq$ 0.8) and Maximum likelihood bootstrap values (ML $\geq$ 70) were obtained for the dendrogram nodes (**Figure 1**).

The 22 *Diaporthe* isolates from *Festuca pruinosa* were set apart from known species, and clustered together to create two novel clades statistically well-supported by ML and PP values. In the multilocus tree, 18 *Festuca pruinosa* isolates clustered together within a well-supported clade (ML/PP = 99/1.00) significantly distinct from *D. sclerotioides*, its closest known species (**Figure 1**). This new species was named *Diaporthe atlantica* sp. nov. Moreover, the single locus phylogeny of the five genes shows that *D. atlantica* was closer but separated from *D. sclerotioides* (**Supplementary Figures 2–7**) and also differed from *D. columnaris* in the ITS and *TEF1* trees (**Supplementary Figures 2, 4**).

Three isolates (S32, TH77, and T6) clustered together to form a new clade (ML/PP = 100/1.00) unrelated to other known *Diaporthe* species, and was named *Diaporthe iberica* sp. nov. Two of these strains were isolated from maritime populations of *Festuca pruinosa*, but strain T6 was isolated several hundred km inland from roots of *Celtica gigantea*. Interestingly, *Festuca pruinosa* strains S32 and TH77 were genotypically closer than strain T6. In addition, the single locus analysis of each of the five genes consistently differentiated the clade formed by *D. iberica* strains (S32, TH77, and T6) from all known species included in the analysis (**Supplementary Figures 2–6**).

The single and multilocus phylogenetic analyses also showed that the strain EB73 was well-separated from closely related species (*D. musigena*, *D. arecae*, and *D. foeniculacae*), and might as well belong to a yet undescribed *Diaporthe* species. As we currently have only a single strain of this taxon, further investigations will have to await further collections.

3.2. Taxonomy

Based on the multi-locus phylogeny and their morphology, the 22 strains from *Festuca pruinosa* were assigned to two newly described taxa, plus a yet undescribed species. All species studied in culture are characterized below.

Diaporthe atlantica Toghueo, Vazq-Alda and Zabalgo, sp. nov.; **Figure 2** and **Supplementary Figure 8**.

MycoBank number: MB845433.

Etymology: Named after the Atlantic ocean, in whose coastal cliffs is found *Festuca rubra* subsp. *pruinosa*, the plant host of this species.

Description: Pycnidial conidiomata globose or irregular, black scattered or aggregated, and irregularly distributed over agar surfaces, with white or cream conidial droplets (50–160 μm) exuding from the ostioles. Conidiophores hyaline, terminal and lateral, cylindrical, 1-3-septate, densely aggregated, 14.3–30.9 × 2.5–4.1 μm, (mean ± SD = 21.84 ± 6.07×3.11 ± 0.61 μm). Conidiogenous cells [4.8–15.1 × 1.9–6.6 μm, (mean ± SD = 9.36 ± 3.84 × 4.44 ± 1.9μm)] hyaline, subcylindrical and filiform, straight to curved, terminal and lateral, with slight taper toward the apex. Alpha conidia were fusiform, hyaline, ellipsoidal, aseptate, rounded at each end, 8.7–12.3 × 3.3–5 μm, (mean ± SD = 10.29 ± 0.9 × 4.3 ± 0.42 μm). Beta and gamma conidia were not observed.

Culture characteristics: Colonies on PDA were white at first, with flattened mycelium and becoming dirty white and umber over time with dark black pycnidia bearing cream conidial droplets distributed over the agar surface. The colony margins had petaloidlike

TABLE 2 Nucleotide substitution models used in the phylogenetic analyses.

Loci/Genes	Analysis	Best-fit model
ITS	ML	Kimura 2-parameter
TUB	ML	Hasegawa-Kishino-Yano
TEF1	ML	Kimura 2-parameter
HIS	ML	Tamura-Nei
CAL	ML	Tamura 3-parameter
Concatenated loci	ML/BI	Hasegawa-Kishino-Yano

BI, Bayesian inference; ML, maximum likelihood.

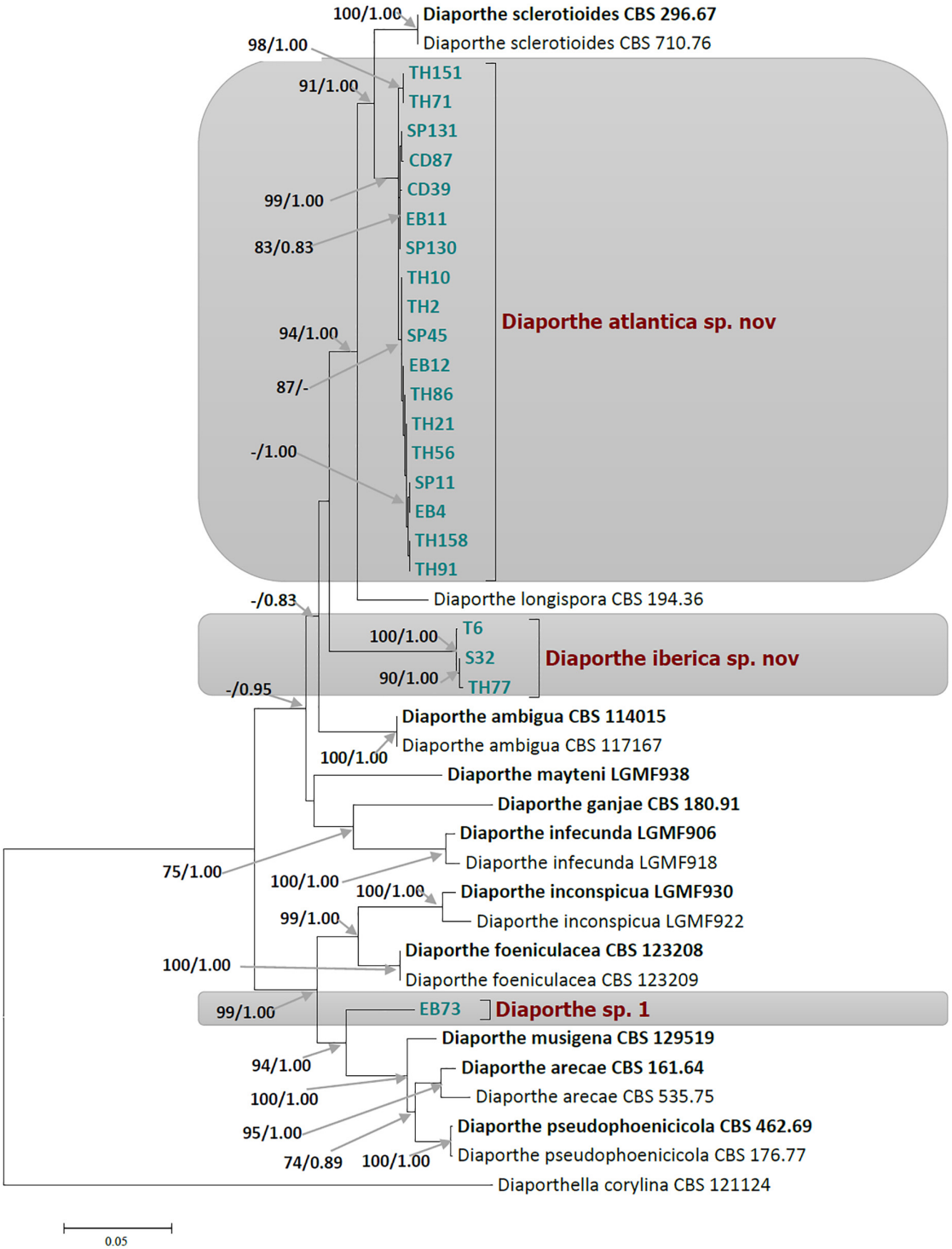

FIGURE 1
Phylogram of *Diaporthe* resulting from a maximum likelihood analysis based on a combined matrix of ITS, *TUB*, *CAL*, *TEF1*, and *HIS3*. Numbers above the branches indicate ML bootstraps (left, ML BS ≥ 70%) and Bayesian Posterior Probabilities (right, PP ≥ 0.8). The tree is rooted with *Diaporthella corylina*. Isolates from present study are marked in colors. The scale bar represents the expected changes per site. The ex-type strains are Bold and strains from this study in green.

shapes. The reverse was graphite gray with black spots uniformly distributed. On MEA, colonies were white at first with flattened mycelium and becoming brownish over time. Dark black and sterile stromata abundantly distributed over the mycelium surface were observed. The colony diameter was 24–30 mm after 3 days at 25°C.

Specimens examined: SPAIN, Asturias, San Pedro de la Rivera (43°34'43"N, 6°13'17"W), from roots of *Festuca rubra* subsp. *pruinosa*, Mar. 2016, E. Pereira, holotype SP11 (CECT 21217); A Coruña, Cedeira, from roots of *Festuca pruinosa*, Mar. 2016, E. Pereira, strains CD39, CD87; A Coruña, Estaca de Bares, from roots of *Festuca pruinosa*, Mar. 2016, E. Pereira strains EB4, EB11,

FIGURE 2
Morphological characteristics of *Diaporthe atlantica*. Colony appearance on potato dextrose agar (PDA) **(a)** and malt extract agar (MEA) **(b)**; conidiomata with conidial droplets on PDA **(c,d)**; conidiomata on pine needle **(e)** and *Festuca pruinosa* leaves **(f)**; conidiogenous cells **(g)**; α-conidia **(h,i)**. Scale bar: panels **(d–f)** = 100 μm; panels **(c,g–i)** = 10 μm.

and EB12; Asturias, San Pedro de la Rivera, from roots of *Festuca pruinosa*, Mar. 2016, E. Pereira, strains SP45, SP130, and SP131; A Coruña, Torre de Hercules, from roots of *Festuca pruinosa*, Mar. 2016, E. Pereira, strains TH2, TH10, TH21, TH56, TH71, TH86, TH91, TH151, and TH158.

Host/Habitat: *Festuca rubra* subsp. *pruinosa* grows on rocky sea cliffs in the Atlantic coasts of Europe. In this habitat, soil and nutrients are very limited, and exposure to salinity is continuous.

Notes: *Diaporthe atlantica* was isolated as an endophyte from surface-disinfected roots of *Festuca pruinosa*. This species was described from a set of 18 strains among which only four produced spores, and this occurred on PNA medium. This species was found in plants from all the four locations investigated. *In vitro*, all 18 strains investigated produced indole-3-acetic acid (IAA), and ammonium, while 16 produced siderophores and cellulase, 13 solubilized phosphate and 11 produced amylase. *Diaporthe atlantica* is phylogenetically close but clearly differentiated from *D. sclerotioides* and *D. columnaris* two species known as pathogens of cucurbits (Shishido et al., 2014) and lingonberry (Farr et al., 2002), respectively. Morphologically *D. atlantica* cannot be reliably separated from *D. sclerotioides* due to overlaps in characteristics such as the culture phenotype or spore shape and size. However, *D. atlantica* is morphologically distant from *D. columnaris* by the size of alpha conidia, and the shape and size of conidiogenous cells (Farr et al., 2002). On the other hand, *D. atlantica* differs from *D. sclerotioides* by 110 nucleotides in the concatenated sequence alignment, 45 of which were distinct in the *TUB* region, 44 in the *TEF1* region, 10 in the ITS region, 9 in the *HIS* region, and 2 in the *CAL* region and differs from *D. columnaris* by 2 and 13 nucleotides in the ITS and *TEF1* regions respectively. Furthermore, a phylogenetic analysis based on the ITS sequences of all *D. atlantica* strains and those of thirteen isolates of *D. sclerotioides* retrieved from GenBank revealed a clear separation between both species. In the ML tree obtained, all *D. atlantica* strains clustered together in a well-supported clade (ML = 70; **Supplementary Figure 7**) clearly separated from the *D. sclerotioides* isolates (ML = 99; **Supplementary Figure 7**). Inoculation of *D. atlantica* strain EB4 in cucumber, melon, and watermelon plants did not cause root necrosis or seedling wilt, as

D. sclerotioides does (Shishido et al., 2014), but shoot and root growth was delayed in the three plant species.

Diaporthe iberica Toghueo, Vazq-Alda and Zabalgo, sp. nov.; **Figure 3** and **Supplementary Figure 8**.

MycoBank number: MB845435.

Etymology: Named after the Iberian Peninsula, where isolates were obtained in different habitats and host plant species.

Description: Sterile stromata were globose or irregular, dark black, irregularly distributed over the agar surface, scattered or aggregated and exposed on the surface of *Festuca rubra* leaves and pine needles. Alpha, beta and gamma conidia were not observed.

Culture characteristics: Colonies on PDA form flattened and white mycelium. Dark sterile stromata uniformly distributed over the agar plate emerged after 2 weeks at 25°C. On MEA, colonies with flattened and white gray mycelium with dark stromata were uniformly distributed over the agar plate. The colony diameter was 22–31 mm after 3 days at 25°C. The dark stromata remained sterile on PDA, MEA PNA, and FLA after 4 months in culture at 25°C, and after 2 weeks of continuous exposure to UV light.

Specimens examined: SPAIN, A Coruña, Torre de Hercules (43°23'09"N, 8°24'23"W), from roots of *Festuca rubra* subsp. *pruinosa*, Mar. 2016, E. Pereira, holotype TH77 (CECT 21218); Asturias, San Pedro de la Rivera, from roots of *Festuca pruinosa*, Mar. 2016, E. Pereira, strain S32; Salamanca, Cuatro Calzadas, from roots of *Celtica gigantea*, Mar. 2015, I. Zabalgogeazcoa, strain T6.

Host/Habitat: *Festuca pruinosa* grows on rocky sea cliffs, with poor nutrient availability, and continuous exposure to salinity, while *Celtica gigantea* grows in nutrient-poor sandy soils in semiarid habitats in the southwestern Iberian peninsula (Vázquez and Devesa, 1996).

Notes: The three isolates of *Diaporthe iberica* studied form a clade with high support (ML/PP = 100/1.00), distinct from other known *Diaporthe* species. This new taxon can colonize at least two distinct host grasses, *Festuca pruinosa* and *Celtica gigantea*, growing in very different habitats. *In vitro*, all three strains produced IAA, ammonium, siderophores and cellulase.

Diaporthe sp. 1

Specimen examined: SPAIN, A Coruña, Estaca de Bares (43°47'25"N, 7°41'16"W), from roots of *Festuca pruinosa*, Mar. 2016, E. Pereira, strain EB73 (CECT 21219).

Culture characteristics: Colonies on PDA form white gray mycelium at first and become dark over time. On MEA, light brown aerial mycelium, cottony and less abundant. The colony diameter was 26 mm after 3 day at 25°C (**Figure 4**).

Host/Habitat: *Festuca rubra* subsp. *pruinosa* grows on rocky sea cliffs in the Atlantic coasts of Europe. Soil and nutrients are very limited, and exposure to salinity is continuous in this habitat.

Notes: Based on phylogenetic data, this endophytic isolate might belong to an undescribed species. Cultures were sterile on various media including PDA, MEA, PNA, and FLA at varied conditions. *In vitro*, strain EB73 produced IAA, ammonium, siderophores, cellulase and solubilized phosphate.

3.3. Genetic diversity of *Diaporthe atlantica*

The genetic diversity of *Diaporthe atlantica* was estimated on the basis of its 18 individuals (**Table 3**). The calculated haplotype diversity of *D. atlantica* was higher than 0.5 for TEF1, CAL, HIS, TUB

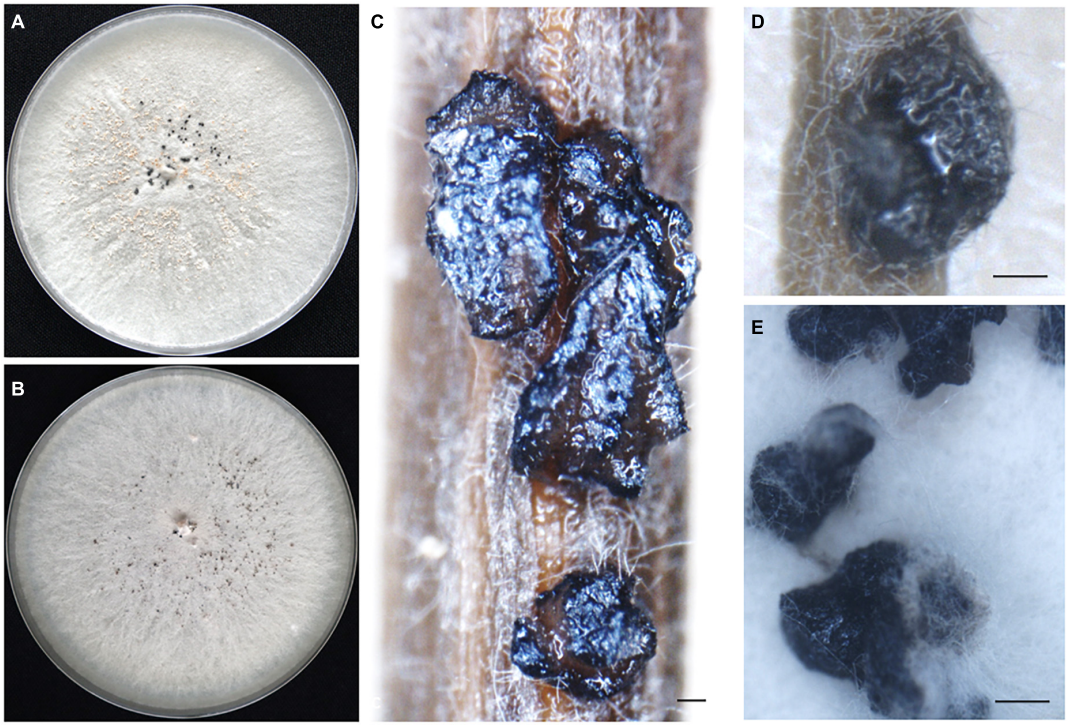

FIGURE 3
Morphological characteristics of *Diaporthe iberica*. Front view of colony appearance on potato dextrose agar (PDA) **(A)** and malt extract agar (MEA) **(B)**; sterile stromata on pine needle **(C)** and *F. pruinosa* leaf **(D)** and on the surface of PDA **(E)**; Scale bar: panels **(C–E)** = 100 μm.

10.3389/fmicb.2023.1105299

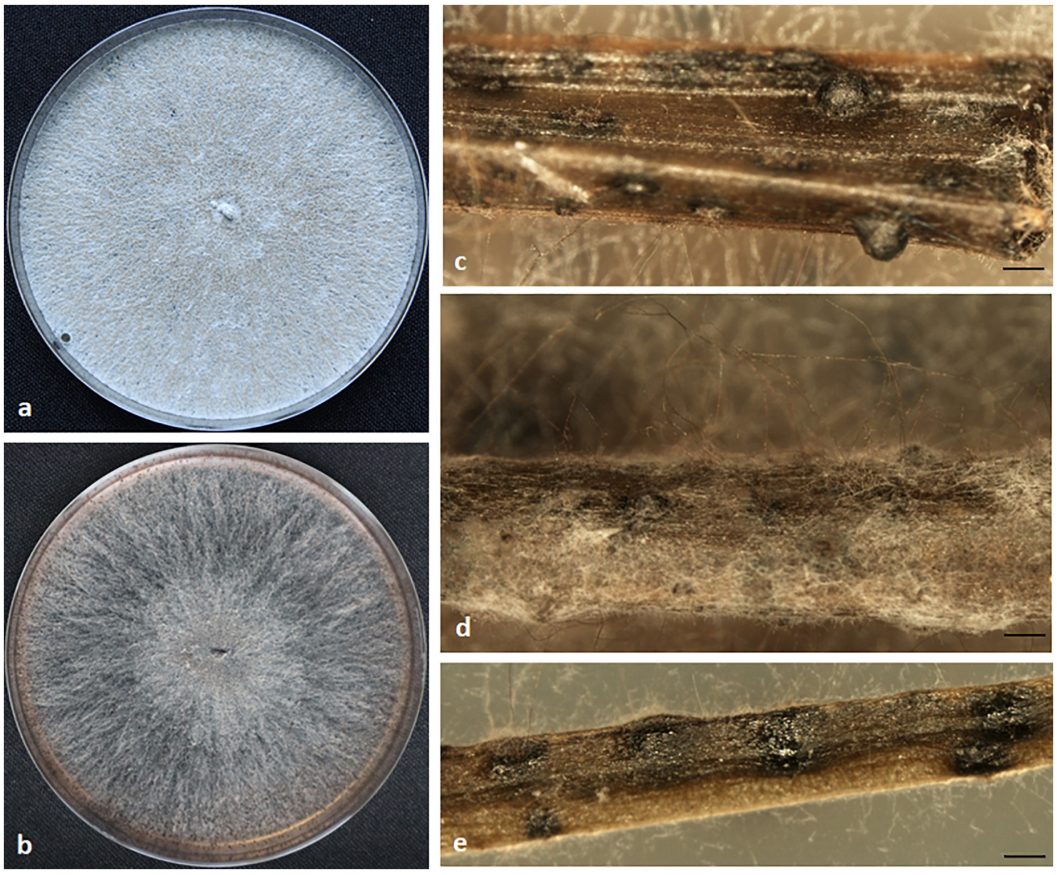

FIGURE 4
Morphological characteristics of *Diaporthe* sp. 1. Colony appearance on potato dextrose agar (PDA) **(a)** and malt extract agar (MEA) **(b)**; sterile conidiomata on pine needle **(c,d)** and *Festuca pruinosa* leaves **(e)**. Scale bar: panels **(c–e)** = 100 μm.

TABLE 3 Polymorphism and genetic diversity parameters of *Diaporthe atlantica* strains associated with *Festuca rubra* subsp. *pruinosa*.

Gene	*n*	bp	θ_w	*S*	*H*	Hd	pi	*k*	TD	Rm
ITS	18	423	0.581	2	2	0.209	0.00099	0.418	−0.68482	0.0
TUB	18	709	2.035	7	7	0.869	0.00256	1.817	−0.36375	0.0
HIS	18	386	2.907	10	9	0.895	0.00598	2.307	−0.74337	0.0
TEF1	18	230	4.070	14	11	0.922	0.01998	4.595	0.20324	2.0
CAL	18	312	0.581	2	3	0.569	0.00297	0.928	1.45481	0.0
Combined	18	2061	8.722	30	17	0.987	0.00451	9.294	0.12562	4.0

n, sample size; bp, total number of sites; S, number of segregating/polymorphic sites (parsimony informative sites); H, number of alleles; Hd, haplotype (allelic) diversity; pi, average nucleotide diversity; k, average number of nucleotide differences; TD, Tajima's D (TD); θ_w, Watterson's theta; Rm, minimum number of recombination events. TD was not significant ($P > 0.10$) for all the genes analyzed.

and the combined data set, reflecting a high genetic diversity of the population analyzed (Nei and Tajima, 1981; Stumpf, 2004).

Overall, 17 haplotypes were detected in the concatenated gene sequence, while eleven, nine, seven, three, and two haplotypes were detected in TEF1, HIS, TUB, CAL, and ITS sequences respectively (**Table 4**). The only common haplotype, H-9, was shared by two strains obtained from different localities, Estaca de Bares (A Coruña) and San Pedro de la Rivera (Asturias). The haplotypes ITS-H1 (n = 16), CAL-H2 (n = 11), HIS-H6 (n = 5), TEF1-H4 (n = 4) and TUB-H5 (n = 5) were the most frequent for each gene. The highest degree of sequence polymorphism (pi = 0.01998) was observed for TEF1, with 11 haplotypes. Details of polymorphism at each locus and unique sequences types for each strain are available in **Supplementary Table 2**. In general, this analysis suggests that the populations of *Diaporthe atlantica* are genotypically diverse, but clonality exists in them.

3.4. Biochemical characteristics of endophytic *Diaporthe* species

All 22 *Diaporthe* strains produced ammonium in peptone water and IAA in potato dextrose broth. On agar-supplemented media, 20 strains were able to produce siderophores and cellulase, 15

TABLE 4 Haplotypes of *Diaporthe atlantica* strains isolated from roots of *Festuca rubra* subsp. *pruinosa*.

Strain	Location	Single locus haplotype					Five-locus haplotype
		ITS	*TUB*	*HIS*	*TEF1-α*	*CAL*	
CD39	CED	ITS-H1	*tub*-H1	*his*-H7	*tef*1-H1	*Cal*-H1	Haplotype 1
TH2	TDH	ITS-H1	*tub*-H5	*his*-H9	*tef*1-H7	*Cal*-H2	Haplotype 2
TH10	TDH	ITS-H1	*tub*-H5	*his*-H8	*tef*1-H8	*Cal*-H2	Haplotype 3
TH21	TDH	ITS-H1	*tub*-H5	*his*-H6	*tef*1-H8	*Cal*-H2	Haplotype 4
TH86	TDH	ITS-H1	*tub*-H3	*his*-H6	*tef*1-H8	*Cal*-H2	Haplotype 5
TH91	TDH	ITS-H1	*tub*-H7	*his*-H4	*tef*1-H11	*Cal*-H2	Haplotype 6
SP45	SPR	ITS-H1	*tub*-H5	*his*-H3	*tef*1-H6	*Cal*-H2	Haplotype 7
CD87	CED	ITS-H1	*tub*-H2	*his*-H5	*tef*1-H2	*Cal*-H1	Haplotype 8
EB4	EDB	ITS-H1	*tub*-H4	*his*-H6	*tef*1-H3	*Cal*-H2	Haplotype 9
SP11	SPR	ITS-H1	*tub*-H4	*his*-H6	*tef*1-H3	*Cal*-H2	Haplotype 9
EB11	EDB	ITS-H1	*tub*-H1	*his*-H1	*tef*1-H4	*Cal*-H1	Haplotype 10
SP130	SPR	ITS-H1	*tub*-H1	*his*-H7	*tef*1-H4	*Cal*-H1	Haplotype 11
SP131	SPR	ITS-H1	*tub*-H1	*his*-H5	*tef*1-H3	*Cal*-H1	Haplotype 12
TH71	TDH	ITS-H2	*tub*-H6	*his*-H1	*tef*1-H10	*Cal*-H3	Haplotype 13
TH151	TDH	ITS-H2	*tub*-H6	*his*-H1	*tef*1-H4	*Cal*-H3	Haplotype 14
TH56	TDH	ITS-H1	*tub*-H5	*his*-H6	*tef*1-H9	*Cal*-H2	Haplotype 15
TH158	TDH	ITS-H1	*tub*-H7	*his*-H4	*tef*1-H4	*Cal*-H2	Haplotype 16
EB12	EDB	ITS-H1	*tub*-H3	*his*-H2	*tef*1-H5	*Cal*-H2	Haplotype 17

The haplotypes observed at each sequenced locus for each strain analyzed ($n = 18$), and the resulting multilocus haplotype are shown. CED, Cedeira; EDB, Estaca de Bares; SPR, San Pedro de la Rivera; TDH, Torre de Hércules; *CAL*, partial sequence of the gene coding for calmodulin; *HIS*, Histone H3; ITS, ITS1-5.8S rDNA-ITS2 internal transcribed spacer region; *TEF1*, partial sequence of the gene coding for translation elongation factor 1-α; *TUB*, partial sequence of the gene coding for β -tubulin.

solubilized phosphate, 13 amylase, and 5 protease (**Supplementary Table 3**). A principal component analysis (PCA) was performed on the whole dataset to investigate if there is a relationship between *Diaporthe* species and some biochemical characteristics. The PCA showed that components I and II accounted for 62% of the total variance. The three *D. iberica* strains (T6, TH77, and S32) clustered in close proximity, within the sparse distribution of *D. atlantica* strains (**Figure 5**).

3.5. Pathogenicity of *Diaporthe*

Neither dark lesions in roots or seedling wilt, symptoms reported for cucurbit root rot disease caused by *D. sclerotioides* (Shishido et al., 2006, 2014) were observed in plants inoculated with *D. atlantica* strain EB4 or *D. iberica* strain S32. However, a significant ($P < 0.05$) inhibition of shoot and root growth occurred in cucumber inoculated with strain EB4, and in roots of melon and watermelon inoculated with strains EB4 and S32 (**Figure 6**).

4. Discussion

The association of *Diaporthe* species with many important plant diseases has stimulated a considerable interest in this genus. On the other hand, *Diaporthe* species are ubiquitous in nature as endophytes, and have been reported as dominant components of the microbiome of various plant species. Therefore, more efforts are still required to increase our understanding of their ecology and biology. In this study, a multi-locus phylogenetic analysis of endophytic *Diaporthe* strains associated with roots of *Festuca pruinosa*, a grass that lives in a very unhospitable habitat, revealed two new species, *Diaporthe atlantica* and *Diaporthe iberica*, and possibly a third undescribed species represented by *Diaporthe* sp. 1. Both species described in this study belong to the *Diaporthe sojae* species complex. This complex is composed of endophytes, saprophytes and pathogens, and is the largest of the thirteen species complexes of *Diaporthe* recently described by Norphanphoun et al. (2022).

Over the past decade, DNA sequence data have become paramount for the accurate definition of species boundaries, and the resolution of important taxonomic questions. In the genus *Diaporthe*, multi-locus phylogenies based on the combined analysis of five genes were found to be better suited for an accurate species delimitation than those based on four or less loci (Santos et al., 2017). Therefore, the five most commonly used loci (ITS, TUB, TEF1, CAL, and HIS) for the separation of species in this genus were used in the present study to resolve the taxonomy of endophytic *Diaporthe* strains from *Festuca pruinosa* (Gomes et al., 2013; Gao et al., 2017; Guarnaccia et al., 2018; Guo et al., 2020; Norphanphoun et al., 2022). Among the *Diaporthe* species identified, *Diaporthe atlantica* was the most abundant, occurred in roots of plants from all four locations examined, and its populations were genotypically diverse. Phylogenetically, *D. atlantica* was close to, but clearly differentiated from *D. sclerotioides*. However, the comparison of morphological characters such as the culture appearance, or the shape and size of alpha conidia make impossible to separate both species (Shishido et al., 2006). Indeed, the difficulties in morphological comparisons have historically made the taxonomy of *Diaporthe* very challenging

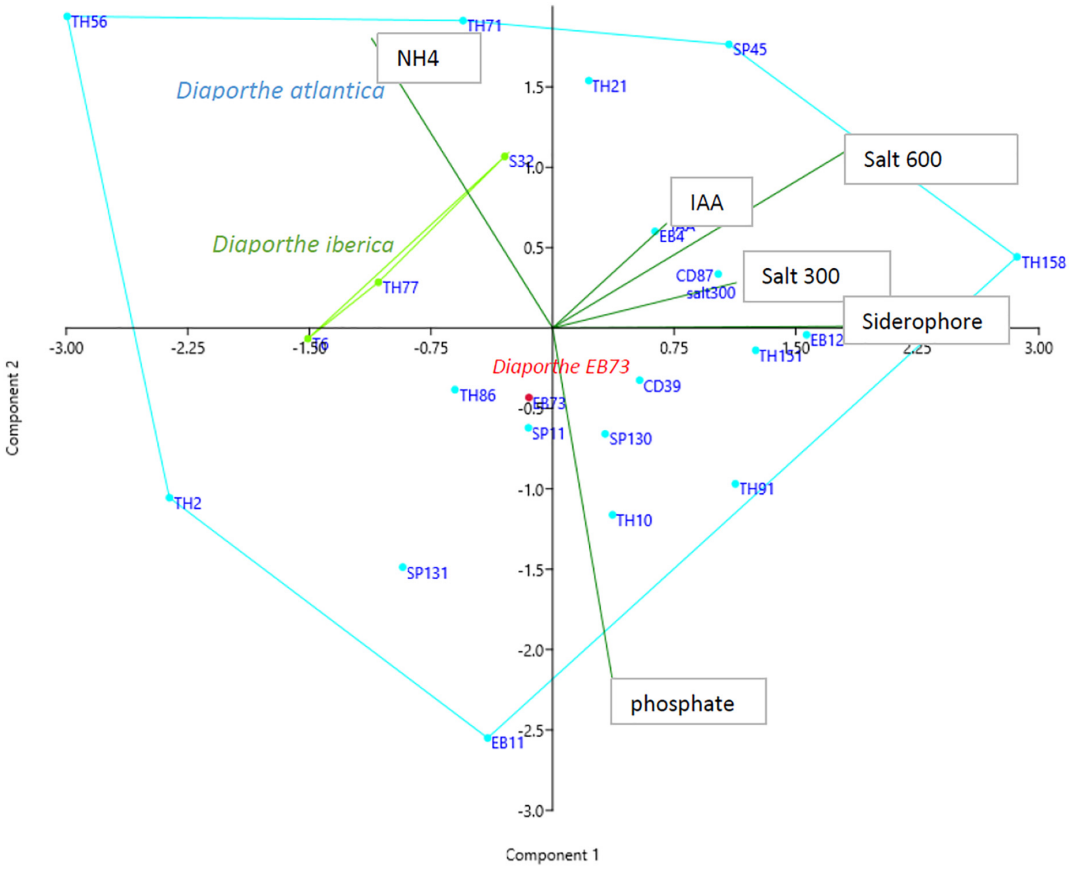

FIGURE 5
Principal component analysis of *in vitro* characteristic of *Diaporthe* strains isolated from *Festuca pruinosa*.

(Gomes et al., 2013). *Diaporthe* species often have similar or overlapping morphological characteristics (Lawrence et al., 2015), and due to their plasticity and inability to properly reflect the evolutionary history of species, these cultural characteristics have been deemed unfit for species delimitation (Rehner and Uecker, 1994; Gao et al., 2015). Therefore, the five gene multi-locus phylogenetic analysis was the key approach used in the present study to classify accurately *Diaporthe atlantica* as new species.

The plants of *Festuca pruinosa* often grow in soilless fissures in the rock, where a compact mass of roots is associated to a complex microbiome (Pereira et al., 2019). In this habitat poor in nutrients, *D. atlantica* could be helping its host to survive by playing a role in the recycling of nutrients from dead Festuca roots and other organic debris. In connection with this, *D. atlantica* cultures had extracellular enzymatic activities capable of degrading protein to ammonium, cellulases which could reduce cell wall material to simpler carbohydrates, and siderophores which help to trap iron. Plant roots can readily absorb ammonium and some simple carbohydrates, thus, the acceleration of organic matter decay by this fungus could increase the nutrient availability in a nutrient poor environment. Therefore, in its host grass *D. atlantica* could be a latent saprophyte, an endophyte that becomes a saprophyte when host roots senesce or die. In addition, *Diaporthe* cultures produced IAA, which can stimulate root growth. This fungus is known to behave as a mutualist in other grasses than its original host, promoting leaf and root growth in tritordeum and perennial ryegrass (*Lolium perenne*) even under salinity conditions (Toghueo et al., 2022).

The other novel species identified in this study, *Diaporthe iberica*, was associated to two plant species inhabiting very different habitats. The strain T6 was isolated from roots of *Celtica gigantea*, a perennial grass that grows in nutrient-poor sandy soils in semiarid zones (Vázquez and Devesa, 1996; Vázquez de Aldana et al., 2021), while strains S32 and TH77 occurred in roots of the perennial grass *Festuca pruinosa* from sea cliffs. Like *D. iberica*, many *Diaporthe* species are known to be multi-host. For instance, *Diaporthe eres*, the type species of the genus has been reported as a pathogen and an endophyte in plant species belonging to several families (Udayanga et al., 2014a). *Diaporthe iberica* did not produce spores on any of the different agar media we tested. *Diaporthe* species like *D. endophytica* (Gomes et al., 2013), *D. infertilis* (Guarnaccia and Crous, 2017), and *D. parvae* (Guo et al., 2020) are also known to be sterile, at least under experimental conditions. Like *Diaporthe atlantica*, *D. iberica* could have a beneficial association with its original host, improving adaptability to nutrient poor environments by contributing to organic matter recycling. Amylase and cellulase activity, ammonium production from protein substrate, and siderophores were observed in its cultures. In addition, *Diaporthe iberica* strain T6 was previously found to exhibit the greatest plant growth promotion capability among 66 fungal endophytes from *Celtica gigantea*, increasing both leaf and root biomass, and the leaf content of several mineral elements in tritordeum (Vázquez de Aldana et al., 2021). Besides, we found that *D. iberica* strain S32 could significantly improve the growth of *Festuca*

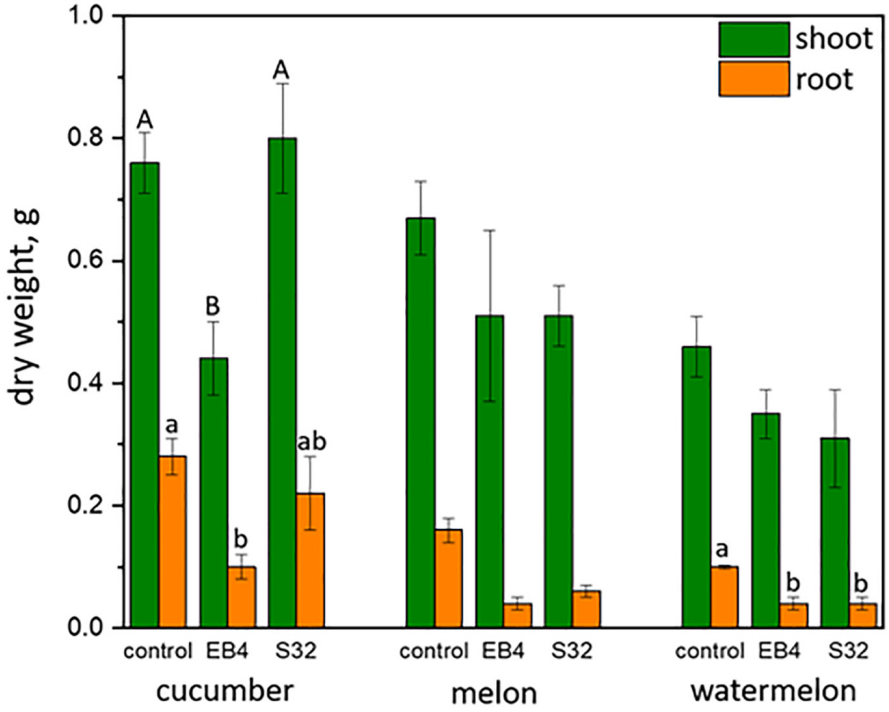

FIGURE 6
Shoot and root dry weight (mean ± standard error) of cucurbitaceous plants inoculated with *Diaporthe atlantica* strain EB4 and *Diaporthe iberica* strain S32. For each plant species and organ, letters on columns indicate significantly different means.

pruinosa clonal line CD8 in greenhouse conditions (data not shown).

In addition to *D. atlantica* and *D. iberica*, *Festuca pruinosa* hosts another undescribed species of *Diaporthe*, *Diaporthe* sp. 1. The single strain representing this taxon also showed *in vitro* characteristics consistent with potential plant growth promotion and nutrient recycling functions, like production of IAA, ammonium, siderophores, cellulase and phosphate solubilization. However, following the recommendations of the International Commission on the Taxonomy of Fungi on the description of new fungal species, the taxonomic status of this strain will have to await further collection, since only one member of this putative species is known so far (Aime et al., 2021).

As above mentioned, virulent *Diaporthe* pathogens can also occur as endophytes in alternative host plant species (Crous and Groenewald, 2005; Gomes et al., 2013). Because of the close relatedness of *Diaporthe atlantica* to *D. sclerotioides*, a well-known pathogen of cucurbits (Shishido et al., 2006), the potential pathogenicity of *D. atlantica* strain EB4 and *D. iberica* strain S32 were investigated on cucumber, melon and watermelon. None of these caused root necrosis or wilt symptoms on cucurbits, as reported for black root rot disease caused by *D. sclerotioides* on these species (Shishido et al., 2006, 2014). However, strains EB4 and S32 caused a decrease in the shoot and root biomass of the three cucurbits. This potentially harmful effect observed in cucurbits contrasts with the beneficial effects observed in plants of tritordeum, perennial ryegrass and tomato inoculated with the same *D. atlantica* strain (Pereira, 2021; Toghueo et al., 2022). A *Diaporthe iberica* strain also showed a beneficial response on tritordeum (Vázquez de Aldana et al., 2021). Therefore, it seems that the benefits imparted by *D. atlantica* and *D. iberica* may be host-dependent, with only particular plant–fungus interactions resulting in positive plant responses (Brader et al., 2017). The host plant species and genotype has been reported to have a significant influence on the symbiotic lifestyles of some *Colletotrichum* species, favoring in some cases a mutualistic, commensal, or pathogenic lifestyle (Redman et al., 2001). Further investigations in this direction are required to understand the bases of the change in lifestyle by these *Diaporthe* species.

5. Conclusion

This study revealed that at least three *Diaporthe* species are associated to roots of *Festuca pruinosa* as endophytes. We identified two new *Diaporthe* species, *Diaporthe atlantica*, *Diaporthe iberica* and a yet undescribed taxon *Diaporthe* sp.1. *Diaporthe atlantica* seems to be the most abundant taxon in *Festuca pruinosa*, while *D. iberica* is a multihost endophyte naturally associated to roots of at least two different species of grasses (*Festuca pruinosa* and *Celtica gigantea*) growing in very different habitats, marine sea cliffs and sandy soils in semiarid inland zones. We propose that these symbiotic fungi might improve nutrient availability for their hosts, living in a habitat where nutrient availability is very limited. Since some *Diaporthe* species are multihost, other plants sympatric with *Festuca pruinosa* in sea cliff habitats, like *Armeria maritima* or *Crithmum maritimum* (Lopez-Bedoya and Pérez-Alberti, 2009), might also be hosts of these *Diaporthe* species. However, the ecological role of the newly described species as well as their interaction with host plants still needs to be better understood. Descriptions, molecular data, *in vitro* activities, and host interactions of the *Diaporthe* species described in the

present study could represent an important resource for agricultural scientists, plant pathologists and taxonomists.

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

RT performed experiments, analyzed the data, and wrote the manuscript. IZ and BV supervised the research and wrote the manuscript. All authors designed the experiments, analyzed the data, and read and approved the final manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1105299/full#supplementary-material

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EDITED BY
Priyanka Chandra,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Pandeng Wang,
Sun Yat-sen University, China
Ake Liu,
Changzhi University, China

*CORRESPONDENCE
Zhijia Cui
✉ zhijiacui@126.com
Ling Jin
✉ gszyxyjl@163.com

†These authors share first authorship

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Structural characteristics and diversity of the rhizosphere bacterial communities of wild *Fritillaria przewalskii* Maxim. in the northeastern Tibetan Plateau

Zhijia Cui[1,2*†], Ran Li[1†], Fan Li[1], Ling Jin[1,2*], Haixu Wu[1], Chunya Cheng[1], Yi Ma[1,2], Zhenheng Wang[1,2] and Yuanyuan Wang[1]

[1]College of Pharmacy, Gansu University of Chinese Medicine, Lanzhou, Gansu, China, [2]Northwest Collaborative Innovation Center for Traditional Chinese Medicine Co-Constructed by Gansu Province & MOE of PRC, Lanzhou, Gansu, China

Introduction: *Fritillaria przewalskii* Maxim. is a Chinese endemic species with high medicinal value distributed in the northeastern part of the Tibetan Plateau. *F. przewalskii* root-associated rhizosphere bacterial communities shaped by soil properties may maintain the stability of soil structure and regulate *F. przewalskii* growth, but the rhizosphere bacterial community structure of wild *F. przewalskii* from natural populations is not clear.

Methods: In the current study, soil samples from 12 sites within the natural range of wild *F. przewalskii* were collected to investigate the compositions of bacterial communities *via* high-throughput sequencing of 16S rRNA genes and multivariate statistical analysis combined with soil properties and plant phenotypic characteristics.

Results: Bacterial communities varied between rhizosphere and bulk soil, and also between sites. Co-occurrence networks were more complex in rhizosphere soil (1,169 edges) than in bulk soil (676 edges). There were differences in bacterial communities between regions, including diversity and composition. Proteobacteria (26.47–37.61%), Bacteroidetes (10.53–25.22%), and Acidobacteria (10.45–23.54%) were the dominant bacteria, and all are associated with nutrient cycling. In multivariate statistical analysis, both soil properties and plant phenotypic characteristics were significantly associated with the bacterial community ($p<0.05$). Soil physicochemical properties accounted for most community differences, and pH was a key factor ($p<0.01$). Interestingly, when the rhizosphere soil environment remained alkaline, the C and N contents were lowest, as was the biomass of the medicinal part bulb. This might relate to the specific distribution of genera, such as *Pseudonocardia*, *Ohtaekwangia*, *Flavobacterium* (relative abundance >0.01), which all have significantly correlated with the biomass of *F. przewalskii* ($p<0.05$).

Discussion: *F. przewalskii* is evidently averse to alkaline soil with high potassium contents, but this requires future verification. The results of the present study may provide theoretical guidance and new insights for the cultivation and domestication of *F. przewalskii*.

KEYWORDS

***Fritillaria przewalskii* Maxim., natural population, rhizosphere soil, bacterial community, characteristics and diversity, northeastern Tibetan Plateau**

1. Introduction

Fritillaria is a genus of herbaceous perennial plants that contains 130–165 species (Hao et al., 2013). *Fritillaria przewalskii* Maxim. is the common species of the genus, and its bulbs possess moistening dryness and clearing lung heat effects as a source of the Chinese traditional cough medicine Fritillariae Cirrhosae Bulbus (Ma et al., 2020). Alkaloids are considered to possess pharmacological activity that reduces lung injury caused by various factors, and are the largest class of photochemical components in *F. przewalskii* (Majnooni et al., 2020). Numerous recent studies indicate that *F. przewalskii* has potential antineoplastic, anti-inflammatory, and anti-tumor effects (Wang Y. et al., 2021). Consequently, the medicinal value of *F. przewalskii* is extremely high. Wild *F. przewalskii* is distributed in high altitude areas in the northwestern part of the Tibetan Plateau, but it has been overharvested and is now classified as an endangered species (Chen T. et al., 2020). To meet market demand, wild *F. przewalskii* are gradually being replaced by cultivated products (Li et al., 2009). Due to the specialized wild habitat of *F. przewalskii*, and its unique soil requirement, cultivated products will likely be of poorer quality than wild *F. przewalskii* (Ma et al., 2020). Several common soil-borne diseases, such as wilt, sclerotinia rot, and root rot, are also causes of production failure (Baayen et al., 2001; Song et al., 2016; Qiao et al., 2022). There is an urgent need to ensure the healthy growth of *F. przewalskii*, and improve the quality of *F. przewalskii* circulating in market.

The rhizosphere is the main area of material exchange between the plant and the soil ecosystem, and it contains a diversity of microorganisms (Raaijmakers et al., 2009). Previous studies suggest that plant-associated microbial function confers environmental fitness advantages to host plants that are crucial to supporting plant growth and health in the natural environment (Mendes et al., 2011; Richardson and Simpson, 2011; Fitzpatrick et al., 2019). For example, enrichment of specific functional microbial taxa can improve plant drought resistance (Fitzpatrick et al., 2019), increase the availability of nutrients in soil (Koranda et al., 2011), and regulate the plant's immune system and enhance resistance to pathogens (Lebeis et al., 2015). Hence, the growth of plants highly depend on their interactions plant-associated microbes (Chaparro et al., 2014; Kong et al., 2019). The abilities of wild progenitors to resist biotic and abiotic stresses are better than cultivated plants (Qu et al., 2020; Gutierrez and Grillo, 2022). These properties, formed under interaction with environment, are regulated by the associated rhizosphere microbiome (Mantelin and Touraine, 2004). Accordingly, understanding the structural characteristics and functional compositions of the rhizosphere microbial community in a wild habitat provides a scientific reference for its artificial cultivation. To date however, the rhizosphere microbial communities of wild *F. przewalskii* have remained elusive.

Rhizosphere microbial communities are dynamically changing due to various influences, which in turn affect material circulation and energy flow in the rhizosphere environment, and ultimately the growth and development of plants (Eisenhauer et al., 2012). Advanced technologies such as culture-independent high-throughput sequencing techniques can reveal the structure of rhizosphere microbial communities, and facilitate investigation of plant–soil-microbiome interactions (Choi et al., 2021). Changes in the diversity and composition of rhizosphere microbial communities are often associated with the host plant and its environment, including root secretions, plant growth stages, soil type, and physicochemical properties (Chaparro et al., 2014; Xue et al., 2018; Broadbent et al., 2021). Host plant associated soil microhabitats influence rhizosphere microorganism enrichment (Xiong and Lu, 2022). For example, in recent studies in lilies the rhizosphere microbial community composition differed significantly in different habitats (Xie et al., 2021). This can be explained by the fact that root secretions and soil physicochemical properties vary in different microhabitats, thus selectively assembling specific microbiomes. Due to the specialized native habitat of *F. przewalskii*, there is currently no comprehensive understanding of the rhizosphere microbial communities involved in its growth.

Fritillaria przewalskii is distributed at 2,400–4,300 m altitude in the alpine grasses of the northeastern part of the Tibetan Plateau. As the highest and largest plateau on Earth, the Tibetan Plateau has a unique alpine ecosystem with complex and diverse microbiota (Chen et al., 2021; Xu et al., 2021). The habitat is extremely vulnerable to natural influences, and human activities including over-harvesting, which have now resulted in *F. przewalskii* being declared endangered (Chen et al., 2013; Jin et al., 2019). Numerous studies indicated that knowing more about rhizosphere microbiota patterns may enhance the effectiveness of measure designed to assist the adaptation of *F. przewalskii* to the environment, and resist stress (Mahoney et al., 2017; Jiao et al., 2022). In particular, rhizosphere microbes can improve tolerance of *Fritillaria* to common diseases, such as wilt and root rot diseases, which can reduce crop yields (Baayen et al., 2001; Song et al., 2016). Nevertheless, to our knowledge, there is no information available on wild *F. przewalskii* root-associated microbial communities, specifically comparative studies of different natural growth areas.

In this current study, 16S rRNA gene amplicon sequencing of soil samples collected from 12 different natural growth areas was conducted to investigate the compositions and functional phenotypes of the soil bacterial communities of wild *F. przewalskii*. The aims were to (1) investigate differences in bacterial community composition and diversity between rhizosphere and bulk soil samples, as well as among sites; (2) elucidate the functional phenotypes of common microorganisms at different sites, and specific microorganisms at each site; (3) indentify suitable soil environments for *F. przewalskii* combined with phenotypic characteristics of *F. przewalskii* at different sites; and (4) provide a comprehensive understanding of the key factors affecting rhizosphere bacterial communities. The results of the study shed new light on the microbial composition of *F. przewalskii*, and may provide theoretical guidance and new insights for resource conservation, artificial cultivation and domestication of *F. przewalskii*.

2. Materials and methods

2.1. Soil collection

Soil samples were collected from 12 areas within the range of wild *F. przewalskii* between late July and early August, including Weiyuan County (WY), Linxia County (LX), Diebu County (DB), Ruoergai County (RG), Ganzi Tibetan Autonomous Prefecture (GZ), Shiqu County (SQ), Luhuo County (LH), Zeku County (ZK), Yushu City (YS), Zaduo County (ZD), Maqin County (MQ), and Ledu District (LD) (Figure 1). At each site, five *F. przewalskii* plants were randomly

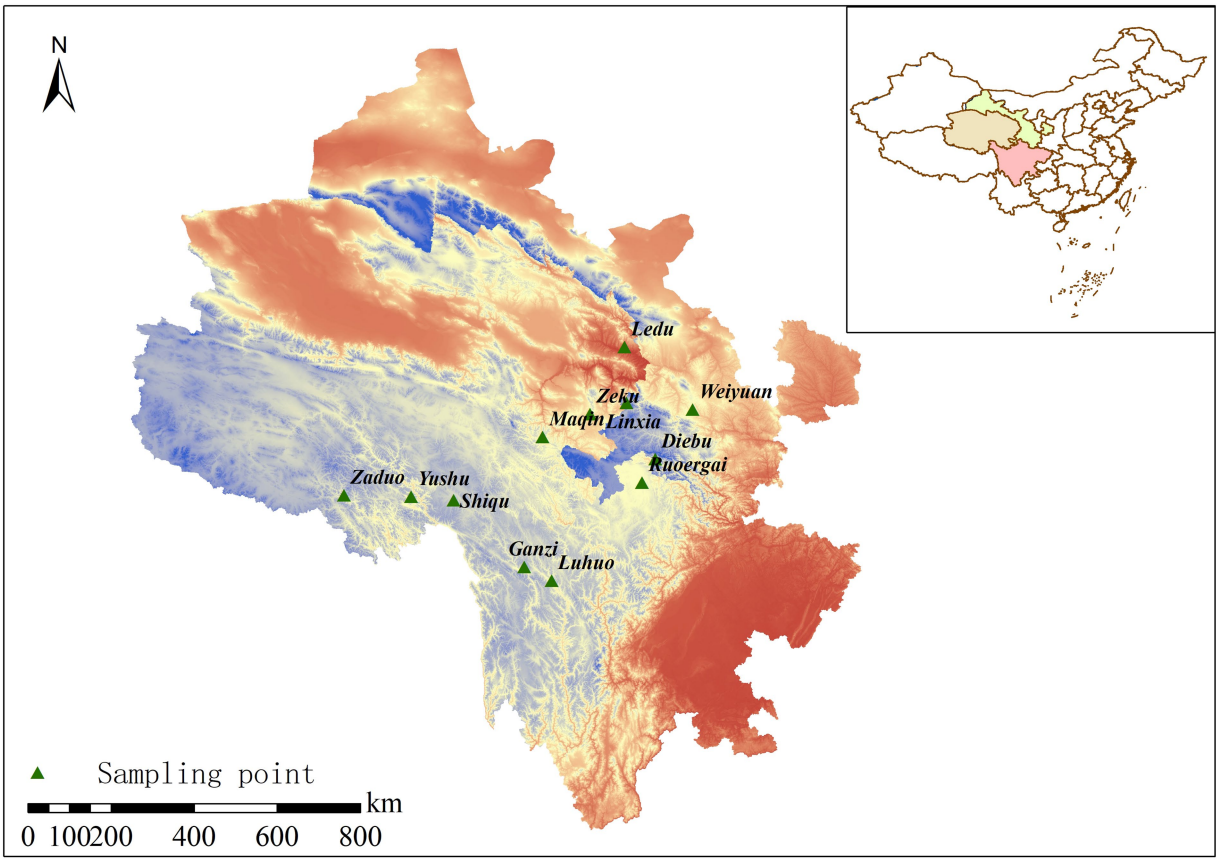

FIGURE 1
Distribution of sampling points.

selected by five-point sampling method (Xie et al., 2021). We only chose plants that had grown for more than 3 years. Rhizosphere soil was carefully collected gently by removing the soil stuck to roots and bulbs using a brush, while bulk soil was collected around the root system with a sterile shovel approximately 5 cm away from each removed plant to a depth of 15 cm (Zhang Z .C. et al., 2021). Each of the five soil samples from the same site was homogenized then sub-sampled three times, resulting in a total of 72 samples (36 rhizosphere and 36 bulk soils). After collecting soil samples, all selected plants were divided into leaves, stems, bulbs, and roots. Leaf length and width, stem length and diameter, bulb horizontal and vertical dimensions, and root length were measured using vernier calipers (Ma et al., 2020). Single bulb fresh weight was measured with an electronic scale. Soil samples were divided into two parts. One was immediately frozen *via* liquid nitrogen submersion, transported to the laboratory, and stored at −80°C for microbial analysis, and the other was air-dried for soil characterization.

2.2. Soil physiochemical analysis

The samples used for soil physiochemical analysis were all rhizosphere soils. Soil pH was measured in a soil solution at a soil: water ratio of 1:5 using a pH meter. Soil was pretreated with $K_2Cr_2O_3$ and mixed with H_2SO_4 to oxidize organic carbon. The combustion method was then used to determine the soil organic carbon (SOC) level (Nishanth and Biswas, 2008; Kim et al., 2021). Soil total nitrogen (TN) was determined *via* the micro-Kjeldahl method using an automatic Kjeldahl instrument (K8400, FOSS Corporation, Denmark) (Bremner, 1996). Nessler reagent spectrophotometry was used to measure soil ammonium-nitrogen (NH^{4+}-N). HCl was used to extract soil total phosphorus (TP) and 0.5 mol/L $NaHCO_3$ was used to extract available phosphorus (AP). Both were quantified by the molybdenum blue method (Fan et al., 2018; Liu et al., 2020). Total soil potassium (TK) was measured using an atomic absorption spectrophotometer (PinAAcle 900 T, United States) (Wang and Huang, 2021).

2.3. DNA extraction and sequencing

Total soil DNA was extracted from rhizosphere and bulk soil *via* a DNeasy PowerSoil Pro kit (Qiang, Germany). For bacterial community diversity and composition, the V3-V4 hypervariable region of the 16S rRNA gene was amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2011). The PCR reaction (50 μL) reagents used were 2 μL template DNA, 2 × 25 μL Phanta Max Master Mix, and 10 μmol/L of each primer. The PCR conditions were predenaturation temperature 94°C (30 s), denaturation temperature 94°C (30 s), annealing temperature 55°C (45 s), and extension temperature 72°C (45 s), then after 30 cycles extension was terminated at 72°C for 10 min. Sequencing was

performed offsite by BGI Genomics Co. Ltd. (Shenzhen, China) using an Illumina MiSeq platform.

2.4. Sequence processing

After quality-filtering using QIIME (v.1.7.0) (Caporaso et al., 2010), chimeric sequences were removed using the UCHIME procedure (Edgar et al., 2011). Raw sequence reads were spliced using FLASH software (Magoč and Salzberg, 2011), then clustered into operational taxonomic units (OTUs) at a 97% similarity level using USEARCH software (v7.0.1090_i86linux32) (Edgar, 2013). Taxonomy assignments were done using RDP classifier software (v1.9.1) at the 80% confidence level against the Greengenes database (DeSantis et al., 2006). Upset diagram was drawn using the R "UpSetR" package (v4.0.4) (Andrysik et al., 2017). Alpha diversity indices such as the Chao index, Ace index, Shannon index, and Simpson index were calculated using Mothur (Schloss et al., 2009). Significant differences in bacterial communities were visualized *via* partial least squares discriminant analysis (PLS-DA) using SIMCA software (v14.1) (Yang et al., 2021) and the unweighted pair group method with arithmetic averages (UPGMA) using the R "vegan" package (Nazish et al., 2017). Species relative abundance was tabulated at each taxonomic level using Qiime software (Caporaso et al., 2010), then plotted with OriginPro 2022. Linear discriminate analysis (LDA) was performed using the R "topicmodels" package (Zhang Y. et al., 2021) to determine the different distributions of bacterial genera at the phylum level in samples. A heatmap of relative abundance at the genus level was generated using the R "pheatmap" package (Mortensen et al., 2016). Different genera and dominant genera in samples were identified and visualized the results by circus plot using the R "circlize" package (Yang et al., 2016).

Co-occurrence networks were inferred for rhizosphere and bulk soil to assess the complexity of interactions among bacterial taxa based on the Spearman's correlation coefficient, calculated using the "Hmisc" package in R (Huang et al., 2018). OTUs found in at least half of the samples were selected to establish co-occurrence networks. The nodes in the network represented OTUs, while the edges represented significant positive or negative correlations between two nodes. The network was visualized using Gephi 0.9.6 (r [absolute value] > 0.6, $p < 0.01$). Measurement properties such as numbers of nodes and edges, average degree, and average clustering coefficient were calculated using Gephi (Tu et al., 2019).

2.5. Statistical analysis

Significant differences were analyzed *via* one-way ANOVA in SPSS (v26.0) (Anderson, 2001) with variables including plant phenotypic characteristics, soil characteristics, and alpha diversity indices. Principal component analysis (PCA) was performed using "ggplot2" and "FactoMineR" packages in R to investigate the distribution of soil characteristics and separate rhizosphere bacterial communities in the samples (Brückner and Heethoff, 2017). Bacterial community composition of rhizosphere and bulk soil samples was analyzed based on the nonmetric multidimensional scaling (NMDS) of the Bray–Curtis dissimilarity using the R "vegan" package (Goslee and Urban, 2007). To identify significant effects of plant phenotypic characteristics and soil characteristics on bacterial communities, a bubble plot was drawn using the R "ggplot2" package (Dussud et al., 2018), and redundancy analysis (RDA) performed using CANOCO (v5.0) (Wu et al., 2021). Correlation analysis was performed using the Mantel test in the R "vegan" package and plotted with the "ggcor" package in R (Xue et al., 2021).

3. Results

3.1. Plant phenotypic characteristics

Fritillaria przewalskii had different phenotypic characteristics in different habitat (Supplementary Table S1). All characteristics except for stem diameter and root length, differed significantly across some sites ($p < 0.05$). In general, aboveground traits were best at the ZK site, whereas at the DB and LD sites they were inferior. *F. przewalskii* at the ZK site was above 45 cm in height, which was significantly higher than that at the DB, SQ, YS, ZD, and MQ sites (all below 30 cm), whereas there were no significant differences detected between other sites ($p < 0.05$). Leaf traits varied little. Leaf length and width data indicated that plants at the ZK site had significantly larger leaf area than those at the LX, DB, and LD sites ($p < 0.05$). Bulb traits differed greatly, and were best at the MQ site. The bulb weight of *F. przewalskii* at the MQ site was 10.87 times greater than that at the lightest site (DB), and it differed significantly from all regions except the ZK site ($p < 0.05$). The bulb weight of *F. przewalskii* at ZK also differed significantly from those at DB, RG, YS, and LD sites ($p < 0.05$).

3.2. Soil characteristics

The soil characteristics of *F. przewalskii* in different habitat were summarized in Figure 2. Across sites, pH ranged from 5.78 to 8.03. Most soil samples were neutral (RG, WY, LH, ZK, MQ, LD, and ZD), a few were sub-acidic (SQ, YS, and GZ), and the remainder were alkaline (DB and LX sites) (Figure 2A). SOC, TN, TK, TP, AP, and NH^{4+}-N were more variable among the 12 sites. TN was high at all sites, whereas TP and NH^{4+}-N were low or scarce (Lin et al., 2017). When the rhizosphere soil environment was sub-acidic, C and N contents were most rich. PCA based on scaled soil characteristics indicated that soil properties varied greatly among habitats excluding ZK, WY, and LH (Figure 2B). The contributions of soil traits to major components of the PCA were differed, and the concentrations of N, C, and P were positively correlated with each other, and all three were significantly negatively correlated with K (Figure 2C).

3.3. Soil bacterial community diversity

Sequencing of 16S rRNA amplicons from 72 soil samples produced 2,549,758 paired-end Illumina MiSeq reads, with an average length of 418 bp. The sequences were clustered into 177,247 bacterial OTUs based on 97% sequence similarity. NMDS based on a Bray–Curtis dissimilarity matrix indicated that different soil samples were separated into two distinct groups of rhizosphere versus bulk, suggesting that the structure of the bacterial communities differed

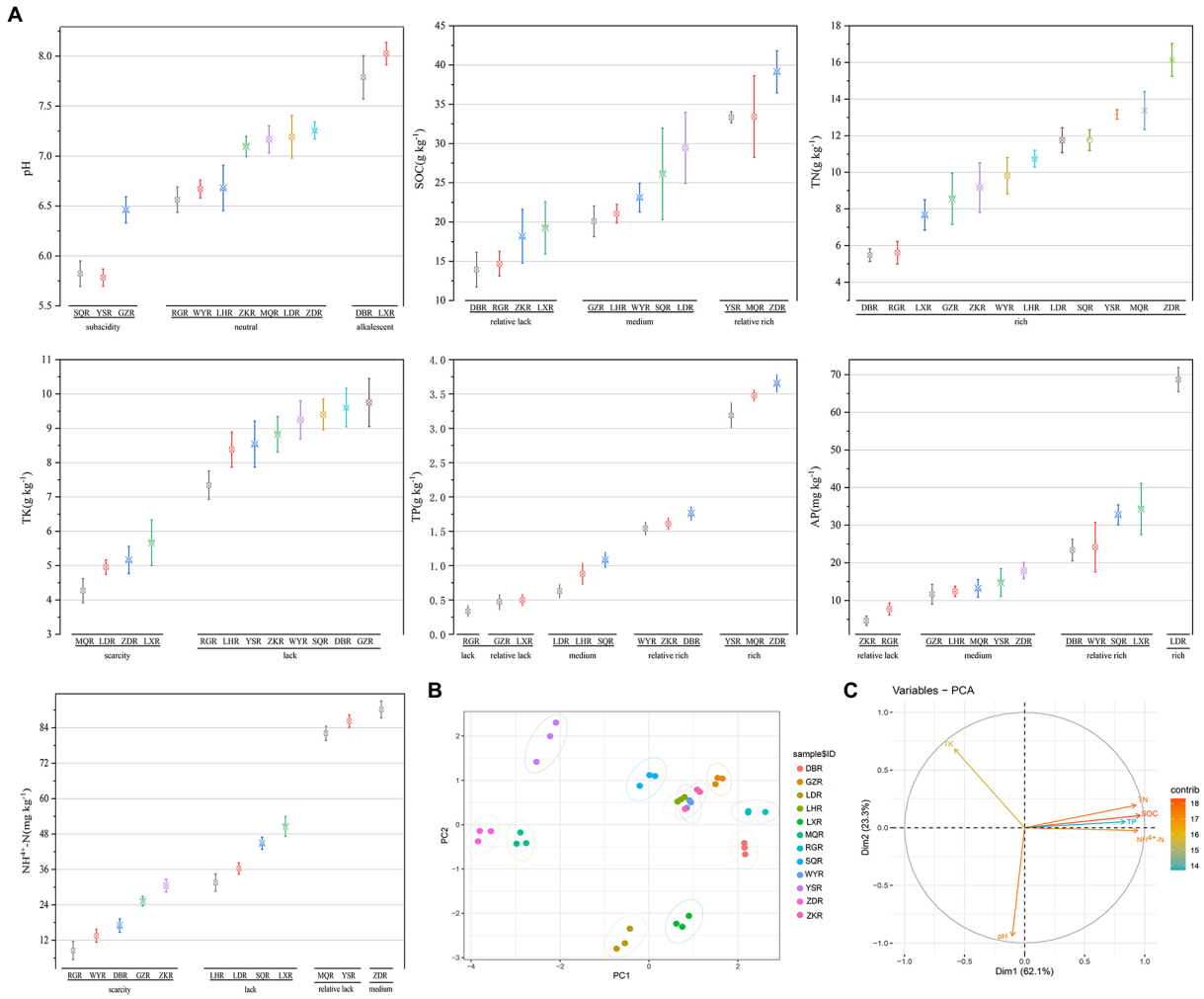

FIGURE 2
Comparative analysis of soil characteristics between different sites. R, Rhizosphere; following the same. **(A)** Grouping interval map of soil characteristics, including pH; SOC; TN; TP; TK; NH^{4+}-N; AP. **(B)** Principal component analysis (PCA) of soil characteristics. **(C)** The contribution of the variables to the major components.

between rhizosphere and bulk soils (Figure 3A). Further analysis found that rhizosphere soil exhibited more complexity (number of nodes 186, edges 1,169, average degree 12.57) than bulk soil (number of nodes 152, edges 676, average degree 8.895) (Figure 3B). Similarly, the interaction between rhizosphere networks was more balanced, with 63.73% positive connections and 36.27% negative connections.

The similarity and specificity of OTUs in rhizosphere soils were displayed in Upset diagram (Figure 3C). The number of OTUs in RG site was the highest, while the SQ site had the lowest number. A total of 330 OTUs were found in all sites, and the numbers of specific OTUs for each site were 211 (MQR), 202 (LXR), 188 (SQR), 186 (DBR), 170 (RGR), 162 (LDR), 160 (YSR), 151 (ZKR), 148 (WYR), 148 (LHR), 147 (ZDR), and 121 (GZR). The alpha diversity of rhizosphere bacterial communities was compared at different sites *via* the Chao1 index, ACE index, Simpson index, and Shannon index (Figure 4A). The Chao1 and ACE indexes of abundance in LD and RG sites were significantly higher than WY, YS, and SQ ($p < 0.05$). Conversely, the Simpson index of diversity at LX was significantly higher than those at WY, SQ, LH, YS, RG, DB, GZ, and LD ($p < 0.05$), and the Shannon index at LD was significantly higher than those at ZD, WY, SQ, LH, MQ, YS, RG, LX, and ZK sites ($p < 0.05$).

PLS-DA analysis, a supervised method, showed that bacterial community composition varied between sites (Figure 4B). Samples with significant differences in community composition were clustered in different quadrants. A dendrogram constructed *via* UPGMA consistently showed that the sites were divided into two main clusters—bulk and rhizosphere soil (Figure 4C). In contrast, bacterial communities were more similar at the site level, particularly when comparing compositions at LX and DB sites, MQ and ZD sites, LD and WY sites, and LH and RG sites.

3.4. Soil bacterial community composition

At the phylum level, Proteobacteria, Bacteroidetes, and Acidobacteria were the three most dominant phyla at all sites, with respective relative abundances of 26.47–37.61%, 10.53–25.22%, and 10.45–23.54%. We considered rare phyla to be those with relative

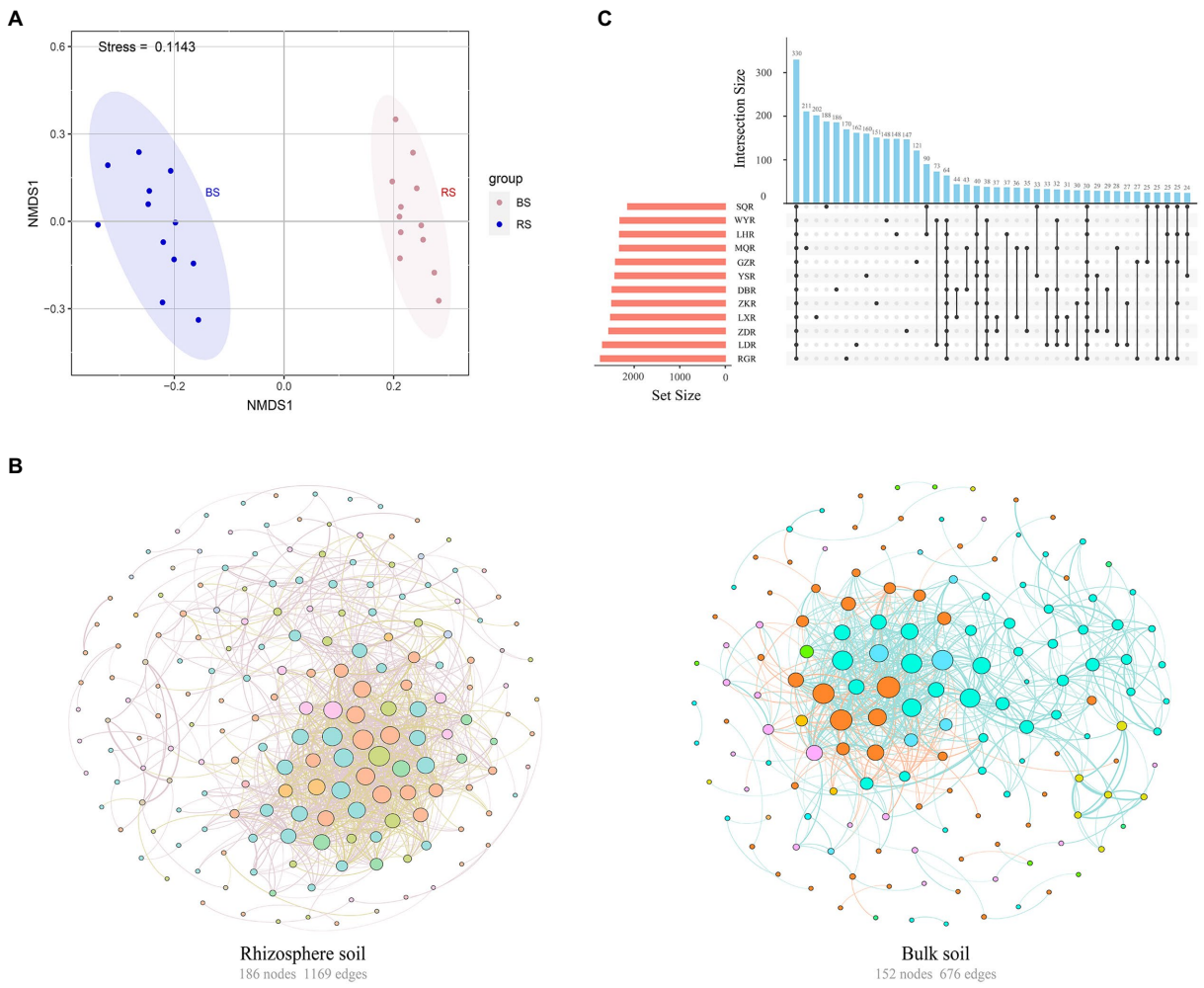

FIGURE 3
(A) Nonmetric multidimensional scaling (NMDS) of the bacterial communities in rhizosphere and bulk soils. RS, rhizosphere soil; BS, bulk soil. **(B)** Co-occurrence network of the bacterial communities in rhizosphere and bulk soils. The colors of edges represented positive correlation (SparCC $r>0.6$, $p<0.01$, pink or blue line) or negative correlation (SparCC $r<-0.6$, $p<0.01$, yellow or orange line). The colors of nodes were based on the phylum to which OTU belong and the size of nodes was proportional to the number of connections (degrees). **(C)** Upset plot of the enriched tax in the rhizosphere soil among different sites.

abundances of <3%, which included Candidatus, Nitrospira, Planctomycetes, and Gemmatimonade (Figure 5A). Cyanobacteria and Firmicutes exhibited significantly higher relative abundance at RG and LH sites, respectively, and could be considered the dominant phyla at individual sites. LDA plot indicated that 12 sites were clustered into 5 groups (i.e., WY, DB, LD and LX sites were clustered into group 1, ZK, NR, GZ, LH and SQ sites were clustered into group 2, and ZD, YS, and MQ sites, respectively, for groups 3, 4, and 5) based on the relative abundance of phyla (Figure 5B). Specifically, Acidobacteria, Bacteroidetes, Actinobacteria, and Verrucomicrobia were critical for distinguishing rhizosphere bacterial communities between sites.

Differences in bacterial community composition were assessed at the order level. The most abundant orders were Rhizobiales, Sphingomonadales, and Actinomycetales which represented >60% of the relative abundance (Figure 5C). The relative abundances of Bacillales, Cytophagales, and Anaerolineales were significantly higher at LH, ZK, LX, and MQ. The top 10 most abundant OTUs in rhizosphere and bulk soil were compared, which together accounted for 11.5 and 11.7% of the respective total OTUs (Figure 5D). In rhizosphere soil, the top 10 OTUs were affiliated with six orders; Rhizobiales, Bacillales, Nitrospirales, Pseudomonadales, Anaerolineales, and Sphingobacteriales. In bulk soil they were affiliated with four orders, including Nitrospirales, Rhizobiales, Rhodocyclales, and Bacillales. This indicated that the bacterial communities in rhizosphere soil were more complex than those in bulk soil, consistent with results shown in Figure 3.

We found that the specific distribution at the genus level was a major factor contributing to the differences of rhizosphere bacterial communities among sites (Figure 6A). When the relative abundance greater than 1% in each sample, we classified the common genera (Xie et al., 2021). In total, there were 9 common genera: *Gp4* (2.57–12.59%), *Spartobacteria* (1.02–6.46%), *Pseudomonas* (1.08–5.27%), *Gp6* (1.70–5.89%), *Aridibacter* (1.02–4.37%), *Chryseolinea* (1.12–3.98%), *Saccharibacteria* (1.23–3.08%), *Nitrospira* (1.23–3.39%), and *Terrimonas* (1.07–2.69%) (Figure 6B). There were also 12 different

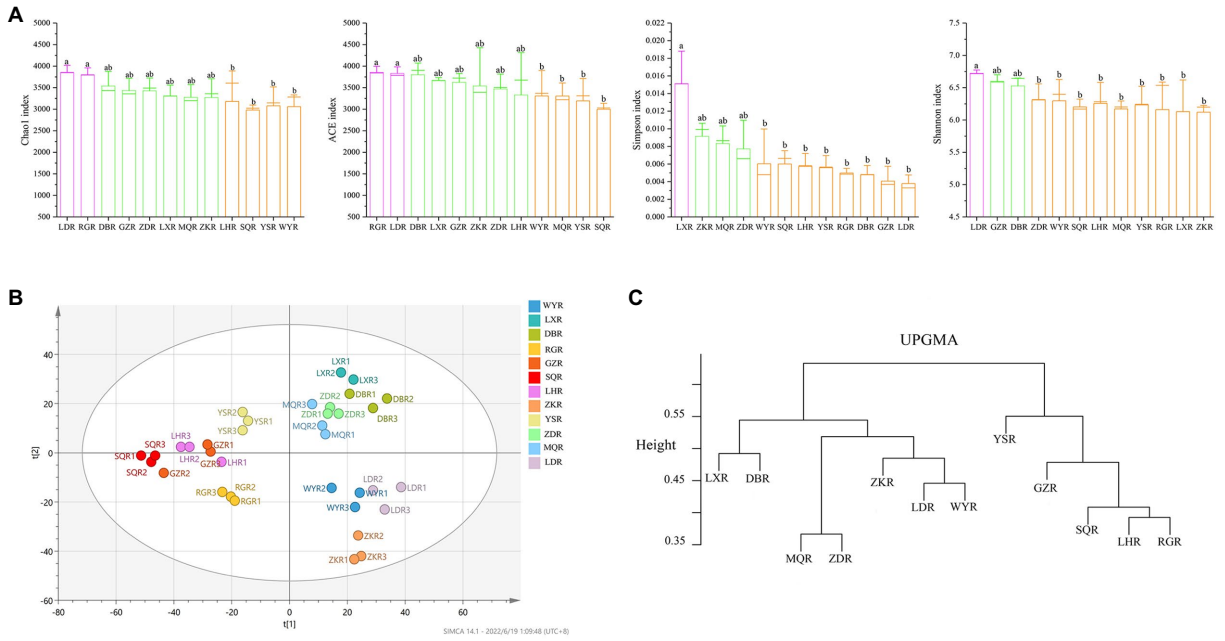

FIGURE 4
Diversity indices of rhizosphere soil bacterial communities. **(A)** Boxplots of the alpha-diversity indices, including Chao1 index; ACE index; Shannon index; Simpson index; different lowercase indicated significant differences between different regions (p<0.05). **(B)** PLS-DA score map of the bacterial community. **(C)** UPGMA clusters of the different bacterial communities.

genera: *Acinetobacter* (GZ: 1.28%, others: 0.00–0.22%), *Pseudonocardia* (WY: 1.06%, others: 0.07–0.84%), *Gp2* (SQ: 2.10%, others: 0.005–0.66%), *Ktedonobacter* (SQ: 1.17%, others: 0.00–0.35%), *Actinoallomurus* (SQ: 1.17%, others: 0.00–0.38%), *GpI* (RG: 9.77%, others: 0.03–0.45%), *Parcubacteria* (LX: 1.43%, others: 0.03–0.53%), *Ohtaekwangia* (LX: 1.38%, others: 0.01–0.43%), *Luteolibacter* (LX: 1.13%, others: 0.03–0.25%), *Flavobacterium* (GZ, WY, and LX: 1.41–2.00%, others: 0.12–0.68%), *Sphingobium* (ZK, WY, and LX: 1.26–1.65%, others: 0.17–0.53%), and *Gaiella* (GZ, LH, NR, and WY: 1.03–1.73%, others: 0.11–0.77%) (Figure 6C).

3.5. Correlation analysis

In different habitats of *F. przewalskii*, plant phenotypic characteristics and soil characteristics were significantly correlated with the diversity, composition, and relative abundance of associated microorganisms, respectively (Figure 7).

Specifically, the ACE index was negatively correlated with stem length, leaf number, leaf length, pH, and SOC. The Chao1 index was positively correlated with leaf length and SOC, and negatively correlated with stem diameter, leaf width, TP, and TN. The Shannon index was positively correlated with stem diameter, root length, and NH^{4+}-N, and negatively with bulb diameter and TK. The Simpson index was positively correlated with bulb length, NH^{4+}-N, and AP, and negatively correlated with bulb horizontal and vertical dimensions.

Correlations between rhizosphere bacterial communities and plant phenotypic characteristics were shown in Figure 7C (axis1 = 21.64%, axis2 = 12.71%), and correlations between rhizosphere bacterial communities and soil characteristics were shown in Figure 7D (axis1 = 31.77%, axis2 = 15.68%). Using the RDA, we found that leaf number, stem diameter, bulb length, pH, TP, AP, NH^{4+}-N, SOC, and TK contributed to the significant correlation with rhizosphere bacterial community structure (p < 0.05), and leaf number and pH were the most strongly contributing factors (Table 1). In contrast, soil characteristics were more related to rhizosphere microbial communities.

Based on the analysis, 6 common microorganisms and 7 different microorganisms were selected at the genus level. Spearman analysis suggested that plant phenotypic characteristics were generally negatively correlated with soil characteristics (Figure 7E). Correlations with the special genera occurred in distinct patterns revealed by the Mental test (Figures 7E,F). All aboveground plant phenotypic characteristics were significantly correlated with different genera, particularly leaf width which was significantly correlated with *Pseudonocardia* and *Parcubacteria* (p < 0.01). Only leaf number, stem diameter, and bulb length were significantly correlated with common genera, with the stem diameter was most significantly correlated with *Aridibacter* (p < 0.01). All soil characteristics were significantly correlated with unique genera. Soil pH was more strongly correlated with unique genera than any other factor, whereas TN was only significantly correlated with common genera. Collectively, these results suggest a higher association between soil characteristics and bacterial community structure, consistent with the above analysis. Plant phenotypic characteristics on different genera were higher than common genera, while the reverse was observed with respect to soil characteristics.

4. Discussion

Due to its high medicinal value considerable interest is devoted to cultivating *F. przewalskii*. The natural *F. przewalskii* habitat is

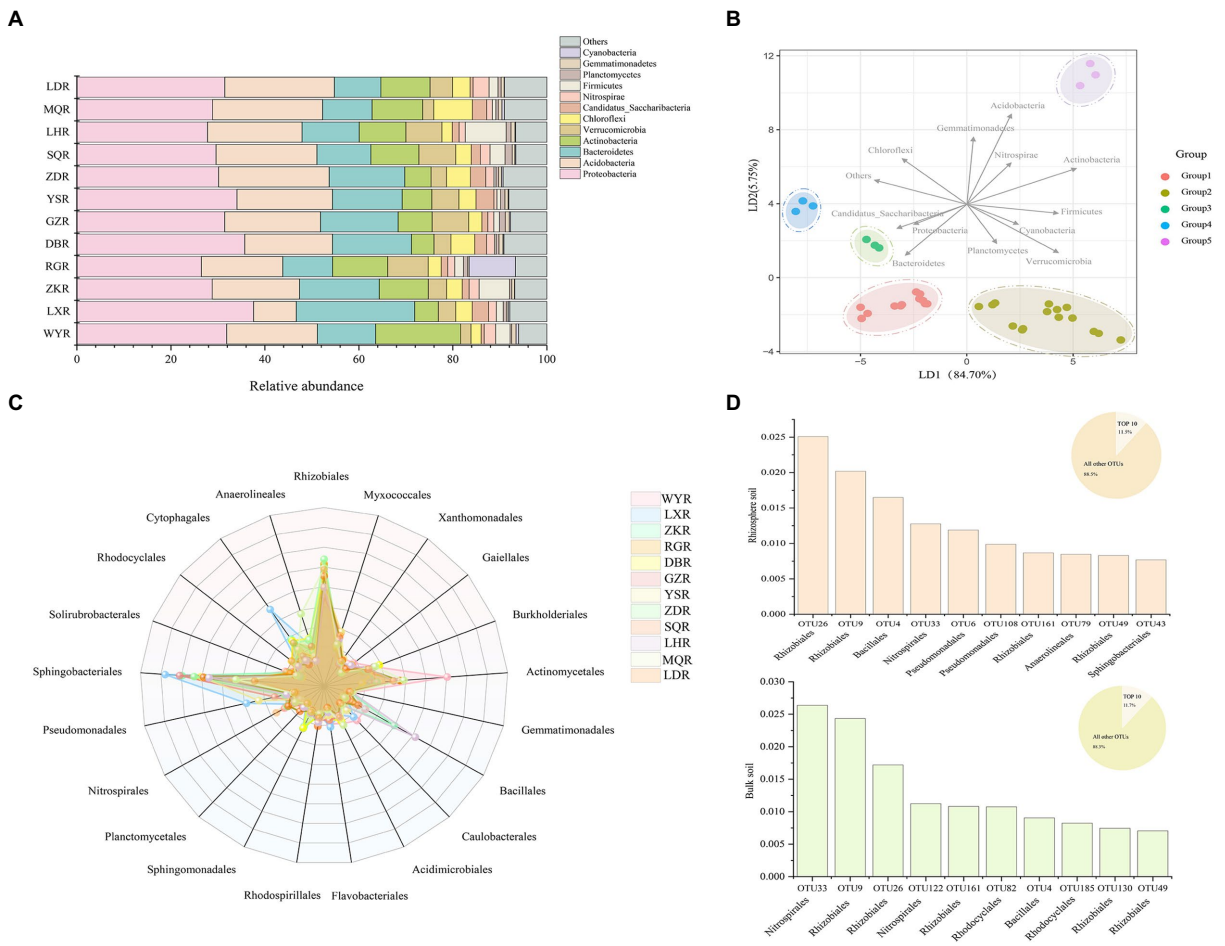

FIGURE 5
Bar chart of relative abundances of bacterial community **(A)** and Linear discriminant analysis (RDA) performed to distinguish the bacterial communities **(B)** at the phylum level; Radar plot displays the relative abundances of bacterial community **(C)** and Top 10 most abundant OTUs from rhizosphere soil and bulk soil at the order level **(D)**.

specialized, and the species is prone to soil-borne disease rendering cultivation more difficult (Cunningham et al., 2018). The associated microbiome can help host plants resist stress and adapt to the environment, promoting healthy growth (Koranda et al., 2011). Different habitats may exhibit diversity of microbiomes. In the current study, the diversity, composition, and structure of bacterial communities were compared at 12 in *F. przewalskii* growth sites, and plant phenotypic characteristics and soil characteristics at different sites based were compared with respect to microbial composition.

Previous studies indicate that plant development and secretory activities in root systems are intertwined, collectively forming a unique rhizosphere microbial community that differs from bulk microbial communities (Bulgarelli et al., 2013; Mendes et al., 2013). The results of the present study are consistent with this, and showed that the rhizosphere bacterial communities were distinctly separated from the bulk bacterial communities (Figure 3A). This was probably due to the strong recruiting effect of plant root on microbial communities (Chaparro et al., 2014). Co-occurrence network plots showed that the community structure of rhizosphere soil was more complex than that of bulk soil (Figure 3B), which is consistent with previous studies (Chen J. et al., 2020; de Albuquerque et al., 2022). This relates to the specific soil microhabitats in the rhizosphere.

Further, Chao1, ACE, Simpson, and Shannon alpha diversity indices varied weakly in rhizosphere soils between sites (Figure 4A), indicating that the rhizosphere bacterial community might be stable across sites (Martiny et al., 2013). Previous studies have hypothesized that environmental factors are critical for determining the structure of microbial communities (de Wit and Bouvier, 2006). Clustering results at the OTU level confirmed this, and sites with similar nutrient contents were more similar in bacterial community composition (Figure 4C). In the current study, the bacterial taxonomic compositions of *F. przewalskii* from different sites exhibited significant differences. Proteobacteria, Acidobacteria, Bacteroidetes, and Actinobacteria phyla formed the dominant microbiota despite habitat fluctuation (Figure 5A), which is consistent with previous studies (Shi et al., 2011; Jiao et al., 2022). Cyanobacteria was only the dominant phylum at RG site. Interestingly, previous studies have shown that Cyanobacteria can fix nitrogen (Elkoca et al., 2007), thus the distribution of Cyanobacteria may relate to the lower N content in RG site (Figure 2A). Most of the OTUs could be further classified at the

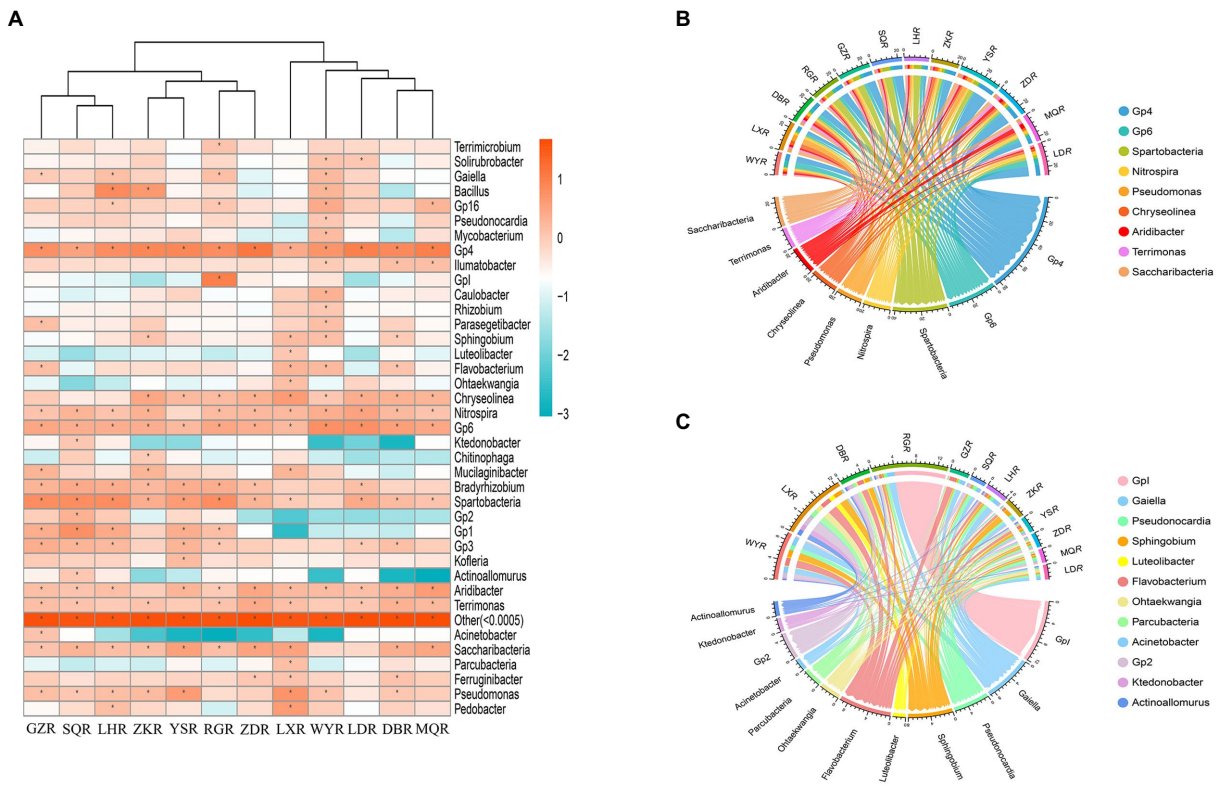

FIGURE 6
Community analysis at the genus level. **(A)** Heatmap of relative abundances, * represents relative abundance of greater than 1%. **(B)** Circos plot of relative abundances of common genera. **(C)** Circos plot of relative abundances of different genera.

order level in this study (Figure 5D). Among the top 10 most abundant OTUs, more than 0.06% of all the sequences were affiliated to Rhizobiales, which can provide specific secondary metabolites to plants, promoting nutrient cycling (Erlacher et al., 2015). Similarly, we found a proportion of both Pseudomonadales and Sphingobacteriales, which have highly antagonistic effects on specific bacterial and fungal pathogens, and may thus contribute to the stability of bacterial communities, ultimately promoting plant growth (Cernava et al., 2015; Larsbrink and McKee, 2020). To date, most research showed that plants can recruit beneficial microorganisms to their defense against environmental damage (Rudrappa et al., 2008; Raaijmakers et al., 2009), the active components in potential root secretory need further study.

Specific microorganisms were significantly enriched at each site, and we defined these enriched groups as core microorganisms (Zhang Y. et al., 2021). In the present study 21 core genera were detected, consisting of 9 common genera and 12 different genera (Figure 6). *Aridibacter*, *Pseudomonas*, *Spartobacteria*, *Gp6*, and *Gp4* were dominant at all sites, consistent with previous reports on rhizosphere soil (Liu W. et al., 2020; Jiao et al., 2022). Dominant microbial communities participate in soil nutrient cycling, and play important roles in ecosystem function regulation (Liu Y. et al., 2020). For example, *Aridibacter* and *Terrimonas* are significantly correlated with N and C due to their ability to degrade carbon and facilitate nitrogen removal (Sheu et al., 2010; Huber et al., 2017). Similarly, soil characteristics can influence the structure of microbial communities. RDA revealed that pH had the strongest effect on rhizosphere bacterial communities (Figure 7D). These results suggested that different habitats of *F. przewalskii* habitats can recruit different microorganisms to form a specific rhizosphere microbiome to adapt to soil microhabitats.

In addition, pH was significantly correlated with several dominant microorganisms (Figures 7E,F). These findings are consistent with previous studies suggesting that pH is the most influential environmental factor for soil microbial communities (Fierer and Jackson, 2006; Tripathi et al., 2018). *Spartobacteria* abundance was significantly correlated with pH. Interestingly, *Spartobacteria* is affiliated with Verrucomicrobia, which was shown to contain diverse acidophilic genera (Pol et al., 2007; Wang L. et al., 2021), thus presumably *Spartobacteria* might be acidophilic or acid tolerant. C and N content are also essential environmental factors that affect microbial communities (Yuan et al., 2014; Praeg et al., 2019), consistent with the results of the current study. In previous studies high SOC content led to significant enrichment of Proteobacteria (Fierer et al., 2008), thus the relative abundance of *Terrimonas* may be related to content of labile carbon substrates. Compared with other elements, P and K content were lower in the rhizosphere soil of *F. przewalskii* (Figure 2), although these could also affect bacterial communities (Figure 7). AP content was highest at the LD site, 10 times higher than at the RG site, which had the lowest AP content (Figure 2A). Further analysis revealed that the distribution of *GPI* was reversed (Figure 6A). This may be related to the role of *GPI* in degrading P, thought the mechanism needs involve requires further research. TK content was at an extremely

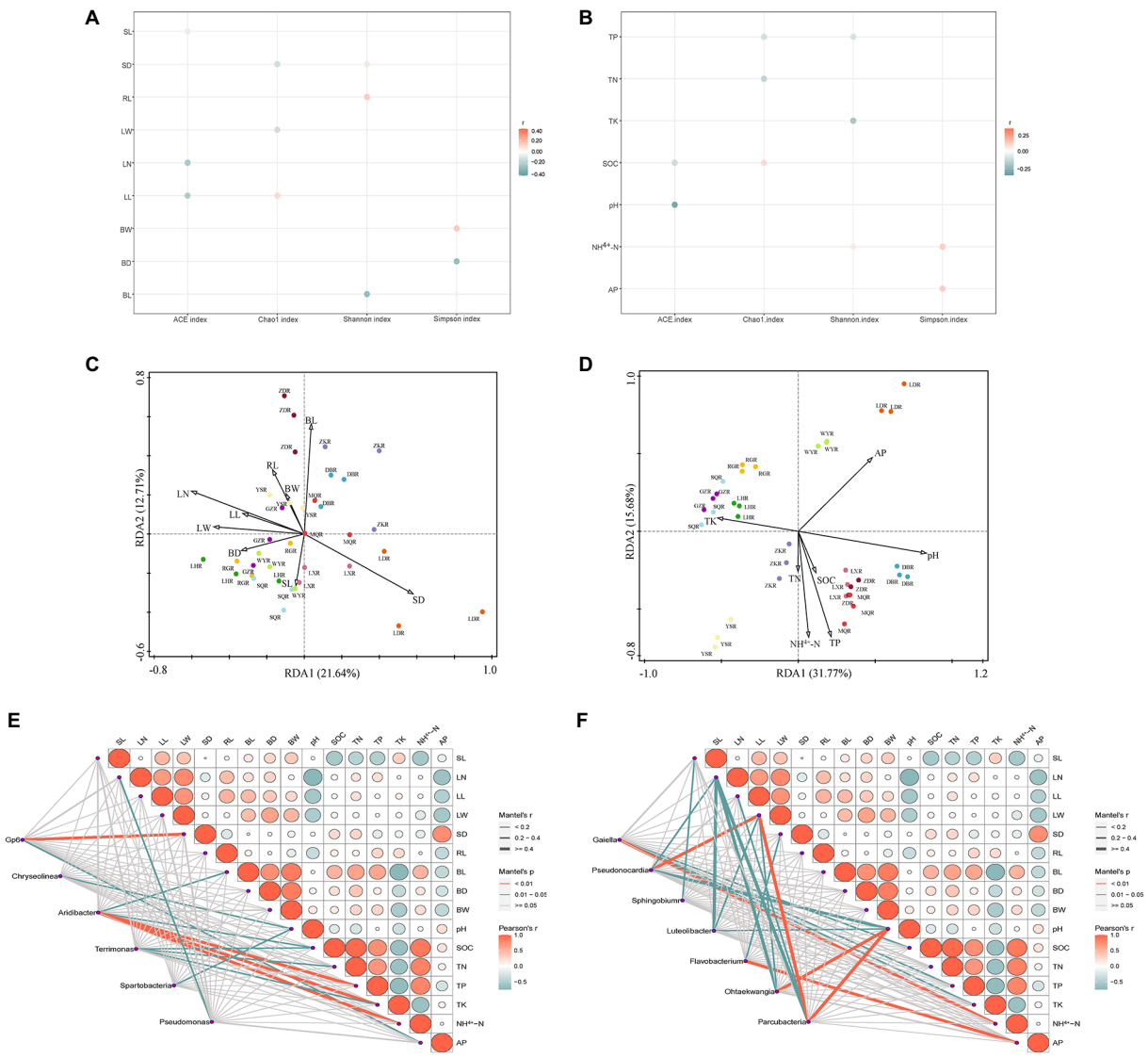

FIGURE 7
Correlation bubble plot between alpha diversity and plant phenotypic characteristics **(A)** or soil characteristics **(B)**. Redundancy analysis (RDA) performed to reveal the correlations between bacterial community structure and plant phenotypic characteristics **(C)** or soil characteristics **(D)**. Mantel analysis of plant phenotypic characteristics, soil characteristics with common genera **(E)** or different genera **(F)**. Color of the line represented the significance of the differences (*p*-value). Size of the line represented correlation coefficients (Mantel's r).

low level in all soils, and was significantly negatively correlated with the Shannon index, and also with other nutrient contents (Figure 7B). The current study indicates that *F. przewalskii* is well adapted to a low potassium soil environment.

Fritillaria przewalskii was found at pH ranging from sub-acidity to alkalescency. The stem-leaf biomass of *F. przewalskii* was lowest in alkaline soil, especially at the DB site, which also had the lowest underground biomass (Supplementary Table S1). C and N content were also lowest in alkaline soil than in sub-acidic and neutral soil. These differences may be explained by the fact that C and N in soil are partly derived from stem and leaf litter which are influenced by root secretions (Postma et al., 2016; Liu Y. et al., 2020), while both above-and under-ground biomass was low at those sites. With the exception of K, overall nutrient availability was positively correlated with plant phenotypic characteristics (Figure 7E), indicating that higher C, N, and P content was beneficial to *F. przewalskii* growth. K was significantly negatively correlated with others (Figure 2C). Therefore, it is possible that excessive potassium content affects the growth of *F. przewalskii* and the uptake of other nutrients, though this premise warrants further study and discussion. As anticipated, there was strong relationship between plant phenotypic characteristics and microbial community structure (Figure 7). The factor that had the greatest effect was leaf number (Figure 7C), probably because leaves are a direct source of litter, and are important for shaping the soil environment (Ralte et al., 2005). As mentioned above, the structure and function of rhizosphere microorganisms directly affected soil nutrient availability, and therefore may also influence *F. przewalskii* growth. The current study was focused on the role played by beneficial microorganisms in the regulation of the soil environment and plant growth. However,

TABLE 1 The relative contribution of RDA analysis of plant phenotypic characteristics and soil characteristics to bacterial community structure.

Factor	Contribution %	*F*	*P*
Leaf number	14.1	1.5	0.004
Stem diameter	13.9	1.5	0.002
Bulb length	12.2	1.3	0.014
Bulb diameter	13.0	1.5	0.004
Stem length	10.9	1.2	0.066
Leaf width	9.6	1.1	0.292
Bulb width	9.2	1.0	0.426
Root length	8.7	1.0	0.558
Leaf length	8.3	0.9	0.656
pH	25.5	3.0	0.002
TP	14.9	1.8	0.002
AP	15.1	1.9	0.004
NH^{4+}-N	14.4	1.8	0.002
SOC	11.8	1.5	0.002
TK	9.6	1.2	0.048
TN	8.5	1.1	0.244

harmful microorganisms are present in the rhizosphere environment (Dinesh et al., 2015). For example, continuous cropping of medicinal plants has limited the development of most Chinese medicine industries, and the accumulation of harmful microorganisms in the rhizosphere is regarded as a major cropping obstacle that we did not address (Xiao et al., 2019). Collectively, *F. przewalskii* plant, rhizosphere soil, and rhizosphere microbial communities form a complex system that is not yet understood, and considerable work is still required in the future.

5. Conclusion

The current study provides a comprehensive assessment of the bacterial communities of wild *F. przewalskii* in different habitats. Herein, we have reported that these bacterial communities varied between rhizosphere and bulk soil, and also between sites. Moreover, the diversity indexes varied little between habitats, and the microbial assembly was consistent with the trend of root-associated soil microhabitat distribution. The dominant phyla were Proteobacteria, Acidobacteria, Bacteroidetes, and Actinobacteria at 12 different habitats. Further, we highlighted the core microorganism species and functions, and concluded that microbiota prefer to enrich in a direction adapted to the environment of the host plant. Meanwhile, soil characteristics, particularly pH, had significant effects on bacterial communities, and it was surmised that these effect are derived from interactions between *F. przewalskii*, soil, and microorganisms. Leaf number was the most influential factor with respect to microbial communities, so we speculate that *F. przewalskii* may also change the microbial community *via* the accumulation of its own litters. These findings enhance our understanding of *F. przewalskii* root-associated soil microhabitats. In addition, the study also suggests that sub-acidic soil more suitable for the growth of *F. przewalskii*. Whether excessive potassium affects the rhizosphere microbial communities of *F. przewalskii*, and whether this has negative effects on *F. przewalskii* growth warrant future investigation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI – PRJNA901426.

Author contributions

ZC and LJ conceived and designed the experiments. ZC, RL, FL, HW, and CC performed the experiments. ZC and RL analyzed the data and wrote the manuscript. ZC, LJ, YM, ZW, and YW revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1070815/full#supplementary-material

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EDITED BY
Luis Raul Comolli,
Independent Researcher, Basel, Switzerland

REVIEWED BY
Rakesh Kumar,
ICAR-Research Complex for Eastern Region, India
Tapan Kumar Adhya,
KIIT University, India

*CORRESPONDENCE
Anil Kumar Khippal
✉ anilkhippal@gmail.com
Kailash Prajapat
✉ kp.agron@gmail.com

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Influence of tillage and residue management practices on productivity, sustainability, and soil biological properties of rice-barley cropping systems in indo-gangetic plain of India

Priyanka Chandra[1], Anil Kumar Khippal[2]*, Kailash Prajapat[3]*, Arijit Barman[1], Geeta Singh[4], Arvind Kumar Rai[1], Om Parkash Ahlawat[5], R. P. S. Verma[2], Kamini Kumari[6] and Gyanendra Singh[7]

[1]Department of Soil and Crop Management, ICAR-Central Soil Salinity Research Institute, Karnal, India, [2]Barley Network, ICAR-Indian Institute of Wheat and Barley Research, Karnal, India, [3]Department of Social Science Research, ICAR-Central Soil Salinity Research Institute, Karnal, India, [4]Department of Agricultural Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi, India, [5]Department of Crop Improvement, ICAR-Indian Institute of Wheat and Barley Research, Karnal, India, [6]Department of Soil Science and Agricultural Chemistry, Lovely Professional University, Phagwara, India, [7]ICAR-Indian Institute of Wheat and Barley Research, Karnal, India

Introduction: Conservation agriculture is a sustainable system of farming that safeguard and conserves natural resources besides enhancing crop production. The biological properties of soil are the most sensitive indicator to assess the short term impact of management practices such as tillage and residue incorporation.

Methods: Nine treatments of tillage and residue management practices [Reduced till direct seeded rice-zero till barley (RTDSR–ZTB); RTDSR–ZTB–green gram residue (Gg); Zero till direct seeded rice–zero till barley–zero till green gram (ZTDSR–ZTB–ZTGg); RTDSR–ZTB + rice residue at 4 t ha 1 (RTDSR–ZTB$_{RR4}$); RTDSR–ZTB$_{RR6}$; un-puddled transplanted rice (UPTR)–ZTB–Gg; UPTR–ZTB$_{RR4}$; UPTR–ZTB$_{RR6}$, and puddled transplanted rice (PTR)–RTB] executed under fixed plot for five years on crop productivity and soil biological properties under rice-barley production system.

Results: The shifting in either RTDSR or ZTDSR resulted in yield penalty in rice compared to PTR. The PTR recorded highest pooled grain yield of 3.61 ha^{-1}. The rice grain yield reduced about 10.6% under DSR as compared to PTR. The ZTB along with residue treatments exhibited significantly higher grain yield over ZTB, and the RTDSR-ZTBRR6 registered highest pooled grain yield of barley. The system productivity (12.45 t ha^{-1}) and sustainable yield index (0.87) were highest under UPTR-ZTBRR6. Biological parameters including microbial biomass carbon, soil respiration, microbial enzymes (Alkaline phosphatase, nitrate reductase and peroxidase), fluorescein diacetate hydrolysis, ergosterol, glomalin related soil proteins, microbial population (bacteria, fungi and actinobacteria) were found to be significantly ($p < 0.05$) effected by different nutrient management practices. Based on the PCA analysis, Fluorescein diacetate hydrolysis, microbial biomass carbon, soil respiration, nitrate reductase and fungi population were the important soil biological parameters indicating soil quality and productivity in present experiment. The results concluded that UPTR-ZTBRR6 was a more suitable practice for maintaining system productivity and soil biological health.

Discussion: The understanding of the impact of different tillage and residue management practices on productivity, soil biological properties and soil quality index under rice-barley cropping system will help in determining the combination of best conservation agriculture practices for improved soil quality and sustainable production.

KEYWORDS

conservation agriculture, principal component analysis, soil biological properties, soil biological index, system productivity

Introduction

The Indo-Gangetic Plain (IGP) of India, spread over 44 million ha, is the most important food-producing region of South Asia. Approximately 76% of its area falls in India, dominated by the states of Punjab, Haryana, Uttar Pradesh, Bihar, and West Bengal (Kumar et al., 2021). The IGP has a wide range of physiographic, climatic, edaphic, and socio-economic production features. The Indian IGP is predominated by a cereal-based production system and contributes to about 40% of the total cereals production of the country.

Rice (*Oryza sativa*)–wheat (*Triticum aestivum* L.) is the main cropping system followed in the IGP covering 13.5 million ha area in Bangladesh, India, Nepal, and Pakistan (Pandey and Kandel, 2020). Despite the fact that the rice–wheat cropping system is the mainstay of the country's food security, its sustainability is at a crossroad due to the development of second-generation problems. It is a high water and nutrient demanding (Ambast et al., 2006; Shahane et al., 2020) crop sequence. The practice of intensive tillage and puddling in standing water for puddled transplanted rice (PTR) results in the breakdown of soil structure, leading to surface and sub-surface soil compaction, poor infiltration and hydraulic conductivity, as well as poor root growth of succeeding crops. Besides, this cropping system leads to a reduction in soil organic matter content, increased greenhouse gas (GHG) emissions (Kakraliya et al., 2021), and a decline in total factor productivity (Kumar et al., 2004).

Other important concerns of this system are the burning of rice residues and the degradation of soil health. Approximately 23 million tons of rice residues are burnt in about 2.5 million farms in the north-western part of India (Meena R.P et al., 2020), which has harmful impacts on the soil and air quality. Large quantities of particulate matter and harmful gases (nitrous oxide, carbon mono oxide, carbon dioxide, methane, etc.) are released into the atmosphere resulting in loss of valuable soil nutrients and deterioration in the air quality leading to ill effects on human health (Jain et al., 2014; Chethan et al., 2020; Venkatramanan et al., 2021). The burning of rice residue results in almost 100% loss of C and N, while 20–60% loss of P, K, and S immersed in the residue (Porichha et al., 2021).

The three main principles of conservation agriculture (CA) (minimum soil disturbance, crop diversification, and permanent soil cover) contribute to protecting the soil from erosion and degradation, improving soil quality and biodiversity, preserving the natural resources, and increasing their use efficiency, optimizing crop yields, while imparting environmental sustainability (Sharma et al., 2022a). Conservation tillage (CT), such as no-tillage or reduced tillage, decreases soil disturbance, protects the soil against erosion, and increases soil organic matter (Busari et al., 2015). Recent studies have also shown that CT protects the life cycle of arthropods, increases their diversity (Rivers et al., 2016), and plays a major role in shaping microbial communities (Morugán-Coronado et al., 2022). Similarly, zero tillage with crop residue management effectively improves soil organic matter (SOM) and maintains crop yields (Bhattacharyya et al., 2012; Singh et al., 2018; Das et al., 2021; Saurabh et al., 2021). Other management practices, such as straw mulching or incorporation, and tillage have significant effects on total SOM and soil enzymes (Song et al., 2016; Zhang et al., 2016; Akhtar et al., 2019; Qin et al., 2021).

Direct seeded rice (DSR) technology, one of the important components of CA, has evolved to overcome the problems associated with soil, water, and the environment under PTR (Kumar and Ladha, 2011). Under the DSR technique, three basic operations, namely, puddling, transplanting, and ponding of water, are omitted. Consequently, DSR helps in reducing the total water requirement in rice production approximately by 30% (Joshi et al., 2013). However, a yield penalty of 10% has been recorded in zero tilled DSR rice but the yield of succeeding zero tilled wheat increased by 21%, showing the net benefit of it in the rice–wheat system (Sharma et al., 2019).

The surface retention of rice residue by direct seeding the Rabi crop with zero till using innovative farm machineries, such as Zero-till Drill, Mulcher, Rotary Disc Drill, and Happy Seeder, are emerging as feasible options for rice residue management (Sidhu et al., 2015). Furthermore, the direct seeding of Rabi crop with these farm machineries consumes less fuel compared to the conventional tillage system (Pratibha et al., 2015). The *in-situ* surface retention of crop residues as full or anchored also boosts soil health by improving soil physical properties (Das et al., 2021; Saurabh et al., 2021), soil organic carbon, and labile carbon fractions (Liu et al., 2013; Gupta et al., 2022); increasing moisture retention; regulating temperature (Fu et al., 2021); enhancing nutrient availability (Moharana et al., 2012) and root absorption; suppressing weeds (Nikolić et al., 2021); decreasing salinity (Prajapat et al., 2020); encouraging soil biological activity (Singh et al., 2018); and controlling crop pests and diseases (El-Shater and Yigezu, 2021). Moreover, crop diversification reduces pressure in current agriculture and maintains or even enhances soil microbial abundance as crop diversification has shown benefits for soil macro- and microorganisms, while maintaining crop yields

(Baldwin-Kordick et al., 2022; Morugán-Coronado et al., 2022; Rai et al., 2022).

Barley (*Hordeum vulgare* L.) is the world's fourth most essential cereal crop after wheat, rice, and maize produced in more than 100 countries (Giraldo et al., 2019). Barley can be successfully grown under adverse climatic conditions of drought, salinity, and alkalinity. It is usually used as food for human beings and feed for animals, and it is considered superior to wheat as it lacks gluten. Furthermore, barley is an industrial crop and the crop of interest for entrepreneurs, farmers, and researchers. The importance of barley as a functional food is mainly due to its potentialities in the production of healthy food, as an excellent source of dietary fiber, and having β-glucan.

The introduction of green gram (*Vigna radiata* L.) in the cereal–cereal cropping system is in use for ages as it is beneficial in sustaining the productivity of the system in several ways. It maintains nitrogen balance in the agroecosystems and provides an opportunity for more grain and protein production. Mungbean-based wheat systems sustain productivity through moisture conservation, stable economic benefits, and improvement in soil nutrition and organic matter over time (Das et al., 2021; Saurabh et al., 2021).

Different tillage and residue management practices and diverse cropping systems have an effect on soil biological health, which include soil organic matter, microbial population, microbial biomass carbon (MBC), microbial biomass nitrogen, and microbial enzymatic activities (Johansen et al., 2012; Singh et al., 2018; Das et al., 2021). Whereas the soil biological indices are the important tools conveyed through several properties and denote the quality of soil in terms of sustainability (Lehman et al., 2015). These indices are dynamic soil properties that are very sensitive to land management, natural disturbances, and chemical contaminants (Herrick, 2000). In general, biological indicators that describe soil organisms mediated soil processes are the most informative about soil function (Paz-Ferreiro and Fu, 2016). In this context, it is important to understand which indicators to test, what information is needed for the appropriate management of soil, and where to get this information.

A soil biological index (SBI), which is an integrated expression of the most sensitive attributes, if found, can monitor soil health after barley in a holistic way for making the most sustainable management choices. To fill this gap, a systematic study was conducted to (1) evaluate the combined effect of cropping systems, tillage, and residue retention on crop yield; (2) identify key biological soil health indicators; and (3) develop a soil quality index to identify the best practices for the rice–barley cropping system in IGP of India.

Materials and methods

Experimental site and soil

The study was carried out at the Research Farm of ICAR-Indian Institute of Wheat and Barley Research, Karnal (29°42'12"N, 76°59'36"E and 245 m msl), from 2013–2014 to 2017–2018. The soil of the experimental farm is sandy loam in texture with 62.2% sand, 26.9% silt, and 10.9% clay. The soil is normal in electrical conductivity ($EC_{1:2}$ 0.24 dS/m), alkaline in reaction ($pH_{1:2}$ 8.3), low in oxidizable organic carbon (0.42), low in $KMnO_4$-N (177.0 kg/ha), and medium in Olsen's–P (18.4 kg/ha) and NH_4OAc–K (222.3 kg/ha). The climate of the region is semi-arid and sub-tropical with hot-dry summer (April to June), hot-humid rainy season (July to September), and cool-dry winter (October to March). The average annual maximum and minimum air temperatures are 29.9°C and 17.1°C, respectively. The average annual rainfall of the region is 670 mm, out of which 75–80% is received during the southwest monsoon (July to September) period. The meteorological parameters recorded during the experimental period are depicted in Supplementary Figure 1.

Experimental setup and treatments

The experiment was initiated in the rainy season of 2013 with nine treatments of tillage and residue management in the rice–barley and rice–barley–green gram cropping systems in randomized block design with three replications. The treatments were T1: Reduced till direct seeded rice–zero till barley (RTDSR–ZTB); T2: Reduced till direct seeded rice–zero till barley–green gram (RTDSR–ZTB–Gg); T3: Zero till direct seeded rice–zero till barley–zero till green gram (ZTDSR–ZTB–ZTGg); T4: Reduced till direct seeded rice–zero till barley + rice residue at 4 t/ha ($RTDSR–ZTB_{RR4}$); T5: Reduced till direct seeded rice–zero till barley + rice residue at 6 t/ha ($RTDSR–ZTB_{RR6}$); T6: Un-puddled transplanted rice–zero till barley–green gram (UPTR–ZTB–Gg); T7: Un-puddled transplanted rice–zero till barley + rice residue 4 t/ha ($UPTR–ZTB_{RR4}$); T8: Un-puddled transplanted rice–zero till barley + rice residue 6 t/ha ($UPTR–ZTB_{RR6}$); and T9: Puddled transplanted rice–reduced till barley (PTR–RTB). The gross plot size was 4 m × 10 m under each treatment. The details of the crop rotation, tillage, and residue management are summarized in Table 1.

At the beginning of the experiment (May–June 2013), the field was plowed and leveled with a laser land leveler. The first crop of rice was grown as per treatments; however, the zero and minimum tillage treatments could not be applied in the first rice crop as the soil was plowed and prepared uniformly to begin the experiment. For subsequent rice crops, two passes of harrows were made followed by planking, and direct seeding was performed with a multi-crop seed drill fitted with inclined seed distribution plates in the reduced till treatments. The seed rate for DSR was 20 kg/ha. For ZTDSR, rice was directly sown in green gram residues in the month of June by using a multi-crop turbo happy seeder. For both the transplanted rice treatments (UPTR and PTR), the nursery was raised in separate plots and transplanted at the age of 30 days in the plots prepared as per tillage. The sowing in the nursery was done on the date of the sowing of DSR. For UPTR, the field was plowed with 2 passing of harrows followed by planking. The plots were ponded with water, and direct transplanting was performed manually without puddling. Under PTR, the field was harrowed two times after harvesting the barley crop. At the time of transplanting, puddling was done with a rotavator in ponded water conditions, and seedlings were transplanted manually in the puddled field. ZT barley and green gram were sown with a turbo

TABLE 1 Detailed description of tillage and residue management practices in rice–barley cropping system.

Treatments depiction	Crop rotations (Rainy–winter–summer)	Tillage and crop establishment method	Residue management	Fertilizer doses (N+ P_2O_5, kg ha^{-1}) and application	Irrigation management
T1: RTDSR–ZTB	Rice–barley	Reduced tillage direct seeded rice (RTDSR)–zero tilled barley (ZTB)	Anchored rice residue retained on soil surface and all barley residue were removed	Rice: 60 + 30; P_2O_5 as basal at the time of sowing through DAP. N in three equal splits at sowing; at 21–25 and at 42–45 days after sowing (DAS). Barley: 90 + 40 Full P_2O_5 and half N as basal. Remaining half N as top dressing after first irrigation through urea	**Rice:** Soil was kept wet for the first 20 days followed by irrigation at an interval of 10–12 days depending on the field conditions. **Barley**: Three irrigations at critical crop growth stages (CRI, panicle emergence and grain formation)
T2: RTDSR–ZTB–Gg	Rice–barley–green gram	Reduced tillage direct seeded rice (RTDSR)–zero tilled barley (ZTB)–conventional tillage green gram (Gg)	Anchored rice residue retained on soil surface, barley residue removed and full green gram residue incorporated	Rice: 60 + 30; P_2O_5 as basal at the time of sowing through DAP. N in three equal splits at sowing; at 21–25 and at 42–45 days after sowing (DAS). Barley: 90 + 40; Full P_2O_5 and half N as basal. Remaining half N as top dressing after first irrigation through urea. Green gram: 20 + 40; applied as basal.	**Rice**: Soil was kept wet for the first 20 days followed by irrigation at an interval of 10–12 days depending on the field condition. **Barley**: Three irrigations at critical crop growth stages. **Green gram:** First irrigation at 20 DAS and thereafter two irrigations as per need.
T3: ZTDSR–ZTB–ZTGg	Rice–barley–green gram	Zero tillage DSR (ZTDSR)– zero tilled barley (ZTB)–zero tillage green gram (ZTGg)	Anchored residue of all the three crops retained on soil surface	Rice: 60 + 30; P_2O_5 as basal at the time of sowing through DAP. N in three equal splits at sowing; at 21–25 and at 42–45 days after sowing (DAS). Barley: 90 + 40; Full P_2O_5 and half N as basal. Remaining half N as top dressing after first irrigation through urea. Green gram: 20 + 40; applied as basal.	**Rice:** Soil was kept wet for the first 20 days followed by irrigation at an interval of 10-12 days depending on the field condition. **Barley:** Three irrigations at critical crop growth stages. **Green gram**: First irrigation at 20 DAS and two more irrigation as per need
T4: RTDSR–ZTB_{RR4}	Rice–barley	Reduced tillage direct seeded rice (RTDSR)–zero tilled barley (ZTB)	Rice residue @ 4 t ha^{-1} retained on soil surface (RR4) and all barley residue were removed	Rice: 60 + 30; P_2O_5 as basal at the time of sowing through DAP. N in three equal splits at sowing; at 21–25 and at 42–45 days after sowing (DAS). Barley: 90 + 40; Full P_2O_5 and half N as basal. Remaining half N as top dressing after first irrigation through urea.	**Rice:** Soil was kept wet for the first 20 days followed by irrigation at an interval of 10–12 days depending on the field conditions. **Barley:** Three irrigations at critical crop growth stages.
T5: RTDSR–ZTB_{RR6}	Rice–barley	Reduced tillage direct seeded rice (RTDSR)–zero tilled barley (ZTB)	Rice residue @ 6 tha^{-1} retained on soil surface (RR6) and all barley residue were removed	Rice: 60 + 30; P_2O_5 as basal at the time of sowing through DAP. N in three equal splits at sowing, at 21–25 and at 42–45 days after sowing (DAS). **Barley:** 90 + 40; Full P_2O_5 and half N as basal. Remaining half N as top dressing after first irrigation through urea	**Rice:** Soil was kept wet for the first 20 days followed by irrigation at an interval of 10–12 days depending on the field condition. **Barley:** Three irrigations at critical crop growth stages.

(Continued)

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TABLE 1 (Continued)

Treatments depiction	Crop rotations (Rainy–winter–summer)	Tillage and crop establishment method	Residue management	Fertilizer doses (N+ P_2O_5, kg ha^{-1}) and application	Irrigation management
T6: UPTR–ZTB–Gg	Rice–barley–green gram	Unpuddled transplanted rice (UPTPR)–zero tillage barley (ZTB)–conventional tillage green gram (Gg)	Anchored rice residue retained on soil surface, barley residue removed and full green gram residue incorporated	Rice: 60 + 30; P_2O_5 as basal at the time of transplanting through DAP. N in three equal splits at 7–10 days after transplanting (DAT), at 21–25 and at 42–45 DAT through urea Barley: 90 + 40; Full P_2O_5 and half N as basal. Remaining half N as top dressing after first irrigation through urea. Green gram: 20 + 40; applied as basal.	**Rice:** Ponding of water (2–3 cm) for one month followed by light irrigations to keep soil moist depending on the field condition. **Barley:** Three irrigations at critical crop growth stages. **Green gram:** First irrigation 20 at DAS and two more irrigations as per need.
T7: UPTR–ZTB_{RR4}	Rice–barley	Unpuddled transplanted rice (UPTPR)–zero tillage barley (ZTB)	Rice residue @ 4 t ha^{-1} retained on soil surface (RR4) and all barley residue were removed	Rice: 60 + 30; P_2O_5 as basal at the time of transplanting through DAP. N in three equal splits at 7–10 days after transplanting (DAT); at 21–25 and at 42–45 DAT through urea. Barley: 90 + 40; Full P_2O_5 and half N as basal. Remaining half N as top dressing after first irrigation through urea.	**Rice**: Ponding of water (2–3 cm) for 1 month followed by light irrigations to keep soil moist depending on the field condition. **Barley**: Three irrigations at critical crop growth stages.
T8: UPTR–ZTB_{RR6}	Rice–barley	Unpuddled transplanted rice (UPTPR)–zero tillage barley (ZTB)	Rice residue @ 6 t ha^{-1} retained on soil surface (RR6)and all barley residue were removed	Rice: 60 + 30; P_2O_5 as basal at the time of transplanting through DAP. N in three equal splits at 7–10 days after transplanting (DAT); at 21–25 and at 42–45 DAT through urea Barley: 90 + 40; Full P_2O_5 and half N as basal. Remaining half N as top dressing after first irrigation through urea.	**Rice:** Ponding of water (2-3 cm) in plots for one month followed by light irrigations to keep soil moist depending on the field conditions. **Barley**: Three irrigations at critical crop growth stages.
T9: PTR–RTB	Rice–barley	Puddled transplanted rice (PTPR)–reduced tillage barley (RTB)	All residue removed	Rice: 60 + 30; P_2O_5 as basal at the time of transplanting through DAP. N in three equal splits at 7–10 days after transplanting (DAT); at 21–25 and at 42–45 DAT through urea. Barley: 90 + 40; Full P_2O_5 and half N as basal. Remaining half N as top dressing after first irrigation through urea.	**Rice:** Continuous flooding of 5 cm depth for 1 month followed by light irrigation at hair line crack in soil. **Barley:** Three irrigations at critical crop growth stages.

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happy seeder using 100 and 25 kg/ha seed rates, respectively. For RT barley, two harrowing and planking were done after rice harvesting in respective plots followed by the sowing of barley with a seed drill. Green gram was knocked down with the spray of 2,4-D at the flowering stage for sowing of succeeding zero-till rice. In RT and UPTR treatments, the green gram was incorporated into the soil at the flowering stage. All the crops were sown/transplanted at 20 cm row spacing. The cultivars used were Basmati CSR30 of rice, DURB52 of barley, and SML668 of green gram.

For controlling the germinated weeds in ZT plots, glyphosate 41% SL at 900 ml a.i./ha was sprayed 7–10 days before the sowing of crops. In DSR and ZT barley plots, pendimethalin 30% EC at 1,000 ml a.i./ha was sprayed just after sowing to control the first flush of weeds. In DSR, post-emergence application of bispyribac sodium 10% SC at 25 ml a.i./ha was done at 20–25 days after sowing (DAS) followed by 2,4-D amine 58% at 500 ml a.i./ha at 30 DAS to control growing weeds. In un-puddled and transplanted rice plots, pre-emergence application of pretilachlor 50% EC at 500 ml a.i./ha followed by post-emergence application of bispyribac sodium 10% SC was done to control the weeds. In barley, post-emergence spray of 2,4-D amine 58% SL at 500 ml a.i./ha and pinoxaden 5% EC at 50 ml a.i./ha was done after the first irrigation. Other standard recommended practices of the region were followed for insect-pest management. At maturity, rice and barley crops were harvested excluding the two border rows. The biomass was sun-dried and threshed to separate the grains. Per plot grain yield of both crops was expressed as grain yield in t/ha.

Soil sampling and storage

Soil samples were collected at the end of the fifth cropping cycle, after the harvesting of barley crops during 2017–18. Samples were (0–15 cm depth) collected with the metal auger (5 cm diameter) near the root zone after the removal of the crop litter and stubble present. Three subsamples were taken from each plot to form one composite sample per plot. The soil was transported to the laboratory immediately after sampling and stored at 4°C until the analyses were conducted.

Soil biological properties

Soil microbial biomass carbon (MBC) was determined using the fumigation-extraction method by fumigating 15 g of soil with ethanol-free chloroform followed by 0.5 M K_2SO_4 extraction (w:v 1:4); additionally, 15 g of soil was extracted with K_2SO_4 without fumigation. MBC flush was calculated using the relationship: MBC = [(1/0.38) × C-flush] (Vance et al., 1987). Soil respiration (SR0) was determined by measuring released CO_2 (Stotzky, 2016). This assay was carried out in glass bottles (300 ml) and plastic cups containing soil with 5 ml of sodium hydroxide (1 mol/L). The C-CO_2 collected in the alkaline solution was determined by titration of the residual NaOH with chloric acid (0.25 mol/L) after the addition of 2.5 ml of $BaCl_2.2H_2O$ (1 mol/L) and phenolphthalein indicator. The C-CO_2 produced was expressed in mg CO_2-C/g soil week. Soil respiration (SR) was also performed after amendment with glucose (100 mg glucose/kg dry soil). The soil enzyme alkaline phosphatase (AP, pH 11.0) (EC 3.1.3.1) was measured by estimating the concentration of p-nitrophenol released on incubation of soil with p-nitrophenyl phosphate, used as substrate, and was expressed as μmol p-nitrophenol (p-NP) produced/g dry weight of soil h (Tabatabai and Bremner, 1969). The nitrate reductase activity (NR; EC 1.7.1.1) of soil was estimated in 0.19 M ammonium chloride buffer (pH 8.5) using 25 mM KNO_3 as substrate (Han et al., 2013). The peroxidase (PO; EC 1.11.1.7) enzymes in soil were estimated colorimetrically as oxidation of L-3,4-dihydroxyphenylalanine in the presence of hydrogen peroxide (Sinsabaugh, 2010). Total microbial activity (TMA) in soil was determined by fluorescein diacetate (FDA) hydrolysis by mixing soil with 60 mM potassium phosphate buffer (pH 7.6) and 3′ 6′-diacetylfluorescein. The concentration of fluorescein released during the assay was measured spectrophotometerically at 490 nm and expressed as μg fluorescein g/soil h (Green et al., 2006). Ergosterol (EG) estimation in soil was done by HPLC using acetonitrile and methanol as eluting reagents with UV detection at 282 nm (Young, 1995). The extraction of ergosterol was carried out by treating the soil with methanol and 2 M sodium hydroxide in a culture tube and heating it for 50 s in a domestic microwave (2,450 MHz and 750 W). Total glomalin-related soil proteins (T-GRSP) estimation was carried out in the soil of all the treatments. The estimation of T-GRSP was carried out and expressed as μg/g dry weight of soil (Wright and Upadhyaya, 1996); 1 g of the air-dried soil was added to 8 ml sodium citrate (20 mM, pH 7.0) and was autoclaved at 121°C for 1 h, and then centrifuged at 5,000 rpm for 20 min. The concentration of T-GRSP was determined by Bradford assay with bovine serum albumin as the standard and was expressed as μg/g dry weight of soil.

Microbial population

Microbial communities in the rhizosphere soils were determined by examining microbial populations by serial dilution method (Chandra et al., 2020). For bacterial (BA) population count, nutrient agar (Himedia®) was used. Potato dextrose agar (Himedia®) was used for the fungal count, while actinobacterial isolation agar (Himedia®) was used for the enumeration of actinobacteria (AC) populations. The collected soils of each treatment were serially diluted, and 1 ml of soil suspension each from 10^{-5} to 10^{-8} dilutions was spread on the respective media-filled plate in triplicate. Petri plates were then incubated at 28 ± 2°C. Bacterial colonies appearing within 48 h were counted, while fungal and actinobacterial colonies were counted after 3–4 and 6–7 days, respectively. Results were expressed in colony-forming units (CFUs)/g of soil sample.

Computation of soil biological index

The principal component analysis (PCA) technique (Bastida et al., 2006; Barman et al., 2017) using SPSS (version 16.0) was used to identify the minimum dataset based on different soil biological parameters analyzed. Through this mathematical procedure, a

(smaller) number of uncorrelated variables (PC) were transformed from several (possibly) correlated variables. The sensitive indicators of SBI were selected based on the score of the factor in PCA analysis carried out with determined parameters (FDA, MBC, SR, NR, and FN). This data reduction analysis provides four principle components; the first component explained the highest variance in the results. Five sensitive parameters with the highest weight from the four principle components of PCA were selected for the development of SBI. The final value of SBI was normalized (0 to 1 scale) because the absolute values of some parameters were lower than other parameters. In this study, the sensitive parameters, namely, FDA, MBC, and FN, functioned on "the more the better," i.e., the more fungi population, the better nutrient availability for the barley crop. In the case of sensitive parameters, SR and NR functioned as "the less the better." For normalization, Eq. 1 was used which followed the sigmoidal curve, between 0–1. SBI consisted of summing these four sensitive parameters but to bring the value between 0 and 1, the final value of SBI was normalized as follows:

$$Y = \frac{a}{1 + (\frac{x}{x_0})^b} \quad (1)$$

where 'a' is the maximum value, in this case, a = 1; "x" is the value of the parameter in question in each case, which is the unknown; "x_0" is the mean value of each sensitive parameter of the rice–barley system in the study; and "b" is the slope of the equation. We used optimized values of "b" that fit the sigmoid curve tending to 1 for 5 sensitive parameters. The value of curves ranged between 0 and 1.

The sensitive variables for each observation were weighted by using the PCA results. Each PC explained a certain amount (%) of the variation in the total dataset. This percentage, divided by the total percentage of variation explained by all PCs with eigen vectors >1, provided the weighted factor for variables chosen under a given PC (Table 2). The weighting of each variable in the principle component was multiplied with each normalized value and then summed up using the following equation:

$$SBI = \sum_{i=1}^{n} w_i s_i \quad (2)$$

where S = indicator score (Equation. 1) and W = the weighing factor obtained from PCA.

Higher index scores were assumed to mean better soil biological quality or greater performance of soil function. The SBI estimated from the above method was validated against the system yield of rice–barley cropping system by computing multiple regression coefficients.

TABLE 2 Performance of soil biological indicators in terms of factor loading/eigen vector values in principal component analysis.

PCs	PC1	PC2	PC3	PC4
Eigen value	5.6	1.7	1.3	1.1
Per cent variance	46.4	14.0	10.8	8.8
Cumulative percentage	46.4	60.4	71.2	80.0
Factor loading/eigen vector				
FDA	**0.91**	0.16	0.11	0.12
MBC	**0.81**	0.23	0.32	0.27
AP	**0.86**	0.33	0.21	0.19
T-GRSP	0.68	0.19	0.58	0.29
EG	0.15	0.36	0.29	0.58
NR	0.03	0.00	**0.89**	−0.01
PO	0.32	0.85	0.27	0.26
SR	0.08	**0.96**	−0.02	−0.05
SR0	0.44	0.43	0.65	−0.18
BA	0.25	0.10	−0.15	0.70
AC	−0.77	0.03	0.09	−0.11
FN	0.10	−0.11	0.01	**0.82**

MBC, microbial biomass carbon; SR0, soil respiration without glucose; SR, soil respiration with glucose AP, alkaline phosphatase; NR, nitrate reductase; PO, peroxides; FDA, fluorescein diacetate hydrolysis; EG, Ergosterol; T-GRSP, glomalin; BA, Bacteria; AC, Actinobacteria; FN, Fungi. Bold values indicate the eigen vectors within 10% of the highest factor loadings.

Sustainable yield index

To express the overall impact of treatments on productivity, the sustainable yield index (SYI) was calculated based on the barley grain equivalent yield (BEY) of the rice–barley cropping system of 5 years. The sustainable yield index (SYI) was computed using the following equation:

$$SYI = i - \sigma / Y_{Max} \quad (3)$$

where i is the mean BEY of the respective treatment, σ is the standard deviation of the treatment, and Y_{Max} is the maximum BEY of any treatment in the experiment in any year.

System productivity

System productivity of the rice–barley system in terms of barley equivalent yield (BEY t/ha) was calculated by taking into account the grain yields and market prices of rice and barley crops as follows:

$$BEY\ (t/ha) = \frac{rice\ grain\ yield\ (t/ha) \times price\ of\ rice\ grain}{price\ of\ barley\ grain} + barley\ grain\ yield\ (t/ha)$$

Statistical analysis

The statistical analysis of data was carried out using the ANOVA technique for randomized block design in the SAS program. The standard error of the mean with respect to each parameter was calculated. For comparisons where significant F probabilities ($p < 0.05$) were found, we used the DUNCAN's

Multiple Range Test (DMRT). Data were tested for normality and transformed suitably if not following a normal distribution. PCA and correlation matrix were carried out in the R computer software. MS Excel was used for normalization models and graphs.

Results

Crop yield and system productivity

Different tillage and residue management practices significantly affected the yield of rice and barley (Figure 1) and system productivity as barley equivalent yield (Figure 2). On the pooled basis, the conventional rice followed by reduced till barley (PTR–RTB) maintained the highest rice grain yield (3.61 t/ha). There was no significant reduction in grain yield of rice grown without puddling irrespective of tillage and residue management practices. The rice grain yield was significantly lower under all the treatments of reduced and zero tillage adopted in rice. Moreover, the mean grain yield of rice was 10.6% lower under DSR than that of PTR.

The RTB after PTR recorded the lowest grain yield of barley crop (4.02 t/ha). Zero tilled barley without residue incorporation also recorded poor grain yield (4.05 t/ha). The mungbean residue incorporation or rice residue retention under ZT significantly improved the barley grain yield and RTDSR–ZTB_{RR6} recorded the highest barley grain yield (4.32 t/ha), followed by UPTR–ZTB_{RR6} (4.29 t/ha) and UPTR–ZTB_{RR4} (4.29 t/ha). The rice residue retention under RTDSR–ZTB_{RR6} enhanced the grain yield of barley by 6.7% over RTDSR–ZTB.

The system productivity calculated in terms of barley equivalent yield showed that tillage and residue management treatment involving UPTR–ZTB_{RR6} proclaimed the highest BEY (12.45 t/ha), followed by UPTR–ZTB_{RR6} (12.35 t/ha), PTR–RTB (12.35 t/ha), and UPTR–ZTB–Gg (12.21 t/ha).

Sustainable yield index

The sustainable yield index of the rice–barley cropping system is depicted in Figure 2. The SYI followed the trends of system productivity, wherein UPTR–ZTB_{RR6} recorded the highest SYI, followed by the UPTR–ZTB_{RR4} and UPTR–ZTB–Gg. Among the conservation tillage, RTDSR—ZTB_{RR6} was recorded with the highest SYI.

MBC and soil respiration

Soil MBC was found to be significantly ($p < 0.05$) affected by different nutrient management practices. Significantly ($p < 0.05$) highest MBC was observed in the UPTR–ZTB_{RR6} (518.78 mg/kg soil), followed by UPTR–ZTB_{RR4} (488.43 mg/kg soil) (Table 3). Similarly, soil respiration (SR0) also significantly ($p < 0.05$) varied in different treatments. Glucose-induced soil respiration (SR) was the highest in UPTR–ZTB_{RR6} (77.78 mg CO_2-C/g soil week), followed by UPTR–ZTB_{RR4} (75.86 mg CO_2-C/g soil week); however, SR0 was significantly ($p < 0.05$) higher in UPTR–ZTB_{RR4} (70.69 mg CO_2-C/g soil week) and PTR–RTB (70.35 mg CO_2-C/g soil week) compared with other management practices (Table 3).

Microbial enzymes

In this study, values of AP varied significantly ($p < 0.05$) with different tillage and residue incorporation practices. The activity of AP was significantly ($p < 0.05$) high in UPTR–ZTB_{RR6} (82.19 μmol p-nitrophenol/g h), followed by UPTR–ZTB_{RR4} (73.5 μmol p-nitrophenol/g h) and PTR–RTB (71.69 μmol p-nitrophenol/g h) (Table 3). The NR activity was significantly ($p < 0.05$) high in UPTR–ZTB–Gg (2,816.32 μg/ml h) and PTR–RTB (2,777.35 μg/ml h) (Table 3). The residue-amended treatment UPTR–ZTB_{RR6} (15.49 units/μg) demonstrated significantly ($p < 0.05$) high PO activity (Table 3).

Microbial indicators

In this study, the FDA was significantly ($p < 0.05$) high in UPTR–ZTB_{RR6} (6.48 μg fluorescein g/soil h), followed by UPTR–ZTB_{RR4} (5.21 μg fluorescein g/soil h), and RTDSR–ZTB_{RR4} (4.51 μg fluorescein g/soil h) (Table 3). EG content was high in residue-amended plots as it was significantly ($p < 0.05$) high in UPTR–ZTB_{RR6} (20.30 μg/g soil) (Table 3). T-GRSP in the soil was significantly ($p < 0.05$) high in treatment UPTR–ZTB_{RR6} (49.22 μg/kg), followed by UPTR–ZTB_{RR4} (44.23 μg/kg) (Table 3).

Microbial population

The significantly ($p < 0.05$) high bacterial population was in treatment UPTR–ZTB_{RR6} (93.3×10^5 CFU/g), while the population of Actinobacteria was significantly ($p < 0.05$) high in ZTDSR–ZTB–ZTGg (54.67×10^4 CFU/g) (Table 4). The significantly ($p < 0.05$) high fungal population was recorded in treatment UPTR–ZTB_{RR6} (47.0×10^3 CFU/g) and UPTR–ZTB_{RR4} (44.0×10^3 CFU/g) (Table 4).

Principal component analysis and soil biological index

Soil biological properties had a significant effect on the productivity of the rice–barley cropping system, which indicates their significance in the determination of SBI. Principal component analysis (PCA) with all the soil biological indicators showed that a variance of 80% was explained in the PCA of 12 variables, and 4 PCs were extracted with eigen values >1 (Table 2). FDA (fluorescein diacetate), MBC (microbial biomass carbon), and AP (alkaline phosphatase) have 46.4% of the total variance, which were highly weighted variables in PC1 (Table 2). To select the minimum variables for the development of soil biological index (SBI), out of the three variables of PC1, FDA and MBC were chosen as sensitive parameters in PC1. Among the two enzyme activity indicators (FDA and AP), AP was removed from the dataset as it has a high

correlation ($p = 0.90$) with FDA, and FDA represents the overall soil enzyme activity (Supplementary Figure 2). PC2 has a 14.0% of the total variance, and SR (soil respiration with glucose) was selected to be the sensitive parameter. The third PC has 10.8% of the total variation, and NR (nitrate reductase) was considered a highly weighted eigenvector, while PC4 has 8.8% of the total variation, and FN (fungi) was selected as a sensitive parameter. The final sensitive biological indicators consisted of FDA, MBC, SR, NR, and FN (Figure 3). Based on the average linear scores of sensitive parameters, the FDA showed the highest scores followed by MBC, fungi, SR, and NR (Figure 4).

Based on these sensitive parameters, SBI was computed for different treatments used in the study. Treatments showed significant ($p < 0.05$) differences for SBI (Figure 5). The traditional practice of rice–barley (PTR–RTB) showed the minimum value of SBI. Rice residue retention improved the SBI under RTDSR and UPTR and had a greater impact than mungbean residues. UPTR–ZTB with rice residue retention of 4 and 6 t/ha and RTDSR–ZTB with rice residue retention of 6 t/ha recorded higher SBI values over other treatments. The regression relationship of SBI with BEY and SYI showed that there was an improvement in system productivity and sustainable yield index with an increase in SBI (Figure 6).

Discussion

Crop yields, system productivity, and sustainability

Conservation agriculture practices have been advocated for mitigating the ill effects of cereal-based intensive agriculture while

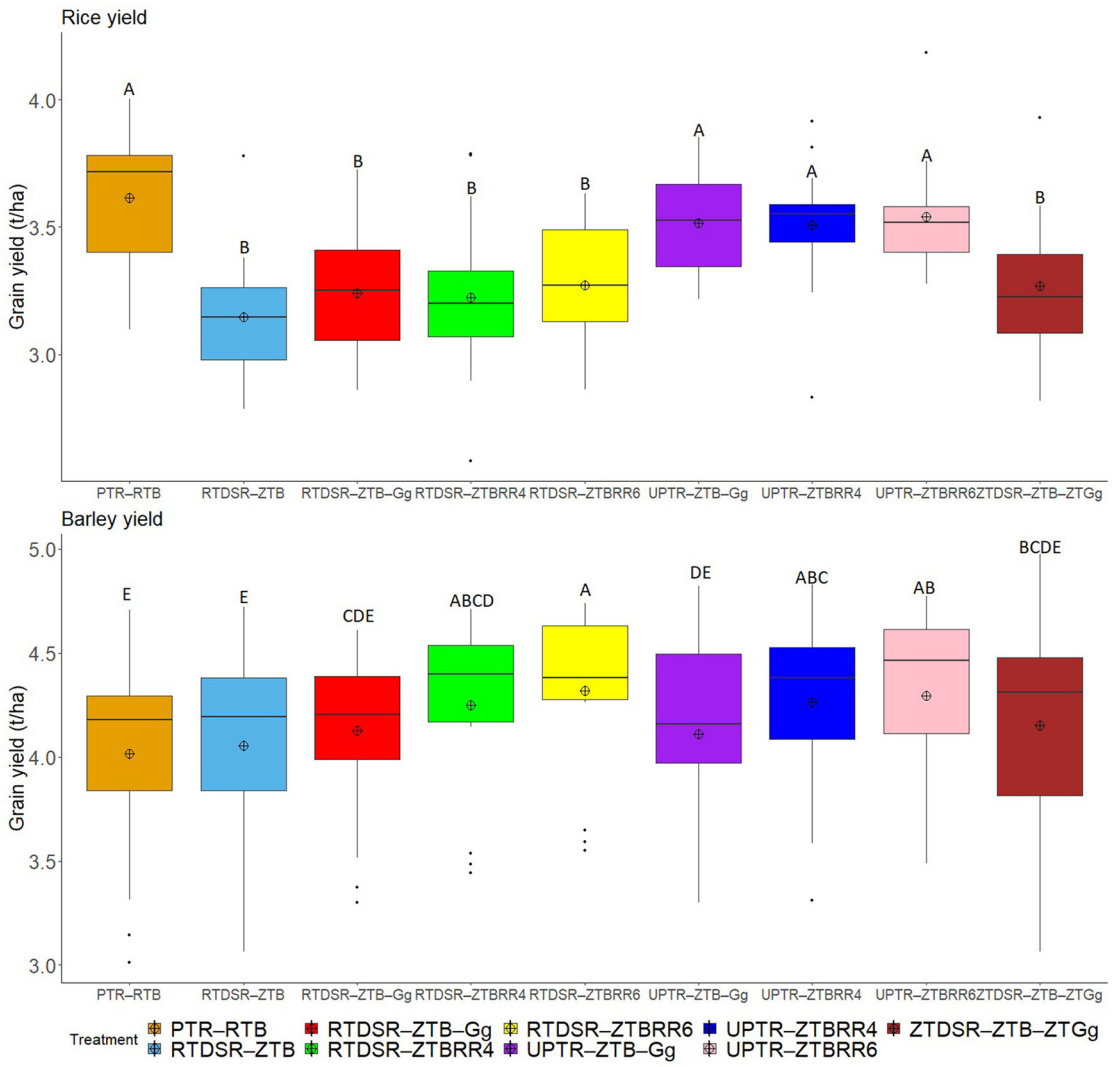

FIGURE 1
Effect of tillage and residue management practices on rice and barley grain yield. RTDSR–ZTB, reduced till direct seeded rice–zero till barley; RTDSR–ZTB–Gg, reduced till direct seeded rice–zero till barley–green gram; ZTDSR–ZTB–ZTGg, zero till direct seeded rice–zero till barley–zero till green gram; RTDSR–ZTBRR4, reduced till–direct seeded rice–zero till barley + rice residue at 4 t/ha; RTDSR–ZTBRR6, reduced till direct seeded rice–zero till barley + rice residue at 6 t/ha; UPTR–ZTB–Gg, un-puddled transplanted rice–zero till barley–green gram; UPTR–ZTBRR4, un-puddled transplanted rice–zero till barley + rice residue 4 t/ha; UPTR–ZTBRR6, un-puddled transplanted rice–zero till barley + rice residue 6 t/ha; PTR–RTB, puddled transplanted rice–reduced till barley; R, residue. Box plots with different capital letters are significantly different ($p < 0.05$) using DUNCAN's multiple range test.

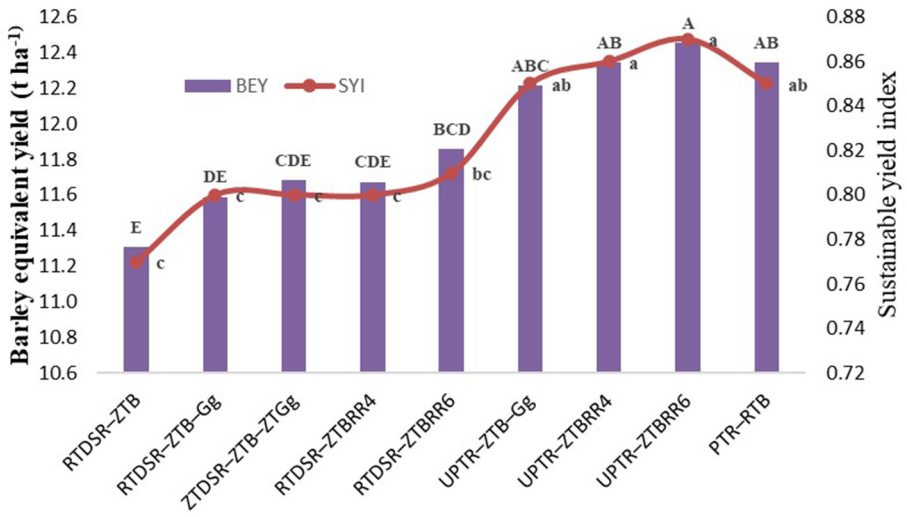

FIGURE 2
Effect of tillage and residue management practices on system productivity and SYI. RTDSR–ZTB, reduced till direct seeded rice–zero till barley; RTDSR–ZTB–Gg, reduced till direct seeded rice–zero till barley–green gram; ZTDSR–ZTB–ZTGg, zero till direct seeded rice–zero till barley–zero till green gram; RTDSR–ZTB_{RR4}, reduced till–direct seeded rice–zero till barley + rice residue at 4 t/ha; RTDSR–ZTB_{RR6}, reduced till direct seeded rice–zero till barley + rice residue at 6 t/ha; UPTR–ZTB–Gg, un-puddled transplanted rice–zero till barley–green gram; UPTR–ZTB_{RR4}, un-puddled transplanted rice–zero till barley + rice residue 4 t/ha; UPTR–ZTB_{RR6}, un-puddled transplanted rice–zero till barley + rice residue 6 t/ha; PTR–RTB, puddled transplanted rice-reduced till barley; R, residue. Bars with different capital letters for BEY and small letters for SYI are significantly different ($p < 0.05$) using DUNCAN's multiple range test.

TABLE 3 Effect of tillage and residue management practices on soil biological properties.

Treatments	MBC	SR	SR0	AP	NR	PO	FDA	EG	T-GRSP
RTDSR–ZTB	106.4^{h}	74.8^{e}	44.6^{g}	39.7^{e}	640.1^{g}	6.7^{c}	1.0^{ed}	14.6^{abc}	17.4^{g}
RTDSR–ZTB–Gg	214.0^{f}	75.5^{c}	46.0^{f}	47.1^{d}	967.3^{f}	7.1^{c}	1.1^{ed}	9.03^{c}	14.3^{h}
ZTDSR–ZTB–ZTGg	264.1^{e}	75.6^{c}	54.9^{e}	42.0^{e}	1011^{f}	9.1^{c}	2.1^{cd}	12.8^{bc}	23.7^{f}
RTDSR–ZTB_{RR4}	398.3^{c}	73.8^{g}	62.1^{c}	54.9^{c}	1225.0^{e}	12.0^{b}	3.0^{c}	19.1^{a}	44.8^{b}
RTDSR–ZTB_{RR6}	398.7^{c}	74.6^{f}	57.6^{d}	71.7^{b}	1752.8^{c}	11.4^{b}	5.2^{b}	17.6^{ab}	38.4^{c}
UPTR–ZTB–Gg	299.1^{d}	71.0^{h}	55^{e}	48.4^{e}	2816.3^{a}	11.2^{b}	1.1^{de}	19.7^{bc}	35.3^{d}
UPTR–ZTB_{RR4}	488.4^{b}	75.8^{b}	70.6^{a}	73.5^{b}	1560.4^{d}	13.0^{b}	4.56^{b}	12.1^{bc}	43.8^{b}
UPTR–ZTB_{RR6}	518.7^{a}	77.7^{a}	65.7^{b}	82.1^{a}	2145.4^{b}	15.5^{a}	6.4^{a}	20.3^{a}	49.2^{a}
PTR–RTB	156.2^{g}	75.1^{d}	70.3^{a}	41.8^{e}	2777.3^{a}	7.0^{c}	0.8^{e}	8.7^{c}	26.6^{e}
Contrast									
RB vs. RBG	0.0061	<0.0001	<0.0001	<0.0001	0.0058	0.0023	0.8291	0.2596	<0.0001
RTDSR vs. ZTDSR	0.2181	<0.0001	<0.0001	<0.0001	0.0194	0.7235	0.0036	0.2399	<0.0001
RTDSR vs. UPTR	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.2824	<0.0001
UTPR vs. PTR	<0.0001	0.0014	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0015	<0.0001
Mulch vs. anchored R	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0037	0.0271	<0.0001

FDA (μg fluorescein g $soil^{-1}$ h^{-1}), MBC (mg kg^{-1} soil), AP (μmol p-nitrophenol g^{-1} h^{-1}), T-GRSP (μg kg^{-1}), EG (μg g^{-1} soil), NR (μg ml^{-1} hr^{-1}), PO (units μg^{-1}), SR (mg CO_2-C g^{-1} soil $week^{-1}$), SR0 (mg CO_2-C g^{-1} soil $week^{-1}$). RTDSR–ZTB, reduced till direct seeded rice–zero till barley; RTDSR–ZTB-Gg, reduced till direct seeded rice–zero till barley–green gram; ZTDSR–ZTB–ZTGg, zero till direct seeded rice–zero till barley–zero till green gram; RTDSR–ZTB_{RR4}, reduced till–direct seeded rice–zero till barley + rice residue at 4 t ha^{-1}; RTDSR–ZTB_{RR6}, reduced till direct seeded rice–zero till barley + rice residue at 6 t ha^{-1}; UPTR–ZTB-Gg, un-puddled transplanted rice–zero till barley–green gram; UPTR–ZTB_{RR4}, un-puddled transplanted rice–zero till barley + rice residue 4 t ha^{-1}; UPTR-ZTB_{RR6}, un-puddled transplanted rice–zero till barley + rice residue 6 t ha^{-1}; PTR–RTB, puddled transplanted rice–reduced till barley; R, residue. Means with different small letters within column are significantly different ($p < 0.05$) using DUNCAN's Multiple Range Test.

preserving soil health with a substantial reduction in the cost of production and minimal environmental footprints (Johansen et al., 2012; Mishra et al., 2022). Different tillage and residue retention or incorporation practices in the rice–barley cropping system had a significant effect on the productivity and soil quality in the present experiment. The conventional practice of

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TABLE 4 Effect of tillage and residue management practices on soil microbial populations.

Treatments	BA	AC	FN
RTDSR–ZTB	93^{a}	50^{ab}	42.3^{bc}
RTDSR–ZTB–Gg	82^{abcd}	46^{abc}	41.3^{bc}
ZTDSR–ZTB–ZTGg	78^{d}	54.6^{a}	41.3^{bc}
RTDSR–ZTB_{RR4}	81^{bcd}	48.6^{ab}	41.3^{bc}
RTDSR–ZTB_{RR6}	81.3^{bcd}	46.6^{abc}	39.3^{c}
UPTR–ZTB–Gg	90.3^{abc}	51.33^{ab}	38^{c}
UPTR–ZTB_{RR4}	92.3^{ab}	43^{bc}	44^{b}
UPTR–ZTB_{RR6}	93.3^{a}	38.6^{c}	47^{a}
PTR–RTB	79^{cd}	48^{ab}	34.3^{d}
Contrast			
RB vs. RBG	0.2131	0.0241	0.2453
RTDSR vs. ZTDSR	0.122	0.0406	0.931
RTDSR vs. UPTR	0.0133	0.1142	0.0156
UTPR vs. PTR	0.0059	0.264	0.0009
Mulch vs. anchored R	0.6927	0.0053	0.8551

BA, Bacteria (CFU $g^{-1} \times 10^5$); AC, Actinobacteria (CFU $g^{-1} \times 10^4$); FN, Fungi (CFU $g^{-1} \times 10^3$). RTDSR–ZTB, reduced till direct seeded rice–zero till barley; RTDSR–ZTB–Gg, reduced till direct seeded rice–zero till barley–green gram; ZTDSR–ZTB–ZTGg, zero till direct seeded rice–zero till barley–zero till green gram; RTDSR–ZTB_{RR4}, reduced till–direct seeded rice–zero till barley + rice residue at 4 t ha^{-1}; RTDSR–ZTB_{RR6}, reduced till direct seeded rice–zero till barley + rice residue at 6 t ha^{-1}; UPTR–ZTB–Gg, un-puddled transplanted rice–zero till barley–green gram; UPTR–ZTB_{RR4}, un-puddled transplanted rice–zero till barley + rice residue 4 t ha^{-1}; UPTR–ZTB_{RR6}, un-puddled transplanted rice–zero till barley + rice residue 6 t ha^{-1}; PTR–RTB, puddled transplanted rice–reduced till barley; R, residue. Means with different small letters within same column are significantly different ($p < 0.05$) using DUNCAN's Multiple Range Test.

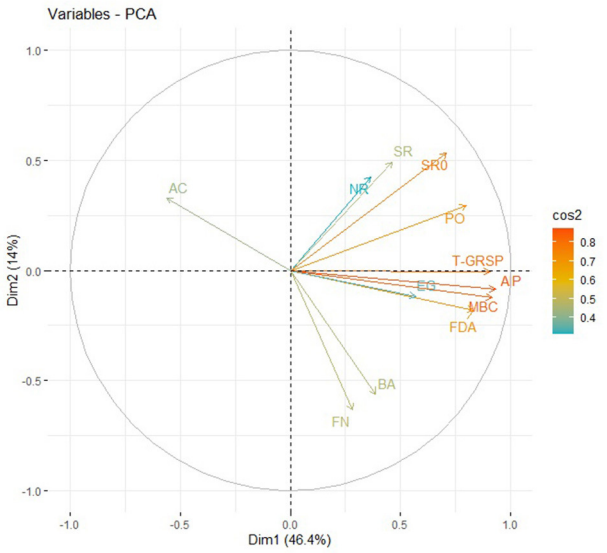

FIGURE 3
Graphic display biplot for soil biological attributes as influenced by tillage and residue management practices. BA, Bacteria; AC, Actinobacteria; FN, Fungi; NR, Nitrate reductase activity; SR0, Soil respiration; PO, Peroxidase; SR, Glucose-induced soil respiration; EG, Ergosterol; T-GSRP, Total glomalin-related soil proteins; FDA, Fluorescein diacetate hydrolysis; MBC, Microbial biomass carbon; AP, Alkaline phosphatase.

puddling and transplanting (puddled transplanted rice) continued to claim higher grain yield in our 5 years cropping cycle. Flooding of rice under puddled transplanting provides several congenial changes suitable for rice to grow faster and healthy. Some of these are suppression of weeds during early growth, availability of micronutrients (Fe and Zn), no infestation of nematodes, etc. (Balasubramanian and Hill, 2002; Kumar and Ladha, 2011; Ismail et al., 2012; Chaudhary et al., 2022). The lower yields in RTDSR and ZTDSR in our experiment are attributed to poor plant stand and infestation of weeds during early vegetative stages (data not presented). The transplanting of rice in un-puddled soil with reduced tillage (UPTR) gave a statistically equivalent yield to that of puddled transplanted rice without adversely affecting the yield of succeeding crop of barley with rice or mungbean residues. Therefore, the un-puddled transplanting of rice with two shallow tillage and direct rice transplanting in shallow water (2–3 cm depth) may be considered as minimum tillage when compared to conventional puddled rice, which involves deep tillage in a dry field, followed by repeated wet tillage for puddling (Haque et al., 2016).

The barley grain yield was significantly affected by the tillage practice of previous rice and residue treatments (Figure 1). The adverse effect of puddling in rice is obvious in barley grain yield, which was recorded as the lowest among all the practices. A number of adverse effects of puddling on the soil after rice such as destruction of soil structure and dispersion of clay, which creates a layer impermeable for root penetration, formation of sub-soil hardpan due to continuous tillage in wet soil, water logging in winter crop in the event of rains, etc., have been reported for lower yield of succeeding winter season crop (Kirchhof et al., 2000; Haque et al., 2016; Kalita et al., 2020; Kumar et al., 2023). The sowing of barley involving zero tillage (ZTB) without residue retention or mungbean residue incorporation was also found to be a poor performer in terms of barley grain yield. Barley yield was higher after RTDSR and ZTDSR practices than that of PTR and UPTR. The better soil conditions in reduced and zero tillage practices might have resulted in a higher yield of the subsequent barley crop. Furthermore, the green gram residue incorporation or rice residue retention substantially improved the grain yield of barley, showing a beneficial effect of conservation agriculture practices. Our results are in accordance with the earlier findings showing the additive effect of residue retention on yields of wheat under zero tillage (Yadvinder-Singh et al., 2004; Jat et al., 2014; Magar et al., 2022). The higher rice yield and comparable barley yield under UPTR–ZTB_{RR6} resulted in overall higher BEY *viś-a-viś* SYI of the rice–barley cropping system.

Soil biological parameters

The MBC is one of the most labile of the carbon pools comprising organic matter, which varies with different

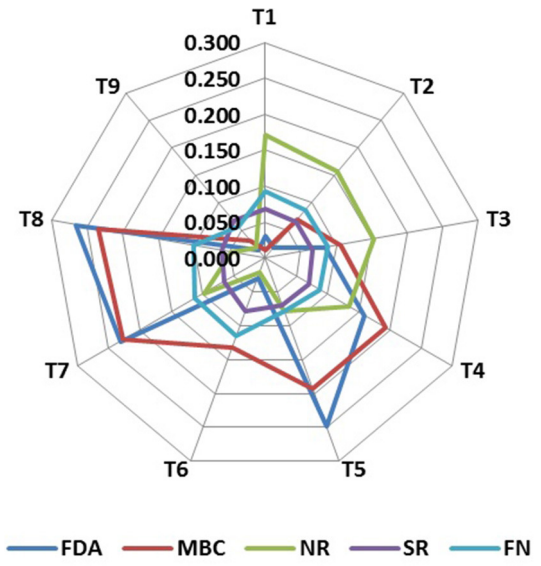

FIGURE 4
Radar graph depicting the average linear scores of key indicators as influenced by soil-nutrient management treatments. T1: reduced till direct seeded rice–zero till barley (RTDSR–ZTB); T2: reduced till direct seeded rice–zero till barley–green gram (RTDSR–ZTB–Gg); T3: zero till direct seeded rice–zero till barley–zero till green gram (ZTDSR–ZTB–ZTGg); T4: reduced till direct seeded rice–zero till barley + rice residue at 4 t/ha (RTDSR–ZTB_{RR4}); T5: reduced till direct seeded rice–zero till barley + rice residue at 6 t/ha (RTDSR–ZTB_{RR6}); T6: un-puddled transplanted rice–zero till barley–green gram (UPTR–ZTB–Gg); T7: un-puddled transplanted rice–zero till barley + rice residue 4 t/ha (UPTR–ZTB_{RR4}); T8: un-puddled transplanted rice–zero till barley + rice residue 6 t/ha (UPTR–ZTB_{RR6}); T9: puddled transplanted rice–reduced till barley (PTR–RTB). FDA, Fluorescein diacetate hydrolysis; MBC, Microbial biomass carbon; NR, Nitrate reductase activity; SR, Glucose-induced soil respiration; FN, Fungi.

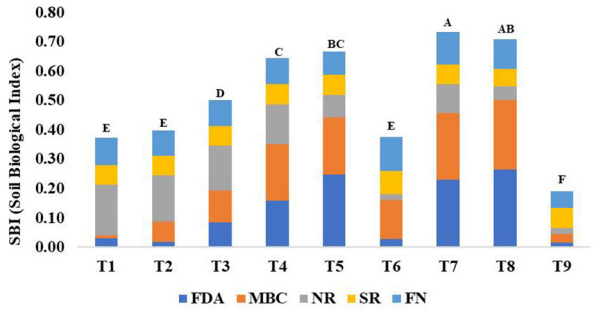

FIGURE 5
Effect of different tillage and residue management practices on soil biological index and the individual contribution of each of the key indicators. T1: reduced till direct seeded rice–zero till barley (RTDSR–ZTB), T2: reduced till direct seeded rice–zero till barley–green gram (RTDSR–ZTB–Gg), T3: zero till direct seeded rice–zero till barley–zero till green gram (ZTDSR–ZTB–ZTGg), T4: reduced till–direct seeded rice–zero till barley + rice residue at 4 t/ha (RTDSR–ZTB_{RR4}), T5: reduced till direct seeded rice–zero till barley + rice residue at 6 t/ha (RTDSR–ZTB_{RR6}), T6: un-puddled transplanted rice–zero till barley–green gram (UPTR–ZTB–Gg), T7: un-puddled transplanted rice–zero till barley + rice residue 4 t/ha (UPTR–ZTB_{RR4}), T8: un-puddled transplanted rice–zero till barley + rice residue 6 t/ha (UPTR–ZTB_{RR6}), T9: puddled transplanted rice–reduced till barley (PTR–RTB). Bars with different capital letters are significantly different ($p < 0.05$) using DUNCAN's multiple range test. FDA, Fluorescein diacetate hydrolysis; MBC, Microbial biomass Carbon; NR, Nitrate reductase activity; SR, Glucose-induced soil respiration; FN, Fungi.

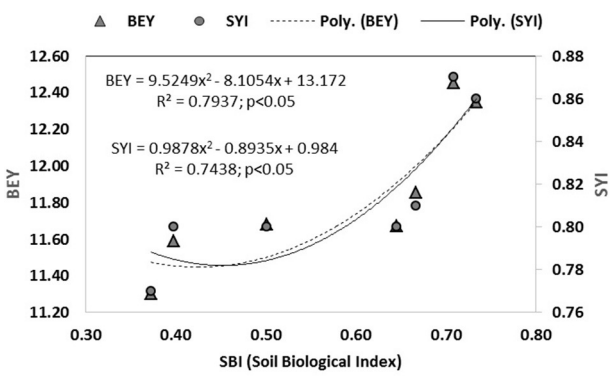

FIGURE 6
Relationship between soil biological index (SBI) with system productivity (BEY) and sustainable yield index (SYI) under tillage and residue management practices.

management practices (Zhang et al., 2021; Cordeiro et al., 2022). Nutrient availability potential represents an increase in MBC than an increase in total organic matter (Sharma et al., 2022b). In this study, residue incorporation enhanced the value of MBC, which aligns with other reports that state that organic sources like farmyard manure, vermicompost, and crop residues decompose slowly and result in the accumulation of organic carbon in soil (Singh et al., 2015, 2018). Several studies have also reported that tillage and crop rotation influence MBC (Zuber et al., 2018; Malobane et al., 2020; Nyambo et al., 2021; Saurabh et al., 2021). In this study, higher MBC in residue-retained treatments may also be due to that residue incorporation modifies microbial distribution in soil which encourages the proliferation of microbial population, supporting higher MBC (Li et al., 2018; Meena R.S et al., 2020). It is also reported that organic amendments stimulate microorganisms to produce enzymes related to the nitrogen and phosphorus cycles and soil organic carbon accumulation to boost soil microorganisms and enzyme activities (Rashid et al., 2016; Jacoby et al., 2017).

SR represents the value of CO_2 released from the soil due to the decomposition of organic matter present in the soil by microbes and the respiration of plant roots and other soil fauna. Soil respiration is very much sensitive to excessive tillage and incorporation of crop residues (Bhowmik et al., 2017; Usero et al., 2021). In this study, SR was also found to vary significantly ($p < 0.05$) in different treatments and was significantly ($p < 0.05$) high in zero till or reduced till plots. Likewise, treatments with residue retention also had high SR, which may be due to the enhancement of soil moisture because of residue retention (Kallenbach et al., 2019) and also due to the conversion of organic matter into available form of nutrients such as phosphate as PO_4, nitrate-nitrogen as NO_3, and sulfate (Fu et al., 2021).

Microbial enzymes represent soil quality as they facilitate diverse functions and are the most sensitive parameters, which change frequently with the physical and chemical properties of the soil (Burns et al., 2013). As reported earlier, fertilization and crop management practices significantly change soil microbial enzymes (Singh et al., 2015; Singh and Sharma, 2020). Crop residue-amended soils were better suited for microbial development, which may have enhanced nutrient mobilization and prevented the soil from fixing the available P (Sharma et al., 2022c). The fluctuations in enzyme activity could easily indicate the changes in the biological dynamics of the soil. Phosphatases in the soil serve important roles in the soil system and are an excellent indication of soil fertility by driving the mineralization of Po to accessible Pi (Saha et al., 2022). As a result of the long-term crop residue management techniques, the soil's microbial population and biomass C or N significantly increased, giving energy and an ideal environment for the development of soil enzymes (Xomphoutheb et al., 2020). In this study, alkaline phosphatase enzymes were found high in residue-incorporated treatments, which is in accordance with earlier studies (Gupta et al., 2022; Sharma et al., 2022a). However, NR is the enzyme that catalyzes the reduction of NO_3^- to NO_2^- in soil and also represents nitrogen immobilization (Canfield et al., 2010; Grzyb et al., 2021). In this study, the activity of NR enzymes was the highest in the treatment incorporated with green gram, which harbors rhizobium in the root nodules that convert atmospheric N_2 to NO_3^-. Hence, the presence of NO_3^- in the soil induces the NR activity of the treatment (Abbasifar et al., 2020). Peroxidase activity was the highest in the treatment incorporated with rice residue of 6 t/ha. Rice straw consists of lignocellulosic material that mainly consists of cellulose (24.0%), hemicelluloses (27.8%), and lignin (13.5%) (Chen et al., 2020). PO enzymes predominantly involve in carbon mineralization processes including humification and lignin degradation (Kumar and Chandra, 2020), and the presence of such a high concentration of lignocellulosic material in soil activates PO enzymes.

Repeated puddling adversely affects soil physical properties by dismantling soil aggregates, reducing permeability in subsurface layers, and forming hardpans at shallow depths. Hence, significantly ($p < 0.05$) higher microbial populations are found in un-puddled plots. Un-puddled conditions were therefore congenial for the proliferation of microbial population in soils, which may be directly linked with the maintenance of aggregates of soil particles (Bhowmik et al., 2017). The population of actinomycetes was the highest in green gram grown in crop rotation as it is a leguminous crop, and its rhizosphere harbors several types of microbes. Their roots secrete exudates encompassing easily available compounds, such as amino acids and carbohydrates, which can also stimulate the microbial population and their activity (Badri and Vivanco, 2009). It is possible that the stronger plant-root association also increased the microbial population, resulting in the use of more root exudate secreted by the plant (Rai et al., 2021; Chandra et al., 2022b). In no-till treatment, fungal biomass is higher than in tilled soils due to less breakage of hyphal networks and less damage to mycorrhizal associations (Schalamuk et al., 2004; Wilkes et al., 2021). The symbiotic association between microbes and roots is generally measured by ergosterol content as it is also called a biomarker for fungi. EG is mainly found in the fungal cell membrane, which controls the activity of membrane-bound enzymes and their permeability (Jordá and Puig, 2020). Similarly, T-GRSP is another biomarker for mycorrhizal associations with roots and is a glycoprotein produced by the hyphae of mycorrhiza, and it is mainly responsible for the formation of soil aggregates (Singh et al., 2015), which were found higher in the un-puddled treatment in the study. FDA hydrolysis enzyme activity corresponds to total microbial activity (Chandra et al., 2022a). FDA hydrolysis enzyme activity was also found to be positively correlated with MBC and AP (Supplementary Figure 2). As microbial population and soil enzymes were high in ZT, un-puddled and rice residue-incorporated treatments; hence, FDA activity was also the highest in these treatments.

Soil biological index

The principal component analysis indicated five sensitive biological parameters such as FDA, MBC, SR, NR, and FN (Figure 3). Among these, FDA and MBC had higher weightage in SBI (Figures 4, 5). FDA related to the total soil microbial activity can be considered an important indicator of soil microbial activity (Chandra et al., 2022a). MBC acts as a sink of soil nutrients and an important indicator of soil quality and biological activity (Srivastava et al., 2020). MBC is also indicative of soil organic matter changes due to soil management practices (Lal, 2020). There was significant variation in SBI under different tillage and residue management practices in the study. The highest SBI was found in the treatments with rice residue retention (Figure 5). These treatments increased the microbial population in the soil, which leads to an increase in the soil biological quality that can be monitored by the assessment of these five sensitive parameters. Agronomic management practices have a greater influence on soil microbial activities (Chen et al., 2021). Enhanced soil microbial activity and microbial populations under residues retained plots have led to improved SBI (Das et al., 2021). Un-puddled transplanted rice followed by zero till barley along with retention of rice residues improved the soil biological quality over the period of 5 years. The SBI values were validated against barley equivalent yield, and the sustainable yield index elaborated that higher SBI was associated with better system productivity and sustainability of rice–barley cropping system (Figure 6).

Conclusion

The adoption of reduced or no tillage in the short term reduced the grain yield of basmati rice more than that of puddled transplanting. The barley yield under zero tillage improved significantly with the incorporation of green gram residue or retention of 4–6 t/ha rice residue. Based on the results of the study, the partial conservation agriculture practice of un-puddled transplanted rice with reduced tillage followed by zero tilled barley and retention of 4–6 t/ha rice residues (UPTR–ZTB $_{RR6}$ or UPTR–ZTB$_{RR4}$) provided the highest system productivity (12.45 t/ha and 12.35 t/ha), sustainable yield index (0.87 and 0.86), and improved soil biological health as evidenced by the highest soil biological index (0.71 and 0.73). For farmers using rice residues for other

purposes, green gram residue incorporation in the UTPR–ZTB may be adopted as it also provided statistically similar system productivity as that of UPTR–ZTB_{RR6}. This study is a step forward toward a better understanding of the impact of tillage and residue management practices on soil biological and biochemical properties under the rice–barley cropping system, and long-term studies are needed for providing more reliable and predictable indicators to monitor the sustainability of semi-arid soils.

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

Conceptualization: PC and AK. Methodology: PC and GeS. Formal analysis: AB, KP, and AR. Writing—original draft preparation: PC and KP. Writing—review and editing: AK, OA, RV, and KK. Editing and supervision: GyS. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1130397/full#supplementary-material

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EDITED BY
Priyanka Chandra,
Central Soil Salinity Research Institute (ICAR),
India

REVIEWED BY
Kailash Prajapat,
Central Soil Salinity Research Institute (ICAR),
India
Evans N. Nyaboga,
University of Nairobi,
Kenya

*CORRESPONDENCE
Henda Mahmoudi
✉ hmj@biosaline.org.ae

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Differential responses in some quinoa genotypes of a consortium of beneficial endophytic bacteria against bacterial leaf spot disease

Ayman Badran[1], Nerhan A. Eid[2], Amr R. Hassan[1] and Henda Mahmoudi[3]*

[1]Department of Genetic Resources, Desert Research Center, Cairo, Egypt, [2]Department of Plant Protection, Desert Research Center, Cairo, Egypt, [3]Directorate of Programs, International Center for Biosaline Agriculture (ICBA), Dubai, United Arab Emirates

Many effective plant-microbe interactions lead to biological changes that can stimulate plant growth and production. This study evaluated the effect of the interaction between quinoa (*Chenopodium quinoa* Willd.) and endophytic bacterial strains on differential responses under biotic stress. Four strains of endophytic bacteria were used to inoculate three quinoa genotypes. Endophytic bacteria, isolated from the endosphere of healthy genotypes of quinoa plants, were used to evaluate their biocontrol activity against *Pseudomonas syringae* on quinoa plants, which causes leaf spot disease, depending on some different parameters. Quinoa genotype plants were treated with four treatments: pathogenic bacteria only (T1), internal bacteria only (T2), pathogenic bacteria + endogenous bacteria (T3), and untreated as the control (T4). The results indicated that there was a significant difference between chlorophyll content index of infected plants without bioagent (untreated) compared to plants bio-inoculated with endophytic bacteria. The highest mean disease incidence was on the plants without bacterial inoculum (90, 80, and 100%) for quinoa genotypes G1, G2, and G3, respectively. The results showed that there were significant differences in the weight of grains/plant, as the value ranged from 8.1 to 13.3g when treated with pathogens (T1) compared to the treatment with pathogens and endogenous bacteria (T3), which ranged from 11.7 to 18.6g/plant. Decreases in total aromatic amino acids appeared due to the pathogen infection, by 6.3, 22.8, and 24.1% (compared to the control) in G1, G2, and G3, respectively. On the other hand, genotype G3 showed the highest response in the levels of total aromatic and total neutral amino acids. The endophytic strains promoted quinoa seedling growth mainly by improving nutrient efficiency. This improvement could not be explained by their ability to induce the production of amino acids, showing that complex interactions might be associated with enhancement of quinoa seedling performance by endophytic bacteria. The endophytic bacterial strains were able to reduce the severity of bacterial leaf spot disease by 30, 40, and 50% in quinoa genotypes G1, G2, and G3, respectively, recording significant differences compared to the negative control. The results indicated that, G1 genotype was superior in different performance indicators (pathogen tolerance index, yield injury %, superiority measure and relative performance) for grain weight/plant under pathogen infection condition when treated with endophyte bacteria. Based on this study, these bacterial strains can be used as a biotechnology tool in quinoa seedling production and biocontrol to diminish the severity of bacterial leaf spot disease.

KEYWORDS

***Chenopodium quinoa*, endogenous bacteria, *Pseudomonas syringae*, amino acids, PGPB**

1. Introduction

Climate change-related land desertification and salinization are increasing at a worrying speed, driving the demand for novel crop cultivation concepts to ensure food security. One prime candidate is the pseudocereala quinoa (*Chenopodium quinoa* Willd.), whose seed has an outstanding nutritional value (Vega-Galvez et al., 2010; Tabatabaei et al., 2022). In 2020, world production of quinoa was 175,188 tons, led by Peru and Bolivia with 97% of the total when combined (FAOSTAT, 2020). Quinoa plants are infected with many bacterial and fungal plant diseases (Li et al., 2017). Fonseca-Guerra et al. (2021) isolated *Pseudomonas* sp. from quinoa leaves; its symptoms appeared in the form of dark brown spots and apical necrosis, thus causing significant damage in crops. The large microbial diversity necessitates the search for different, more efficient bacterial strains to enhance plant capability to colonize quinoa roots (Labanca et al., 2020). Endogenous bacteria decrease the severity of the bacterial pathogen, reduce the rate of disease, and thus diminish the economic loss of the crop.

Bacterial diseases are among the most critical pathogens that may lead to great collapse in the quinoa crop and affect the quantity and quality of the crop. Bacterial leaf spot is remarkable disease of foliage plants as well as vegetables plants. These bacterial diseases may molder leaves, petioles and stems rendering infected plants unsightly and unsalable. The disease is characterized by circular, gray to black, water-soaked lesions on the leaves. Symptoms on leaves included leaf blight and white and brown spots on the leaf surface (Myung et al., 2011). Eid et al. (2019) evaluated leaf spot disease and isolate *pseudomonas* as causative agent from different plants in Egypt.

Bacterial leaf spot disease is important disease of foliage and flowering ornamental plants as well as vegetables plants. This bacterial disease may destroy leaves, petioles and stems rendering infected plants unsightly and unsalable. The disease is characterized by circular, gray to black, water-soaked lesions on the leaves. The lesions coalesce, become irregular in shape, dry and dark brown with age (Burgess et al., 1986). Among them, bacterial spot is the most problematic devastating disease in most of the tomato growing regions around the world (Sharma and Bhattarai, 2019). The incidence of bacterial disease ranged from 48 to 95% on lettuce in Turkey (Ozyilmaz and Benlioglu, 2018). Bacterial leaf spot of onion (*Allium cepa* L.) was observed in fields of Korea with incidence varying from 95 to 100%. Symptoms on leaves included leaf blight and white and brown spots on the leaf surface (Myung et al., 2011).

One of the biggest challenges in quinoa production is maintaining high productivity and minimizing the harmful environmental effects of low fertilization efficiency and long-term use of the soil (Cárdenas-Castillo et al., 2021). The identification of beneficial microorganisms that can improve agricultural production is one of the solutions. In this context, inoculants based on endophytic bacterial strains suggest a sustainable alternative.

Quinoa seedling preparation, one of the main stages of its production, is conducted in nurseries. These conditions can favor beneficial bacterial inoculation *via* micropropagation, which improves crop production at the early stage.

Crop protection is still largely associated with the use of chemical products, despite its negative effects on the environment (Pilet-Nayel et al., 2017). Therefore, the gradual integration of new practices must take into account the environmental and social dimension (Purvis et al., 2019). The continuous growth in productivity and international trade leads to Increased incidence of some diseases, which resulted in the use of more pesticides, which in turn increases environmental pollution (Pilet-Nayel et al., 2017). Therefore, the use of microorganisms is a possible way to reduce pollution and inconveniences associated with the use of synthetic chemicals and reduce their negative impact on the environment clearly (Compant et al., 2005). So, it has become evident the importance of using multiple economic approaches to control plant pathogens while preserving the environment.

Throughout their evolution, plants have developed a complex set of mechanisms for environmental adaptation. One mechanism is association with beneficial microorganisms, such as endophytic and rhizosphere bacteria known as plant growth-promoting bacteria (PGPB). PGPB have been explored as pathogen antagonists and bio-stimulants of plant growth, suggesting an ecofriendly alternative to pesticides and chemical fertilizers in sustainable agriculture (Olanrewaju et al., 2017). The plant-bacteria interaction, through a complex array of mechanisms, can result in plant growth promotion due to increased nutrient uptake, nitrogen fixation, or phytohormone production, or indirectly due to phytopathogen suppression (Berg, 2009). Plant receptors of bacterial signals are known to recognize phytopathogenic bacteria or be involved in the identification of beneficial microorganisms by plants (Carvalho et al., 2016). Likewise, the role of free amino acids and polyamines has been shown to alter during the interaction between plants and microorganisms. Both plant-phytopathogenic bacteria and plant-beneficial microorganism interaction could result in significant changes in polyamine metabolism of the host and/or microbe partners, thus revealing this to be a complex and dynamic process (Terra et al., 2019).

Plant genetic factors might contribute to the increased efficiency of plant-bacteria interaction, causing plant physiological changes that culminate in the modulation of plant growth and development (Carvalho et al., 2016). The endophytic bacterial strains with different characteristics differently alter the plant response regarding increasing amino acid content. To test this hypothesis, we designed an experiment to evaluate whether quinoa seedlings inoculated with endophytic bacteria could induce pathogen resistance reactions and reduce the need for chemical fertilization. Increasing environmental concerns and the search for a more sustainable agriculture have led research to intensify the development of biofertilizers. However, the correct and efficient use of microorganisms as inoculants requires more knowledge about their benefits and impacts. Thus, the objective of this study was to evaluate the effect of consortium of bacterial inoculants on the development of quinoa plants and resistance to bacterial leaf spot disease using the physiological and biochemical aspects of plant-bacteria interaction.

2. Materials and methods

2.1. Tested genotypes

Three genotypes of quinoa were selected among a large group of genetic material provided by the International Center for Biosaline Agriculture (ICBA): genotype G1 (CO-KA-1873), genotype G2 (CO-KA-2300), and genotype G3 (CO-KA-1901).

2.2. Isolation of endophytic bacterial strains

Four endophytic bacterial strains were isolated from the endosphere of healthy genotypes of quinoa plants, QF3 and QF4 were isolated from quinoa genotype (CO-KA 1982), QC5 was isolated from quinoa genotype 132 (D 12123) and QD4 was isolated from quinoa genotype 64 (CO-KA 1830). The age of the plants used for isolation was 60 days. For isolation of endophytic bacteria, the quinoa roots, stems and leaves (1 cm pieces) were washed by running tap water with two drops of tween 20 (as wetting agent) then surface sterilized by ethanol 70% for 2 min. Followed by sodium hypochlorite 2% for 1 min. After that, the samples were washed in sterile distilled water for 3 times, drying on sterilized filter paper under aseptic conditions (laminar air flow). The last washing distilled water was plated onto nutrient agar medium for 48 h, incubated at 28 ± 2C° to confirm that the surface of plant pieces was efficacious purified the parts of sterilized roots, stems, and leaves were macerated in 5 ml of 12.5 mM potassium phosphate buffer (pH 7.0) with sterile mortar and pestle. Tissue extracts were then sequent diluted in potassium phosphate buffer (pH 7.0) and plated on tryptic soy agar (TSA) plates. Incubation was carried out at 28°C for 1–7 days to allow growth of endophytic bacteria. The plates were incubated at 28 ± 2°C for 1–7 days or pending growth was observed (Das et al., 2007).

2.3. Characterization of endophytic bacterial strains

Four endophytic bacterial strains were isolated from the endosphere of healthy genotypes of quinoa plants and characterized by their antagonistic activities against *Pseudomonas syringeae*, bacterial strains incubated at 28–30°C for 4 days. To determine production of indole-3-acetic acid (IAA) according to Bric et al. (1991), Cell free culture filtrate was gained by centrifuging 10 ml culture at 10000 rpm for 20 min at 4°C., The amount of gibberellic acid (GA3) in the ethyl acetate phase was determined by the UV spectrophotometer at 254 nm versus control blank (Mitter et al., 2002), % antioxidant (Burits and Bucar, 2000), total phenols (Kumar and Min, 2011), hydrogen cyanide production, the filter padding in each tube was soaked with 2 ml of sterile picric acid solution (Picric acid 2.5 g/l + Na_2Co_3 12.5 g/l) under aseptic condition and the lids were closed. The tubes were sealed with parafilm in order to contain gaseous metabolites produced by the antagonists and to allow for chemical reaction with picric acid present in the filter paper padding. Wei et al. (1991), and siderophore production according to Alexander and Zuberer (1991), standard solution of deferoxamin mesylate (DFOM) with concentrations (15,30,45,60,90,105,120 and 135 mM) was prepared to calibrate the assay of the collected samples. A standard curve for the modified Chrome Azurol S (CAS) assay was prepared by analyzing the absorbance (630 nm) of each standard solution. The concentration of siderophores in the culture filtrate was measured by modified CAS assay method.

2.4. Evaluation of the antagonistic interaction among selected isolates of endophytic bacterial strains

The inhibitory effect of the selected isolates was measured based on the formation of an inhibition zone around the organism on King's medium agar plate. The test was carried out among selected strains according to Berga et al. (2001).

2.5. Identification of endophytic bacterial strains

The BLAST database of the National Center for Biotechnology Information was used to compare the resolved sequences of the most efficient bacterial isolates with known 16S rDNA sequences (Altschul et al., 1997). Determination of phylogenetic relationships was analyzed by the program Phylogenetic Analysis CLC Genomics Workbench version 4.5.1 (Vinnere et al., 2002).

The most efficient antagonistic isolates were identified by 16S rRNA sequence. Isolation of cellular DNA was performed as described by Ausubell et al. (1987) and amplification of 16S rDNA according to Lane et al. (1985) using the universal 16S primers [F1 5′ AGAGTTT(G/C) ATCCTGGCTCAG 3′ R1 5' ACG(G/C) TACCTTGTTACGACTT 3′]. PCR was run on a Gene Amp PCR System 2400 thermal cycler (Perkin Elmer) and then DNA was amplified according to Lane et al. (1985). The resulting PCR product sizes ranged from 1450 to 1500 bp. The PCR products were purified using QIA Quick PCR Purification Kit (Qiagen). The sequencing was performed in two directions using the previously described primers (Lane et al., 1985) in GATC Company (Germany). Sequencing data were analyzed by two different computer alignment programs, DNAStar (DNASTAR, Inc., United States) and Sequence Navigator (Perkin Corp., United States).

2.6. Evaluation of endophytic bacteria against bacterial leaf spot of quinoa *in vivo*

A greenhouse trial was conducted during 2021 in Al-Ismailia Governorate, Egypt, using mixed culture of the most efficient endophytic bacterial strains: *Pseudomonas taiwanensis* QF3, *Bacillus velezensis* QC5, *Bacillus subtilis* QF4, and *Pseudomonas putida* QD4.

The response of the three quinoa genotypes to the previous four treatments was evaluated in a randomized complete block design using three replicates.

2.6.1. Inoculum preparation of antagonistic bacteria

The antagonistic bacteria were inoculated in nutrient broth medium (individually) and inoculated at 28 ± 2°C for 2 days. After the incubation period, the bacterial cultures were mixed. The cell density of the mixed culture was ~10^9 cfu/ml to use as a standard inoculum.

2.6.2. Inoculum preparation of pathogenic bacteria

The 48-h-old culture of *Pseudomonas syringae* was inoculated in nutrient broth medium individually for 48 h at 28 ± 2°C. The optical

density (OD) was adjusted at ~10^6 cfu/mL for the inoculum to be used as a standard inoculum.

2.6.3. Inoculation treatments

Our study was conducted to investigate the effect of antagonistic endophytic bacterial strains on the severity of bacterial leaf spot disease. The seeds were soaked in the mixed culture of antagonistic bacteria and then cultivated in sapling trays. After 2 weeks, the seedlings were transferred to pots and the inoculation treatments were applied as follows:

Quinoa genotype plants treated using pathogenic bacteria only (T1), plants treated using endogenous bacteria only (T2), plants treated using pathogenic bacteria + endogenous bacteria (T3), and sterile media was used in untreated plants. Untreated quinoa plants as the control (T4).

2.6.4. Antagonistic bacterial treatments

Five mL of mixed liquid culture consisting of 5 g peptone, 3 g beef extract, and 10 g glucose of four isolated endophytic bacteria were added to the pots by supplementing to the soil in a 2-cm hole beside the seedlings after transplanting.

2.6.5. Pathogenic bacterial treatment

The pathogenic bacterium *Pseudomonas syringae* was used as foliar application on the seedlings after 48 h of antagonistic bacterial treatment.

Re-inoculation of the antagonistic bacteria was carried out for quinoa plants (1 month after transplanting). Incidences of leaf spot disease were recorded after 10 days of infection according to Li et al. (2008).

2.6.6. Compatibility test between the selected antagonists *in vitro*

Cross interaction between selected bacterial isolates (QF3, QC5, QF4, and QD4) was examined for each other to know whether any antibiosis was presented among them. We concluded that no antibiosis was noticed for any combination of tested isolates.

2.7. Growth parameters

2.7.1. Vegetative growth and agronomic characteristics

Sixty days after planting, vegetative growth parameters such as plant height (cm), fresh and dry weight of shoot (g/plant), leaf area (cm^2), and chlorophyll content index were estimated, three CCI readings per leaf, including one reading around the midpoint of leaf blade and two readings 3 cm apart from midpoint. Chlorophyll was measured using chlorophyll Meter SPAD-502 (MINOLTA C., LTD JAPAN 78923067). After harvesting, grain yield/plant and 100-grain weight (g) were estimated.

2.7.2. Determination of disease incidence

The disease incidence percentage was calculated by using the following formula:

Disease incidence (%) = (Number of diseased leaves in treatment/Total number of leaves/treatment) × 100.

2.7.3. Determination of amino acids

The determination of free amino acids was performed by ultra-performance liquid chromatography mass spectrometry (UPLC-MS). Samples of young leaves were collected, macerated in liquid nitrogen, and lyophilized. Extraction was performed from 100 mg of lyophilized material, homogenized with 1 ml of methanol water (80,20, v:v) in an ultrasonic bath at 30°C for 15 min, centrifuged at 10,000 rpm for 5 min, and the supernatant collected for analysis. The extracted samples were analyzed directly on an Acquity UPLC-MS (QTOF, Micromass-Waters, Manchester, United Kingdom). The chromatographic separation was done in a Waters Acquity C18 BEH analytical column (150 mm × 2.1 mm i.d, 1.7 μm) according to Tezotto et al. (2016). Output data were obtained in the range of 50 at 300 m/z. For quantification of amino acids, calibration curves were produced by the injection of a known concentration of the standards. In our investigation free amino acids in leaves were quantified to monitor the changes due to plant-pathogen interaction. Such infection, with prolonged exposure, may result in changes in amino acids profile in the seeds, particularly in terms of storage protein quantity. However, seed protein, and amino acids profile were not examined in our project.

2.7.4. Tolerance indices of tested genotypes against pathogen

Tolerance Indices of tested genotypes against pathogen were calculated as follow:

(1) Pathogen tolerance index (PTI): PTI = (Yp) × (Yd)/(Ýp)2 according to Fernandez (1992).
(2) Yield injury % (YI): YI = (Yp-Yd)/Yp × 100 according to Blum et al. (1983).
(3) Superiority measure (SM): SM = Yd/Yp according to Lin and Binns (1988).
(4) Relative performance (RP): RP = (Yd/Yp)/R according to Abo-Elwafa and Bakheit (1999).

Where, Yp = grain weight/plant using pathogen & endophytic bacteria treatment (T3); Yd = grain weight/plant using pathogen (T1); Ýp = Mean grain weight/plant of all genotypes using pathogen & endophytic bacteria treatment (T3); Ýd = Mean grain weight/plant of all using pathogen; R = (Ýd/Ýp).

2.8. Statistical analysis

Statistical significance was calculated using the analysis of variance procedure in SAS program (version 9.0). Differences between the means of tested genotypes in treatments were separated by the least significant difference (LSD) test at the 0.05 *P* level.

3. Results and discussion

3.1. Identification of antagonistic bacteria by molecular analysis

The most potent antagonistic bacteria were identified by amplifying and sequencing the 16S rDNA using techniques of Sigma

Scientific Services. The results showed that the 16S rDNA sequence of QF3, QC5, QD4, and QF4 isolates had 99% identity with *Pseudomonas taiwanensis* strain Pst1 (OP984768), *Bacillus velezensis* strain Bv1 (OP984765), *Pseudomonas putida* strain Psp1 (OP984769), and *Bacillus subtilis* strain Bs1) OP984766), respectively. Construction of a phylogenetic tree based on comparative analysis of the 16S rRNA genes was performed with the use of various algorithms implemented in CLC Genomics Workbench version 4.5.1. The phylogenetic analysis based on 16S rRNA gene sequences indicated that strains QF3, QC5, QD4, and QF4 formed a phyletic lineage within *Pseudomonas taiwanensis* strain Pst1 (OP984768), *Bacillus velezensis* strain Bv1 (OP984765), *Pseudomonas putida* strain Psp1 (OP984769), and *Bacillus subtilis* strain Bs1)OP984766), respectively (Figures 1–4).

3.2. Performance of endophytic bacteria upon different genotypes of quinoa

3.2.1. Vegetative and agronomic characteristics

The effects of plant growth-promoting endophytic bacteria (PGPEB) as bioagents in quinoa plants in the presence of phytopathogenic bacteria are presented in Tables 1–4.

The plants that were inoculated with bioagents showed a positive effect on growth parameters. The data indicated that the inoculation treatments with biocontrol agents had the highest significant plant growth in the presence of pathogenic bacteria compared with the control treatments. Quinoa plant bacterization with endophytic bacteria registered a highly significant increase in shoot and root length (cm), fresh and dry weight of shoot (g/plant), fresh and dry weight of root (g/plant), leaf area (cm), and 1,000-grain weight (g). This means that the biocontrol agents suppress the activity of *Pseudomonas syringae* as well as stimulate the growth of quinoa plants.

Infected plants without bioagent showed a significant reduction in plant growth traits (i.e., shoot and root length (cm), fresh and dry weight of shoot (g/plant), fresh and dry weight of root (g/plant), leaf area (cm), and 100-grain weight (g)). The highest significant increase in growth traits was observed with the endophytic bacterial treatment. The increment in previously mentioned attributes for endophytic bacteria-treated plants was particularly significant in most cases vis-à-vis all other treatments. Endophytic bacteria play an essential role in improving crop growth during biotic stress conditions. The endophytic bacterial treatment increased shoot and root length (cm), fresh and dry weight of shoot (g/plant), fresh and dry weight of root (g/plant), leaf area (cm^2), and 1,000-grain weight (g) of quinoa plants compared with un-inoculated plants in the presence of phytopathogenic bacteria. The fresh weight of roots was determined to give 1.1, 1.2, and 1.0 g/plant for quinoa genotypes G1, G2, and G3, respectively, with endophytic bacterial inoculum, against 0.3, 0.6, and 0.6 g/plant for quinoa genotypes G1, G2, and G3, respectively, with un-inoculated plants in the presence of phytopathogenic bacteria. Similar results were observed by Mahdi et al. (2020), who found that *Bacillus licheniformis* QA1 and *Enterobacter asburiae* QF11 treatment significantly increased the fresh and dry yield and plant height of quinoa plants.

3.2.2. Photosynthetic pigments

Chlorophylls are considered the main photosynthetic pigments that harvest light energy used to start photosynthesis processes. Such processes are the main motive force for plant growth and development. The total chlorophyll content index (CCI) of fresh leaves of quinoa plants is shown in Figure 5 for each genotype. The CCI of infected plants without bioagent (untreated) was significantly less than that of plants bio-inoculated with endophytic bacteria. Decreases in photosynthetic pigments might be due to the destruction of chlorophyll by increased activity of chlorophyll-degrading enzymes, which results in leaf necrotic areas under stress conditions. The highest significant values of CCI were recoded with endophytic bacterial inoculum treatment. At the cellular level, the bacterial infection causes oxidative damage resulting from higher levels of reactive oxygen species (ROS), which negatively affect photosystem integrity and decrease chlorophyll content, thus affecting the development and productivity of the plant (Osdaghi et al., 2021). A

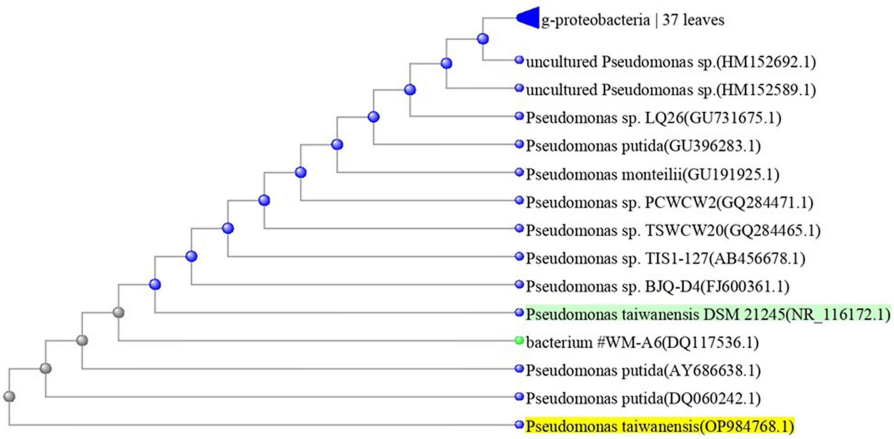

FIGURE 1
Overview of phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between isolate QF3 and related taxa, which had high identity with *Pseudomonas taiwanensis* strain Pst1 (OP984768). Unrooted phylogenetic tree of isolate QF3 rDNA seq. This topology was obtained using all known complete or almost complete sequences from *Pseudomonas* strains and the percentage values indicating the results of a bootstrap analysis (500 replications, values above 50%) and maximum-parsimony analyzes are shown by $p<0.01$.

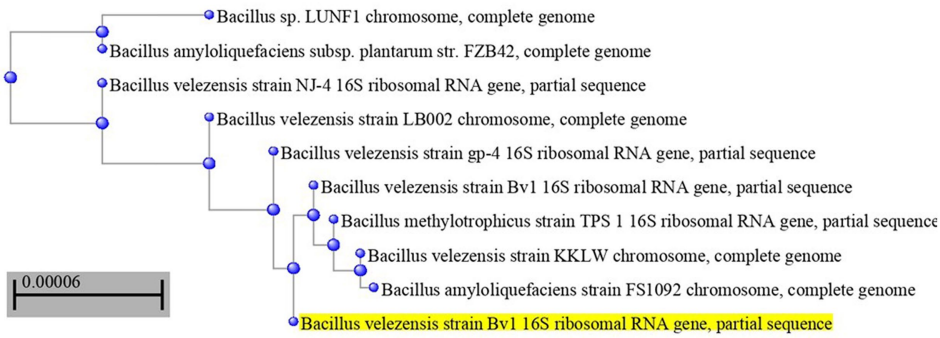

FIGURE 2
Overview of phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between isolate QC5 and related taxa, which had high identity with *Bacillus velezensis* strain Bv1 (OP984765). The tree was reconstructed from the core genomes of type strains of species from the *Bacillus* group (350 genes). Bootstrap values >50%, based on 1,500 replicates, are indicated on branch points.

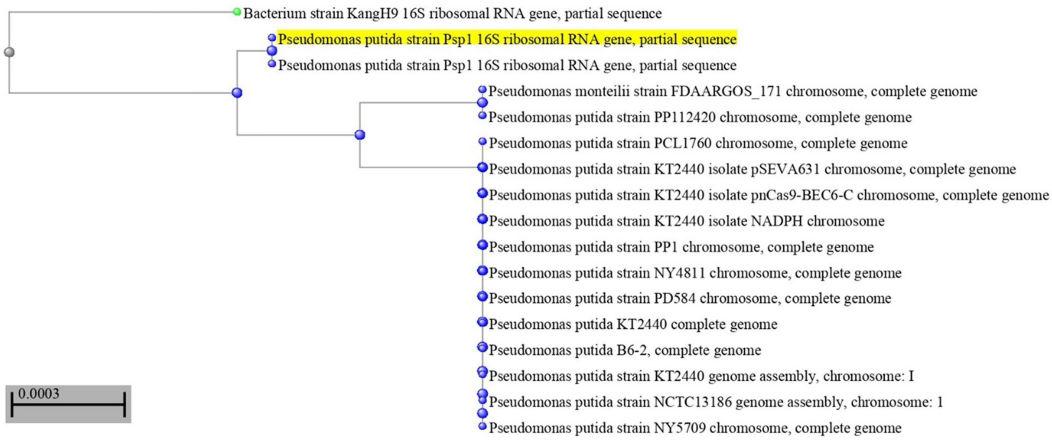

FIGURE 3
Overview of phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between isolate QD4 and related taxa, which had high identity with *Pseudomonas putida* strain Psp1 (OP984769). The percentage of trees in which the associated taxa clustered in the bootstrap test (1,000 replicates) is shown next to the branches.

similar result was reported by Mahdi et al. (2020), who observed that inoculation with *Bacillus licheniformis* QA1 significantly increased the leaf CCI in quinoa plants.

3.3. Leaf spot disease expression

Disease incidence in different genotypes of quinoa plants with different treatments is presented in Figure 6. The typical symptoms of leaf spot were developed on the leaves 10 days after inoculation. The averages of disease incidence with endophytic bacteria as bioagent were 30, 40, and 50% for quinoa genotypes G1, G2, and G3, respectively, and there was a significant difference compared with the control treatment (five plants as replicates). The highest mean disease incidence was on the plants without bacterial inoculum (90, 80, and 100%) for quinoa genotypes G1, G2, and G3, respectively, whereas the results indicated that the mixture of bacteria improved the induced plant resistance against the pathogenic bacteria. Backer et al. (2018) reported similar results. Some bacteria support plant growth indirectly by improving growth-restricting conditions either *via* the production of antagonistic substances or by inducing host resistance toward plant pathogens. Since associative interactions of plant and microorganisms must have come into existence because of convolution, the use of bioinoculants forms one of the vital components for a long-term sustainable agricultural system (Backer et al., 2018). Le et al. (2020) stated that the application of *Paenibacillus elgii* JCK-5075 caused effective suppression of the development of red pepper bacterial leaf spot in pot experiments with control values of 67%. According to Lalitha (2017), various plant growth-promoting rhizobacteria bring about induced systemic resistance (ISR) and therefore provide resistance against plant pathogens. ISR was reported to be associated with many defense enzymes, including ascorbate peroxidase (APX), β1,3-glucanase, catalase (CAT), chitins, lipoxygenase (LOX), peroxidase (PO), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), proteinase inhibitors, and superoxide dismutase (SOD).

Our study recognized the endophytic bacteria *Pseudomonas taiwanensis* QF3, *Bacillus velezensis* QC5, *Bacillus subtilis* QF4, and *Pseudomonas putida* QD4 as suitable inoculants for quinoa cultivation

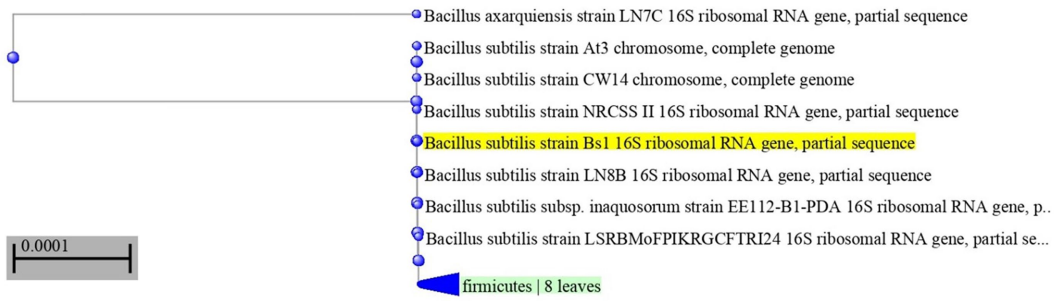

FIGURE 4
Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between isolate QF4 and related taxa, which had high identity with *Bacillus subtilis* strain Bs1 (OP984766). Phylogenetic tree of 16S rRNA sequences from endophytic bacterium strain QF4 compared with representative members of *Bacillus* genus with more than 98% identity.

TABLE 1 Effects of endophytic bacteria on root length, shoot length, and leaf area of the different quinoa genotypes.

Treatment	Shoot length (cm)			Root length (cm)			Leaf area (cm^2)		
	G1	G2	G3	G1	G2	G3	G1	G2	G3
Pathogen	20.3	24.0	22.0	11.03	11.16	10.66	10	9	10
Endophytic bacteria	39.3	42.0	39.6	16.26	16.23	15.20	19	19	21
Pathogen and endophytic bacteria	30.3	28.6	28.3	13.90	14.10	14.03	17	17	19
Control	28.0	29.0	27.3	11.73	12.30	12.30	14	15	14
LSD $_{(0.05)}$	1.82			4.47			1.46		

LSD = least significant difference.

TABLE 2 Effects of endophytic bacteria on shoot fresh and dry weight of the different genotypes of quinoa plants.

Treatment	Shoot fresh weight (g)			Shoot dry weight (g)		
	G1	G2	G3	G1	G2	G3
Pathogen	5.5	5.2	6.1	0.6	0.8	1.0
Endophytic bacteria	11.6	10.0	10.2	1.4	1.6	1.3
Pathogen and endophytic bacteria	8.5	7.9	8.2	1.2	1.2	1.2
Control	7.9	7.9	7.3	1.0	1.0	1.1
LSD $_{(0.05)}$		1.01			0.65	

LSD = least significant difference.

TABLE 3 Effects of endophytic bacteria on root fresh and dry weight of the different genotypes of quinoa plants.

Treatment	Root fresh weight (g)			Root dry weight (g)		
	G1	G2	G3	G1	G2	G3
Pathogen	0.3	0.6	0.6	0.1	0.1	0.1
Endophytic bacteria	1.1	1.2	1.0	0.9	1.1	0.9
Pathogen and endophytic bacteria	0.6	0.9	0.8	0.5	0.6	0.5
Control	0.8	0.7	0.8	0.4	0.4	0.4
LSD $_{(0.05)}$	0.23				0.02	

LSD = least significant difference.

with potential for alleviating biotic stress in quinoa plantlets. These bacteria possess desirable characteristics of PGPB, such as antagonistic activities against phytopathogens, siderophores, auxins, and cyanide production.

These obtained results are in harmony with those obtained by many investigators such as Cipriano et al. (2021), who confirmed that using endophytic bacteria promoted sugarcane seedling growth mainly by improving nutrient efficiency. Previously, numerous reports studied

TABLE 4 Effects of endophytic bacteria on 1,000-grain weight and grain weight/plant of some different quinoa genotypes.

Treatment	1,000-grain weight (g)			Grain weight/plant (g)		
	G1	G2	G3	G1	G2	G3
Pathogen	1.00	1.11	1.01	13.3	8.1	11.2
Endophytic bacteria	2.90	3.10	3.00	19.0	13.0	18.3
Pathogen and endophytic bacteria	1.90	1.70	1.70	18.6	11.7	17.4
Control	2.10	1.49	2.01	15.1	10.3	14.3
LSD $_{(0.05)}$	0.122			0.670		

LSD = least significant difference.

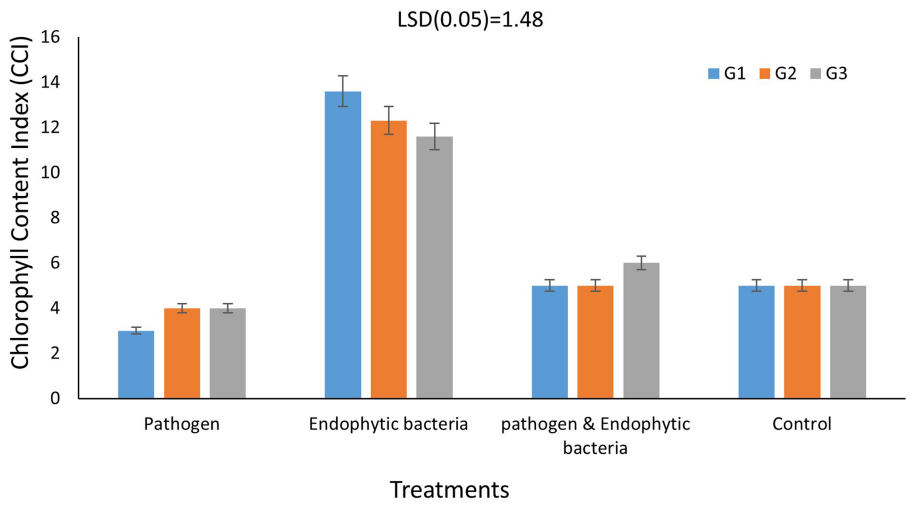

FIGURE 5
Effects of endophytic bacteria on chlorophyll content index of different genotypes of quinoa (G1, G2, G3). Data are means of three replicates. Differences between the means of tested genotypes under treatments were differentiated by least significant difference (LSD) test at the 0.05 *P* level.

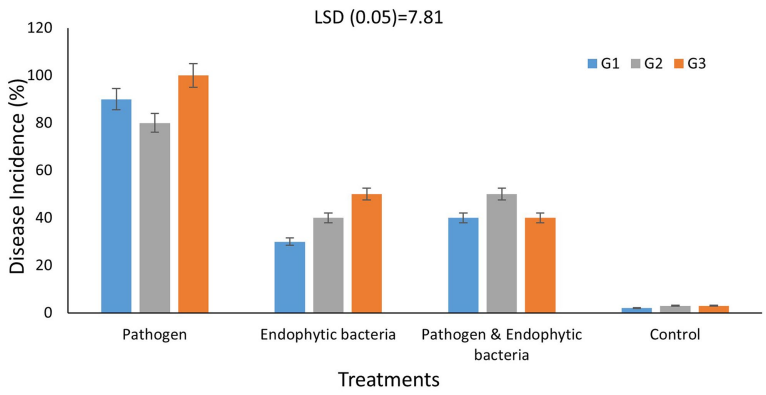

FIGURE 6
Effects of endophytic bacteria on disease incidence (%) of some different genotypes of quinoa plants (G1, G2, G3). Values of disease incidence (as a percentage) data were transformed by arcsine before analysis of variance. Data are means of three replicates. Differences between the means of tested genotypes under treatments were differentiated by least significant difference (LSD) test at the 0.05 *P* level.

the endophytic bacterial community isolated from leaves, stems, and roots of quinoa plants, including *Paenibacillus* sp., *B. megaterium*, and *Pseudomonas* sp., which has been previously characterized as a quinoa leaf endophyte (Ortuño et al., 2013; Zahoor et al., 2022).

3.3.1. Amino acid production

The free amino acid (FAA) profiles in the leaves of the quinoa genotypes under study as affected by the pathogen and the endophytic bacteria (individually or in combination) are shown in Table 5

TABLE 5 Effects of endophytic bacteria on amino acid content of the different genotypes of quinoa seeds.

Type		G1*				G2*				G3*			
		Amino acid weight (g/100g)											
		T1*	T2*	T3*	T4*	T1*	T2*	T3*	T4*	T1*	T2*	T3*	T4*
Acidic	Aspartic acid	9.7	10.2	9.5	9.3	8.9	10.0	9.3	9.1	9.5	10.3	9.4	9.3
	Glutamic acid	13.0	13.5	13.4	13.2	12.9	13.6	13.3	13.1	13.2	13.7	13.5	13.4
Aromatic	Phenylalanine	3.7	4.8	4.5	4.0	3.2	5.0	4.9	4.2	3.0	5.4	4.8	4.1
	Tryptophan	0.9	1.2	1.0	1.0	0.7	1.1	0.9	0.8	1.0	1.3	1.2	1.1
	Tyrosine	2.9	3.9	3.2	3.0	2.2	3.9	3.1	2.9	2.6	4.3	3.2	3.5
Basic	Arginine	7.8	8.3	8.1	8.0	7.8	8.4	8.2	8.1	7.5	8.4	8.0	7.8
	Histidine	3.1	4.2	3.8	3.9	3.0	4.0	3.6	3.8	3.4	4.4	3.9	3.4
	Lysine	5.0	6.7	6.0	5.5	4.8	6.3	6.1	5.2	5.2	7.0	6.7	5.7
Neutral	Alanine	7.0	7.5	7.3	7.2	6.9	7.4	7.2	7.0	7.0	7.4	7.1	7.1
	Cysteine	2.1	3.0	2.5	2.7	2.1	3.2	2.4	2.9	2.0	3.8	2.7	3.0
	Glycine	10.5	11.3	11.0	10.7	10.3	11.6	11.3	11.0	10.7	11.4	11.1	11.0
	Isoleucine	3.2	4.8	4.1	3.8	3.3	5.0	4.2	3.9	3.1	5.2	4.8	3.5
	Leucine	5.1	6.9	6.1	5.8	5.3	7.0	6.4	5.5	5.0	7.1	6.7	5.8
	Methionine	1.7	2.9	2.2	2.0	1.9	2.3	2.0	1.9	1.7	2.9	2.2	1.5
	Proline	4.0	4.6	4.2	4.1	4.1	4.8	4.4	4.3	4.2	4.6	4.4	4.5
	Serine	6.0	6.5	6.3	6.1	6.1	6.7	6.4	6.2	6.0	6.4	6.2	6.3
	Threonine	3.0	4.2	3.7	3.2	3.5	4.8	3.1	3.0	3.1	5.2	3.0	3.0
	Valine	4.0	5.1	4.6	4.2	3.9	5.8	4.2	4.0	3.4	6.7	4.7	4.3
Total acidic AA		22.7	23.7	22.9	22.5	21.8	23.6	22.6	22.2	22.7	24.0	22.9	22.7
Total aromatic AA		7.5	9.9	8.7	8.0	6.1	10.0	8.9	7.9	6.6	11.0	9.2	8.7
Total basic AA		15.9	19.2	17.9	17.4	15.6	18.7	17.9	17.1	16.1	19.8	18.6	16.9
Total neutral AA		46.6	56.8	52.0	49.8	47.4	58.6	51.6	49.7	46.2	60.7	52.9	50.0

T1*: pathogen, T2*: endophytic bacteria, T3*: pathogen and endophytic bacteria, T4*: control.
G1*: genotype (CO-KA-1873), G2*: genotype (CO-KA-2300), G3*: genotype (CO-KA-1901).

compared to the control. The pathogen negatively affects the FAA content (compared to the uninfected plants) among all the quinoa genotypes. The total FAA decreased in the pathogen-treated plants by 5.1, 6.2, and 6.8% in the leaves of G1, G2, and G3, respectively, compared to the control plants. In this context, total acidic amino acids exhibited negligible changes due to the pathogen (compared to the control) in all the studied genotypes. Notable decreases occurred in the total basic and total neutral amino acid levels. Moreover, dramatic decreases in total aromatic amino acids appeared due to the pathogen infection, by 6.3, 22.8, and 24.1% (compared to the control) in G1, G2, and G3, respectively. In this context, the levels of phenylalanine of the infected plants showed the most remarkable decreases: by 7.5% in G1, by 23.8% in G2, and by 26.8% in G3 (compared to the uninfected ones). Phenylalanine, tyrosine, and phenolics are biosynthesized through the shikimate pathway. The shikimate pathway, in plants, is localized in the chloroplast. These aromatic molecules have roles as pigments, antioxidants, signaling agents, electron transport, communication, and the structural element lignan, and as a defense mechanism (Santos-Sánchez et al., 2019). Inhibition of one or more of the enzymes controlling this pathway by pathogen interactions might be a mechanism of pathogenicity.

However, inoculation with the endophytic bacteria resulted in consistent increases in leaf FAA content of the three quinoa genotypes compared to the control. Dual inoculation (pathogen and endophytic bacteria) not only counteracted the negative effects of the pathogen infection in terms of leaf FAA, but also by over the healthy untreated plants. In terms of amino acid fractions, acidic, basic, neutral, and especially aromatic amino acid levels increased in response to the dual inoculation treatment for all the genotypes. Noteworthy was that genotype G3 showed a higher response to the dual inoculation as its levels of neutral, basic, and aromatic amino acids increased by 21.4, 17.2, and 26.4%, respectively, compared to the untreated plants. Within the aromatic amino acid group, the prominent increases were related to the levels of tyrosine (22.9–34.5% increase) and phenylalanine (19.0–31.7% increase) vis-à-vis the control. Interestingly, specific amino acids exhibited marked increments due to dual inoculation: lysine, methionine, threonine, leucine, and valine. In general, the superiority of genotype G3 was observed in its response to the pathogen and endophytic bacteria (T3) against the pathogen (T1), and this is evident by the increase in the proportions of most essential amino acids.

Quinoa is a strategic crop because of its high N content and its adaptability to adverse conditions, as nitrogen is one of the elements that plants need in large quantities. Quinoa responds positively to fertilization by nitrogen (Badran et al., 2020; Cárdenas-Castillo et al., 2021). Quinoa is a strategic partner crop for food security as a

TABLE 6 Tolerance indices of tested quinoa genotypes under the condition of pathogen (T1) and pathogen and endophytic bacteria (T3) for grain weight/plant.

Genotype	Yp	Yd	PTI	YI (%)	SM	RP
G 1	18.60	13.30	0.98	28.49	0.72	1.05
G 2	11.70	8.10	0.37	30.77	0.69	1.01
G 3	17.40	11.20	0.77	35.63	0.64	0.94
Mean	15.90	10.87	0.71	31.63	0.68	1.00

Yp = grain weight/plant using pathogen and endophytic bacteria treatment (T3); Yd = grain weight/plant using pathogen (T1); PTI = pathogen tolerance index; YI = yield injury; SM = superiority measure; RP = relative performance.

plant-based protein source (Alandia et al., 2020) and its adaptability to unfavorable growing conditions (Rodriguez et al., 2020; Badran, 2022). As a result of previous research, based on the plant's response to nitrogen fertilization, bacterial treatments were used that effectively provided nitrogen for quinoa plants through their effect on the synthesis of amino acids within the plant tissues after bacterial treatment.

Our results are in harmony with Almuhayawi et al. (2021), who showed that endophytic bacterium inoculation can increase amino acid levels of the sprouts of three *Chenopodium* species: *C. ambrosoides*, *C. ficifolium*, and *C. botrys*, Endo 2 (strain JSA11).

3.3.2. Tolerance indices of tested genotypes against pathogen

Tolerance indices were calculated to evaluate the tested genotypes of quinoa against Pseudomonas syringae. Weight/plant (g) of the three genotypes under treated with pathogens (T1) compared to the treatment with pathogens and endogenous bacteria (T3) were measured to assess the tolerance indices of the tested genotypes (Table 6). Pathogen tolerance index (PTI) data indicates that the genotype G1 was the most tolerant according to the grain weight of the plant (0.98), while the G2 genotype was the least tolerant to infection with the pathogen (0.37) during the study.

With regard to the general mean of yield injury (31.63%), the G1 genotype recorded the lowest infection rate (28.49) of pathogen, followed by the G2 genotype (30.77%), while the G3 genotype was the most sensitive to infection with the pathogen (35.63%). The same results for the scale of superiority (SM) and relative performance (RP) are in harmony with the previous result of yield injury (Table 6).

The results indicate that the classification of the G1 genotype according to different indicators can be said to be distinct under all conditions compared to the other two genotypes (G2 and G3).

An environmental stress-tolerance genotype can be defined as one that gives a yield that is significantly above average under environmental stress conditions. Therefore, the results in Table 6 can separate the tested genotypes according to the grain yield / plant under pathogen infection condition (T1) and under pathogen infection condition when treated with endophyte bacteria these results are consistent with Majidi et al. (2011) and Badran and Moustafa (2014). Therefore, based on the results presented in Table 6, it is possible to divide the genotypes into three groups based on their performance, which is indicated by Fernandez (1992), that the genotypes can be classified into a number of groups based on their performance: genotype that expresses performance good under any conditions (cluster 1), genotypes that performed only well under optimal conditions (cluster 2), genotypes that gave relatively higher productivity under environmental stress conditions (cluster 3), and genotypes that performed poorly in both cases (Group 4).

4. Conclusion

The results indicated that there was a significant difference between chlorophyll content index of infected plants without bioagent (untreated) compared to plants bio-inoculated with endophytic bacteria. The highest mean disease incidence was on the plants without bacterial inoculum for quinoa genotype G3. A significant differences in the weight of grains/plant, was obvious when treated with pathogens (T1) compared to the treatment with pathogens and endogenous bacteria (T3), and a decreases in total aromatic amino acids appeared due to the pathogen infection, compared to the control. The genotype G3 showed the highest response in the levels of total aromatic and total neutral amino acids. The endophytic strains promoted quinoa seedling growth mainly by improving nutrient efficiency. This improvement could not be explained by their ability to induce the production of amino acids, showing that complex interactions might be associated with enhancement of quinoa seedling performance by endophytic bacteria.

The endophytic bacterial strains were able to reduce the severity of bacterial leaf spot disease by 30, 40, and 50% in quinoa genotypes G1, G2, and G3, respectively, recording significant differences compared to the negative control. The results indicated that, G1 genotype was superior in different performance indicators (pathogen tolerance index, yield injury %, superiority measure and relative performance) for grain weight/plant under pathogen infection condition when treated with endophyte bacteria.

Based on this study, these bacterial strains can be used as a biotechnology tool in quinoa seedling production and biocontrol to diminish the severity of bacterial leaf spot disease.

In fact, plant growth-promoting endophytic bacteria can offer incredible benefits to plants and can support the environmentally friendly approaches for sustainable agriculture. They can be used as tools that could be an alternative way to diminish the use of chemicals. It is obvious that, under the current climate changes and increasing world population, the sustainability of agriculture should be based on innovative environment-friendly approaches and should consider crop biodiversity and highly nutritious crops such as quinoa and other non-conventional crops.

Our study focused on the effect of PGPEB on quinoa growth and resistance to bacterial leaf spot disease. It proved that PGPEB can be used as promising bioagents to limit this disease and can be used

as a biotechnology technique in quinoa seedling production in Egypt and other countries with similar climate conditions.

However, further investigation of the beneficial impacts of the tested PGPEB is required and should focus on the implications of such beneficial bacteria in plant protection 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 at: https://www.ncbi.nlm.nih.gov/nuccore/, OP984768, OP984769, OP984765, and OP984766.

Author contributions

AB and NE designed the research and wrote the initial draft of this manuscript and conceived the original ideas. NE conducted the experiment and analysis. AB, NE, AH, and HM contributed to idea refinement, writing, and revising of the manuscript. HM supervised the work and revised and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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EDITED BY
Upendra Kumar,
National Rice Research Institute (ICAR), India

REVIEWED BY
Sandeep Kumar Sihag,
Indian Agricultural Research Institute (ICAR), India
Deepranjan Sarkar,
Banaras Hindu University, India

*CORRESPONDENCE
Nirmalendu Basak
✉ nirmalendubasak@rediffmail.com;
✉ Nirmalendu.Basak@icar.gov.in
Anoop Kumar Dixit
✉ Anoop.Dixit@icar.gov.in

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Changes in soil microbial biomass and organic C pools improve the sustainability of perennial grass and legume system under organic nutrient management

Arvind Kumar Rai[1,2], Nirmalendu Basak [2]*, Anoop Kumar Dixit[1]*, Suchit Kumar Rai [1], Sanjoy Kumar Das[1,3], J. B. Singh[1], Sunil Kumar[1], T. Kiran Kumar[1,4], Priyanka Chandra[2], Parul Sundha [2] and Sandeep Bedwal[2]

[1]ICAR–Indian Grassland and Fodder Research Institute, Jhansi, Uttar Pradesh, India, [2]ICAR–Central Soil Salinity Research Institute, Karnal, Haryana, India, [3]ICAR-Central Inland Fisheries Research Institute, Kolkata, West Bengal, India, [4]ICAR–Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India

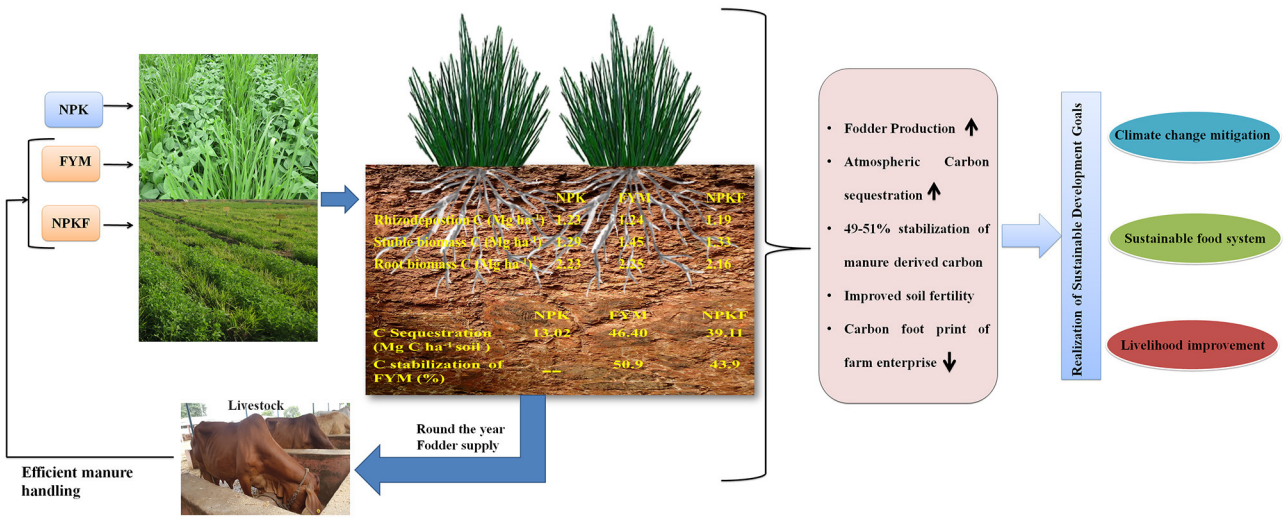
Introduction: The perennial grass–legume cropping system benefits soil because of its high biomass turnover, cover cropping nature, and different foraging behaviors. We investigated the response of soil organic carbon (SOC) pools and their stock to organic and inorganic nutrient management in the Guinea grass and legume (cowpea-Egyptian clover) cropping system.

Methods: Depth-wise soil samples were collected after harvesting the Egyptian clover. Based on the ease of oxidation with chromic acid, different pools of SOC oxidizable using the Walkley–Black C method, very labile, labile, less labile, non-labile; and dissolved organic C (DOC), microbial biomass C (MBC), and total organic C (TOC) in soils were analyzed for computing several indices of SOC.

Result and discussion: After 10 years of crop cycles, FYM and NPKF nutrient management recorded greater DOC, MBC, SOC stocks, and C sequestration than the NPK. Stocks of all SOC pools and carbon management index (CMI) decreased with soil depth. A significant improvement in CMI, stratification ratio, sensitivity indices, and sustainable yield index was observed under FYM and NPKF. This grass–legume intercropping system maintained a positive carbon balance sequestered at about 0.8Mg C ha^{-1} after 10 years without any external input. Approximately 44–51% of the applied carbon through manure was stabilized with SOC under this cropping system. The DOC, MBC, and SOC in passive pools were identified for predicting dry fodder yield. This study concludes that the application of organics in the perennial grass–legume inter cropping system can maintain long-term sustainability, enhance the C sequestration, and offset the carbon footprint of the farm enterprises.

KEYWORDS

carbon input, dissolved organic carbon, microbial biomass C, semi-arid agroecosystem, SOC indices, SOC sequestration, stratification ratio



GRAPHICAL ABSTRACT
Nutrient management affects biomass production and SOC sequestration.

1. Introduction

Soil organic carbon (SOC) is key to soil health management influencing crop production, climate change mitigation, and other ecosystem services (Srinivasarao et al., 2016; Oldfield et al., 2019; Hasegawa et al., 2021; Doblas-Rodrigo et al., 2022). The accrual and hoarding of SOC are determined greatly by the set of biophysical variables such as temperature, rainfall, potential evapotranspiration, soilscape, physiography, vegetation, cropping systems, and management practices (Schmidt et al., 2011; Lal, 2015; Poffenbarger et al., 2018; Alavi-Murillo et al., 2022). The cropping systems and associated management practices utilizing inorganic fertilizer and organic manures alone or in combination are the key drivers influencing the SOC stock of soil in a given agroecosystem (Mandal et al., 2008; Ghosh et al., 2012; Luo et al., 2014; Sainju et al., 2014; Yadav et al., 2017; Poffenbarger et al., 2018; Ngangom et al., 2020; Basak et al., 2021a). These management practices affect the separation of SOC in pools of varied stability (Basak et al., 2021a) characterized by the susceptibility to oxidation and residence period (Zimmermann et al., 2007). A faster turnover rate of labile C fraction of SOC (Sanderman et al., 2017) and its oxidation maintain the productivity of soil and CO_2 flux to the atmosphere. Conversely, the passive pool has a long residence time because of physical and biochemical protection, making it resistant to microbial actions (Zimmermann et al., 2007; Weil et al., 2017; Datta et al., 2018). Previous studies reported the effect of the cropping system on the distribution of active and passive pools in soils of subtropic (Chaudhary et al., 2017; Yadav et al., 2017, 2019; Anantha et al., 2020; Babu et al., 2020; Basak et al., 2021a), arid (Moharana et al., 2017), and semi-arid (Srinivasarao et al., 2013, 2016; Mitran et al., 2018; Basak et al., 2021b) regions. The application of organic fertilizers is also reported to improve the yield of grain crops through their influence on SOC and associated soil properties (Mandal et al., 2007). However, similar studies involving perennial grass-based intensive forage production in semi-arid climatic conditions are lacking (Ghosh et al., 2010; Kim et al., 2022).

In India, 57% of the net sown areas are rainfed under different agroecosystems (Bal et al., 2022). A significant proportion of the area under semi-arid regions in India had been brought under canal irrigation by developing dams on the rivers with seasonal water flow. As a result, cropping intensity in these canal commands increased. The limited supply of canal water only during the winter makes it a rainfed (monsoon) rotation followed by irrigated (winter) crops in these regions. Guinea grass (*Panicum maximum* cv. Hamil) intercropped with seasonal legumes is an important intensive forage production system in rainfed-irrigated areas in semi-arid regions of India (Kumar et al., 2012). Fast growth and high biomass production make it an ideal crop for fodder and biofuel production (Mohapatra et al., 2019) and SOC sequestration in pastures and grassland (Ghosh et al., 2009; Sarkar et al., 2020). Cowpea and Egyptian clover (*Trifolium alexandrinum* L) are leguminous crops commonly incorporated in grass-based cropping systems to improve forage quality and enhance forage productivity during the poor growth of grass components (Kumar et al., 2012). The cowpea and Egyptian clover also complement the cropping system's soil SOC pools and productivity because of spatial and temporal annidation and different rooting behaviors (Rai et al., 2013).

The SOC stock and stability indices developed from SOC pools were found better in assessing the impact of management practices and cropping systems in several cropping systems in different agro-ecologies (Blair et al., 1995; Franzluebbers, 2002; Mandal et al., 2008). But, there is limited information on the perennial grass + legumes-based intensive intercropping system for biomass production under inorganic and organic nutrition with rainfed-irrigated water management. These indices in a perennial grass–legume-based intensive cropping system will capture the impact of different nutrient management on SOC and their interactions with soil properties in rainfed-irrigated

agroecosystems of the semi-arid region. This information is essential from an agronomic and environmental point of view for crop planning and carbon budgeting at the farm scale. Therefore, this study was conducted to assess the impact of the organic and inorganic nutrients alone or in combination with the stock and stability of SOC in perennial guinea grass + the (cowpea-Egyptian clover) cropping system under rainfed (monsoon)—irrigated (winter) water management. The objective of the study was to (i) assess the impacts of organic, inorganic, and integrated nutrient management on depth-wise SOC stock and its sequestration potential; (ii) measure the effect of nutrient management on the pool-wise allocation of SOC of different stability, and (iii) to establish a relationship between SOC pools and cropping system sustainability.

2. Materials and methods

2.1. Experimental site

This experiment was conducted in the semi-arid, continental, and monsoonal climate at the Central Research Farm (25°31′ 01.73″ N, 78°33′ 32.84″ E, at 224 m mean sea level), ICAR-Indian Grassland and Fodder Research Institute, Jhansi, Uttar Pradesh, India. The region's annual rainfall is 906.5 mm (781 mm during the rainy season and 52 mm during the winter), with an annual potential evapotranspiration of 1,512 mm. The soil of the experimental site is classified under hyperthermic *Typic Haplustepts* with silty loam in texture (sand, silt, and clay with 36.6, 43.2, and 20.2%, respectively). The soil of the experimental site had $pH_{1:2}$ of 7.9 and Walkley–Black organic carbon (Walkley and Black, 1934) of 4.2 g kg^{-1}; and $KMnO_4$ oxidizable N (Subbiah and Asija, 1956), Olsen's extractable P (Jackson, 1967), and ammonium acetate extractable K (Jackson, 1967) of 192, 9.9, and 135 kg ha^{-1}, respectively.

2.2. Field experiment: treatment detail and layout

The field experiment was conducted following a randomized complete block design with three replications. The fertilizers treatments were as follows: control, the recommended dose of N, P, and K (NPK) fertilizers, farmyard manure (FYM), and integrated application of FYM and NPK (NPKF) were allocated randomly in triplicate in plots of 4 × 5 m^2 separated by 1-meter buffer strip from all sides to prevent treatment interference. Details of the treatments imposed, including source, application schedule, and other cultural practices followed, are given in Table 1. The first crop Guinea grass (*Panicum maximum* cv. Hamil) rooted slips were planted in July 2004 at a 100 × 50 cm distance. Cowpea (*Vigna unguiculata cv*. EC-4216) and Egyptian clover (*Trifolium alexandrinum cv*. Wardan) were planted in the inter-row space in summer (June–July) and winter season (November–March), respectively. In their respective season, two rows of cowpea and berseem were planted in the inter-row space at a 30 cm distance. In FYM and NPKF treatments, FYM was applied in the rainy (July–October) and winter (November–June) seasons on a fresh weight basis with an average moisture content of 35% (w/w). The FYM contained ∼8.7 ± 0.4, 2.0 ± 0.3, and 12.1 ± 0.2 g kg^{-1} of N, P, and K, respectively. The mean C: N ratio of the manure was 70:1. Rainy (July-October)-season crop was grown rainfed, while in winter season 7–8, irrigation was applied depending upon the winter rains and the crop's need in the cropping system. The above-ground biomass of the Egyptian clover was harvested at 30-day intervals, while cowpea was harvested 60 days after sowing. Guinea grass was harvested 5–7 cm above the ground surface at 30 and 60 days intervals in rainy (July–October) and the rest of the season (November–June), respectively.

2.3. Soil analysis

Soil samples were collected randomly from three representative sites in each plot at the field moist condition from four depths *viz*., 0–0.15, 0.15–0.30, 0.30–0.50, and 0.50–0.70 m after harvesting the Egyptian clover in the last week of April 2014. One set of soil samples was immediately preserved at 4°C for microbial biomass carbon (MBC) estimation (Vance et al., 1987). Another sample set was air-dried and processed to pass through a 2 mm sieve for further analysis of SOC pools. Depth-wise bulk density of undisturbed soil was measured using a core sampler (Blake and Hartage, 1986). A pH meter (Eutech pH 700) was used for estimating soil pH in a 1:2 soil: water suspension (Jackson, 1967). Soil texture (Gee and Bauder, 1986) and calcium carbonate (Allison and Moodie, 1965) were determined using standard procedure. Total carbon (TC) was estimated using CHNS Elemental Analyzer (Vario EL III, Germany). The TOC was computed by deducting the carbon derived from calcium carbonate (Allison and Moodie, 1965) from TC.

2.3.1. Microbial biomass carbon, microbial quotient, and dissolved organic carbon

Chloroform fumigation and extraction method were used to determine microbial biomass carbon (MBC) in fresh soil samples (Vance et al., 1987). The difference in the C flux from fumigated to unfumigated samples was used to calculate the MBC (Mg ha^{-1}) using equation (1) proposed by Voroney and Paul (1984). The dissolved C in the extract was determined using the method of McGill et al. (1986). The microbial quotient (MQ) was calculated from MBC and TOC (Equation 2).

$$MBC = \frac{Microbial\ carbon\ flush}{0.41} \quad (1)$$

$$MQ\ (\%) = \frac{MBC}{TOC} \times 100 \quad (2)$$

2.3.2. Oxidizable soil organic carbon and its pools

The SOC pools were determined using the modified method suggested by Chan et al. (2001). Briefly, soils were treated with 12, 18, and 24 *N* H_2SO_4 and 0.5 *N* $K_2Cr_2O_7$. The obtained TOC was separated into four pools of reduced oxidability: very labile (C_{VL}), labile (C_L), less labile (C_{LL}), and non-labile (C_{NL}) following the procedure described in Majumder et al. (2007). $C_{VL} + C_L$ and

TABLE 1 Description of the management practices for guinea grass + (cowpea-Egyptian clover) cropping system.

Treatments detail		Nutrient management		Tillage
		Rainy season (July-October)	Winter season (November–February)	
Control	Unfertilized control (without NPK or organics)	-	-	One harrowing followed by leveling and line sowing of cowpea and Egyptian clover at 30 cm spacing in rainy and winter seasons respectively
NPK	100% RDF (recommended dose of fertilizer (N: P : K kg ha^{-1})	200:50:50	20:80:0	
FYM	Farmyard manure (60.0 Mg ha^{-1})	37.5	22.5	
NPKF	FYM (52.5) + 25% RDF in rainy season	37.5 Mg ha^{-1} + (50:12.5:12.5 kg ha^{-1})	15.0 Mg ha^{-1}	

Fertilizers N, P, and K were applied in the form of urea, single super phosphate, and muriate of potash, respectively.

$C_{LL} + C_{NL}$ were expressed as the active (C_{AP}) and passive pools (C_{PP}), respectively.

2.4. Crop-derived C inputs into the soil

After harvesting multi-cut guinea grass, cowpea, and Egyptian clover, the leftover biomass and root samples were collected. The protocol described by Thangaraj and O'Toole (1986) was used to sample guinea grass, cowpea, and Egyptian clover roots. The C content of these samples was analyzed. The net rhizodeposition of grass and legume crops was derived from roots' biomass, as described by Pausch and Kuzyakov (2018). A detailed assessment was made for crop residue C added from guinea grass, cowpea, and Egyptian clover. Total carbon input to the soil from rhizodeposition, roots, stubbles, and leaves of the component crops was measured annually during the last 3 years using respective biomass addition and carbon content.

2.5. Soil carbon stock

The soil carbon stock (Mg ha^{-1}) was calculated using bulk density (Mg m^{-3}), depth of soil layer (m), and C content (g C g^{-1}) in the respective soil layer using Equation (3) (Batjes, 1996).

$$Carbon\ \ stock\ in\ soil\ = (Carbon\ content)\ \times Bulk\ density \times Depth \quad (3)$$

2.6. Recalcitrant index

To assess the effect of FYM, inorganic fertilizer, and control, the recalcitrant index (RI_1 and RI_2) of SOC was calculated using Equations (4) and (5) (Datta et al., 2018). The RI_{mean} was computed by averaging the RI_1 and RI_2.

$$RI_1 = \frac{C_{LL} + C_{NL}}{C_{VL} + C_L} \quad (4)$$

and

$$RI_2 = \frac{C_{NL}}{TOC} \quad (5)$$

2.7. Carbon lability index

The carbon lability index (CLI) of SOC was developed using SOC pools of comparative oxidability using Equation (6) (Majumder et al., 2007).

$$CLI = \frac{C_{VL}}{TOC} \times 3 + \frac{C_L}{TOC} \times 2 + \frac{C_{LL}}{TOC} \times 1 \quad (6)$$

2.8. Carbon management index

The CMI highlights the management-induced changes in soil quality (Basak et al., 2021a). It is derived from the carbon pool index (CPI) and lability index (LI) as shown in the following equations (Blair et al., 1995):

$$CMI = CPI \times LI \times 100 \quad (7)$$

$$CPI = \frac{TOC\ (NPK/FYM/NPKF)}{TOC\ (unamended\ control)} \quad (8)$$

$$LI = \frac{LC\ (NPK/FYM/NPKF)}{LC\ (unamended\ control)} \quad (9)$$

$$Lability\ of\ C\ (LC) = \frac{C_{VL}}{TOC - C_{VL}} \quad (10)$$

2.9. Carbon build-up and sequestration

The total carbon addition in the soil through organic manure was calculated based on oven-dry weight. Carbon budgeting was done for 0–0.70 m depth. The percent C build-up over control, C build-up rate, per cent stabilization of applied carbon, carbon sequestration, and carbon sequestration potential for different nutrient management strategies

were estimated using the following equations as described by Mishra et al. (2015):

$$\%\ C\ build-up = \frac{[TOC_{Treatment} - TOC_{Control}]}{TOC_{Control}} \times 100 \quad (11)$$

$$C\ build-up\ rate\ (Mg\ C\ ha^{1}\ yr^{-1}) = \frac{[TOC_{Treatment} - TOC_{Control}]}{Year\ of\ experiment} \quad (12)$$

$$Percent\ \ stabilization\ of\ applied\ C = \frac{[C_{Trt} - C_{background}]}{C_{applied\ through\ manure}} \times 100 \quad (13)$$

Here, C_{Trt} is the TOC in a particular treatment; TOC in control treatment was considered a background contribution of TOC for FYM treatment; while for NPKF treatment considering the additive effect, $C_{control} + 0.25\ (C_{NPK-}\ C_{control})$ was considered as the background contribution to soil TOC,

$$C\ sequestration\ \left(Mg\ C\ ha^{1}\right) = TOC_{Current} - TOC_{Initial} \quad (14)$$

2.10. Stratification ratio

The stratification ratio (SR) specifies the sub-surface influence of surface-imposed management practices. The SR of SOC and its pools was derived as the ratio of respective carbon fractions using the following equation described by Franzluebbers (2002):

$$SR = \frac{TOC/WBOC/C_{VL}/C_{AP\ in\ surface\ soil\ (0-15\ cm)}}{TOC/WBOC/C_{VL}/C_{AP\ in\ sub-surface\ soil\ (15-30\ cm)}} \quad (15)$$

The average stratification ratio of all the carbon fractions was denoted as SR_{mean}.

2.11. Sensitivity index

The SI (%) of 0–0.15 m soil layer was calculated to assess the changes in different C pools in treated soils in comparison to control using Equation (16) proposed by Banger et al. (2010) as follows:

$$SI = \frac{\{TOC/WBOC/C_{VL}/CAP\ (NPK/FYM/NPKF) - TOC/WBOC/C_{VL}/CAP\ (unamended\ control)\}}{TOC/WBOC/C_{VL}/CAP\ (unamended\ control)} \times 100 \quad (16)$$

The average SI of all the carbon fractions was denoted as SI_{mean} for the respective treatments.

2.12. Biometric observation and biomass yield

Total green biomass yield and dry biomass yield (oven-dry weight basis at 60°C) of the guinea grass, cowpea, and Egyptian clover were recorded plot-wise during 2004–2014. The sustainable yield index (SYI) was calculated using dry biomass yield using the following formula (Singh et al., 1990):

$$Sustainable\ yield\ index\ (SYI\ ) = \frac{[(Mean\ dry\ biomass\ yield\ of\ guinea\ grass,\ cowpea\ and\ Egyptian\ clover\ during\ the\ ten\ year)\ -\ \sigma]'}{Maximum\ dry\ biomass\ yield\ of\ guinea\ grass,\ \ cowpea\ and\ Egyptian\ clover\ during\ the\ ten\ year} \quad (17)$$

Where σ estimated the standard deviation in the experiment during the 10 years of cultivation.

2.13. Statistical analysis

All the data were tested for normality and homogeneity of variance using the Bartlett and Shapiro–Wilk tests, respectively. Analysis of variance (ANOVA) was carried out using SPSS statistical software (SPSS Inc., 2004, Chicago, USA). The pairwise comparison of the means of all the parameters was performed using Duncan's multiple range test ($P < 0.05$). The relationship between the soil parameter dry fodder yield and sustainable yield index was determined using Pearson's correlation coefficient. Multiple linear regression models were developed to establish a relation between the yield and SYI with different response variables.

3. Results

3.1. Nutrient management and SOC pools

Farmyard manure (FYM), when applied alone or in conjunction with chemical fertilizer (NPKF), increased the soil pH and decreased bulk density (BD) compared to the unamended control and sole application of NPK (Table 2). The soil pH and BD increased with soil depth. FYM had a more pronounced ($P < 0.05$) effect on the increase in Walkley–Black organic carbon (WBOC), TOC stocks, and dissolved organic C (DOC). The NPKF had greater WBOC and TOC stocks than NPK and control. The WBOC, TOC, and DOC stocks decreased at a lower depth.

Carbon allocation in different pools varied with nutrient management (NM) strategies and soil depth (Table 3). Very labile (C_{VL}), labile (C_L), and non-labile (C_{NL}) SOC stocks in FYM and

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TABLE 2 Changes in soil physicochemical properties with nutrient management (mean over depths) and soil depth (mean over treatments).

Treatment	$pH_{1:2}$	BD (Mg m^{-3})	WBOC (Mg ha^{-1})	TOC (Mg ha^{-1})	DOC (Mg ha^{-1})
Control	7.65^{B}	1.38^{AB}	5.6^{D}	14.7^{C}	0.23^{C}
NPK	7.40^{C}	1.39^{A}	7.8^{C}	17.7^{B}	0.31^{A}
FYM	7.90^{A}	1.35^{C}	10.4^{A}	26.1^{A}	0.31^{A}
NPKF	7.85^{A}	1.37^{B}	8.8^{B}	24.8^{A}	0.27^{B}
Depth (m)					
0–0.15	7.38^{c}	1.34^{d}	10.2^{a}	23.5^{a}	0.32^{a}
0.15–0.30	7.72^{b}	1.36^{c}	7.6^{b}	21.4^{b}	0.25^{c}
0.30–0.50	7.79^{b}	1.39^{b}	8.1^{b}	20.5^{b}	0.28^{b}
0.50–0.70	7.90^{a}	1.41^{a}	6.7^{c}	17.9^{c}	0.27^{bc}
0–0.7	7.96	1.42	32.6	81.4	1.12
Treatment × Depth	*	ns	***	***	ns

Soil pH, (soil: water, 1:2 ratio); BD, bulk density; WBOC, Walkley–Black organic carbon; TOC, total organic carbon; DOC, dissolved organic C; uppercase for nutrient management and lowercase for depths within a column differ significantly by Duncan's multiple range test ($P < 0.05$); ns, * and *** represent non-significant and significance at $P < 0.05$ and 0.001 for interaction effect (treatment × depth) in experimental sites with nutrient management (mean over depths) and soil depth (mean over treatments).

TABLE 3 Changes in soil organic C pools (Mg ha^{-1}) with nutrient management in soil depth.

Treatment	C_{VL}	C_L	C_{LL}	C_{NL}	C_{AP}	C_{PP}
Control	5.11^{C}	3.68^{B}	2.49^{C}	3.42^{B}	8.80^{C}	5.90^{B}
NPK	6.00^{C}	3.33^{B}	4.05^{A}	4.30^{B}	9.33^{C}	8.35^{A}
FYM	10.01^{A}	6.30^{A}	3.62^{AB}	6.20^{A}	16.31^{A}	9.83^{A}
NPKF	8.93^{B}	6.33^{A}	3.34^{B}	6.23^{A}	15.26^{B}	9.57^{A}
Depth (m)						
0–0.15	9.78^{a}	3.54^{b}	3.48^{a}	6.73^{a}	13.32^{a}	10.21^{a}
0.15–0.30	7.77^{b}	5.80^{a}	3.15^{a}	4.67^{b}	13.57^{a}	7.82^{b}
0.30–0.50	6.80^{b}	5.98^{a}	3.22^{a}	4.54^{b}	12.78^{a}	7.76^{b}
0.50–0.70	5.72^{c}	4.31^{b}	3.66^{a}	4.21^{b}	10.04^{b}	7.87^{b}
0–0.7	30.1	19.6	13.5	20.2	49.7	33.7
Treatment × Depth	***	***	***	ns	***	ns

C_{VL}, very labile; C_L, labile; C_{LL}, less labile; C_{NL}, non-labile; C_{AP}, active; C_{PP}, passive pool of soil organic carbon; uppercase for nutrient management and lowercase for depths within a column differ significantly by Duncan's multiple range test ($P < 0.05$); ns and *** represent non-significant and significance at $P < 0.001$ for interaction effect (treatment × depth) in experimental sites with nutrient management (mean over depths) and soil depth (mean over treatments).

NPKF were greater than unamended control and NPK. However, the less labile (C_{LL}) SOC pool was greater in NPK than in NPKF and control. Irrespective of NM, approximately 25 and 37% of TOC in 0–0.70 m depth were allocated in C_L and C_{VL} pools, respectively (Supplementary Table S1). The per cent of C_{VL} to TOC stock remained at par for all treatments, but C_L and C_{LL} proportion to TOC stock was greater for NPKF and NPK, respectively. The proportion of C_L to TOC increased with soil depth; however, at the 0.50–0.70 m depth, the proportion of C_{NL} to TOC was greater than the upper soil layers. The active (C_{AP}) and passive (C_{PP}) pools constitute 61 and 42% of the TOC in 0.70 m soil depth, respectively. The FYM and NPK maintained relatively higher proportions of active and passive pools, respectively. The MBC was 8 and 37% greater for FYM and NPKF compared to NPK and control. On the contrary, microbial quotient (MQ) decreased under FYM and NPKF compared to NPK and control. Both MBC stocks and MQ decreased with an increase in soil depth (Figure 1).

3.2. Lability index, recalcitrant index, stratification ratio, carbon management index, and sensitivity index

A lower lability index (LI) and higher recalcitrant index (RI_{mean}) appeared for NPK compared to FYM (Table 4). Soils of

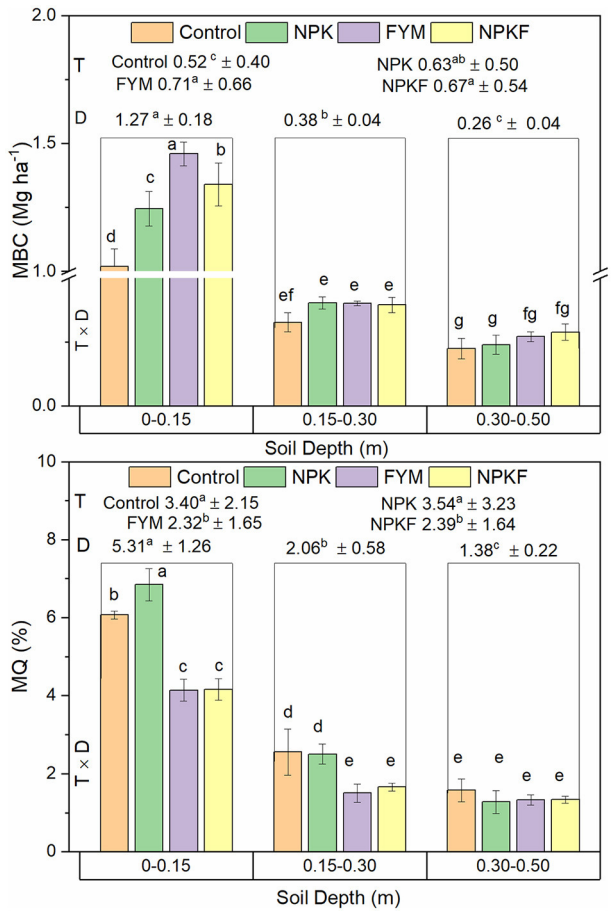

FIGURE 1
Effect of nutrient management (T), depth (D), and their interaction (T × D) on microbial biomass carbon and microbial quotient; capped lines on bars are standard deviations; $n = 9$, 12, and 3 for T, D, and T × D, respectively; numbers and bars followed by different letters (a–f) are significantly different ($P < 0.05$).

lower depth had low LI and higher RI_{mean} values. A significant improvement in the carbon management index (CMI) was observed for FYM and NPKF compared to NPK and control (Table 4). The values of CMI decreased at lower soil depths. The stratification ratio (SR) of C_{VL} was greater under FYM and NPKF. Except for active pools, the stratification ratio of different pools was greater for FYM. The sensitivity index for different pools was also greater for FYM (Table 5).

3.3. Fodder yields, C input, and its sequestration and stabilization

The dry forage yield and SYI were greater than FYM (Table 6). The annual return of C through crop residues of guinea grass, cowpea, and Egyptian clover in the form of root, stubble, and rhizodeposition was estimated for different NM practices for the entire 10 years of the cropping cycle (Supplementary Table S1). The return of root, stubble, and rhizodeposition C was greater when NM practices were adopted (NPK/FYM/NPKF) than in control. Besides, crop residue C return, a 4.0 and 3.46 Mg ha^{-1} C $year^{-1}$ are supplied through organic amendments FYM and NPKF treatment, respectively. The annual total C turnover, TOC stock, C builds-up per cent, C build-up rates, and C sequestration rate were greater with FYM (Table 6). The conspicuously cropping system alone, without any external nutrient supply, maintained a positive carbon balance and sequestrated 0.8 Mg C ha^{-1}. Approximately 50.9 and 43.9% of the C applied through manure were stabilized with SOC under FYM and NPKF, respectively. The remaining 49.1 to 56.1% of applied carbon escaped from the soil.

3.4. Soil properties and system productivity

A strong positive correlation was reported among DOC, MBC, C_{VL}, C_{NL}, C_{PP}, WBOC, and TOC ($r \geq 0.59^{***}$); these indices had a significant effect on CPI and CMI and eventually affected the DFY and SYI (Figure 2). The SI_{mean} also showed a strong positive correlation with MBC, C_{VL}, WBOC, TOC, CMI, and CPI, while a strong negative correlation was observed between BD and CPI, RI_{mean} and MBC, C_{AP}, C_{VL}, CMI; MQ with C_{VL}, C_{AP}, C_{NL}, WBOC, TOC, CPI, CMI, SYI; and LI with C_{NL}, C_{CP}, and RI_m. Multiple regression analyses showed that WBOC and SImean explained approximately 94 and 96% variability in SYI and DFY, and MBC, DOC, and C_{pp}, respectively (Table 7).

4. Discussions

Soil health and agricultural sustainability largely depend on the stock and stability of SOC in agroecosystems. Continuous cropping in semi-arid regions often decreases SOC pools, and the net depletion of TOC stock accelerates because of hot summer, perturbation of the system, and photo-oxidation of SOC (Srinivasarao et al., 2021). Soil disturbances because of tillage and other cultural practices hasten the SOC oxidation by exposing the protected C to an ultimate reduction in SOC (Six et al., 2002). Several studies reported a decline in TOC because of long-term cropping with annual crops under semi-arid and sub-tropical regions (Majumder et al., 2008b; Srinivasarao et al., 2012; Anantha et al., 2022). Contrarily, the guinea grass-based round-the-year forage production systems provided a protective covering to the soil and improved aggregation, SOC, and soil quality in pastures and grassland because of perennial growth habit, profuse rooting of the tussocks, adaptation to conservation tillage practices, and greater biomass turnover (Sanderson and Adler, 2008; Das et al., 2014; Kibet et al., 2016; Chen et al., 2022). The positive build-up of SOC in control plots in the present experiment without the application of any external carbon except through crops also supports the hypothesis of the SOC protection against oxidation loss because of the protective covering of the soil surface and reduced tillage (Rai et al., 2013; López-Vicente et al., 2021).

TABLE 4 Effect of nutrient management practices on lability index (LI), recalcitrant indices (RI), and carbon management index (CMI) in soil depth.

Treatment	LI	RI_1	RI_2	RI_{mean}	CMI (%)
Control	1.76^{AB}	0.69^{B}	0.23^{A}	0.46^{B}	57.2^{B}
NPK	1.66^{B}	0.91^{A}	0.24^{A}	0.58^{A}	66.8^{B}
FYM	1.85^{A}	0.61^{B}	0.24^{A}	0.42^{B}	116.5^{A}
NPKF	1.81^{AB}	0.65^{B}	0.26^{A}	0.46^{B}	99.6^{A}
Depth (m)					
0–0.15	1.57^{c}	0.82^{a}	0.27^{a}	0.54^{a}	95.1^{ab}
0.15–0.30	1.86^{ab}	0.61^{b}	0.23^{a}	0.42^{b}	99.9^{a}
0.30–0.50	1.91^{a}	0.64^{b}	0.24^{a}	0.44^{ab}	77.4^{bc}
0.50–0.70	1.74^{b}	0.80^{a}	0.25^{a}	0.52^{ab}	67.9^{c}
0–0.7	1.75	0.70	0.25	0.47	83.9
Treatment × Depth	*	ns	ns	ns	ns

Uppercase for nutrient management and lowercase for depths within a column differ significantly by Duncan's multiple range test ($P < 0.05$); ns and *represent non-significant and significance at $P < 0.05$ for interaction effect (treatment × depth) in experimental sites with nutrient management (mean over depths) and soil depth (mean over treatments).

TABLE 5 Effect of nutrient management practices on SOC stratification ratio (SR) and sensitivity indices (SI, %).

Treatment	SR_{TOC}	SR_{VL}	SR_{AP}	SI_{WBOC}	SI_{TOC}	SI_{VL}	SI_{AP}	SImean
Control	1.31^{A}	1.18^{AB}	1.22^{A}					
NPK	1.14^{A}	1.07^{B}	1.04^{AB}	0.32^{B}	0.13^{B}	0.69^{B}	0.51^{B}	0.41^{B}
FYM	1.32^{A}	1.35^{A}	0.89^{B}	81.63^{A}	114.7^{A}	95.1^{A}	54.8^{A}	86.5^{A}
NPKF	1.36^{A}	1.35^{A}	0.97^{B}	0.36^{B}	0.36^{B}	0.44^{B}	0.25^{B}	0.35^{B}

ns, *, ** and ***represent non-significant, significance at $P < 0.05$, 0.01, and 0.001; values followed by different letters (uppercase for different treatments) within a column differ significantly by Duncan's multiple range test ($P < 0.05$).

The long-term application of chemical fertilizer (NPK) resulted in increased SOC content because of greater return of root biomass and rhizodeposition (Srinivasarao et al., 2012; Datta et al., 2018; Pausch and Kuzyakov, 2018). With the application of FYM, the SOC pool was increased further because of the positive impact of FYM on root biomass production and its direct contribution to SOC. The integrated nutrient management under NPKF provided favorable conditions for microbial proliferation (Majumder et al., 2008a). The DOC is derived from root exudates, above-ground residues, microbial biomass debris, soluble organic carbon and liberated from decomposition of added organic matter (Christ and David, 1996; Liang et al., 2011). Therefore, NM practices NPK, FYM, and NPKF increased the DOC stock compared to the control. Earlier findings also reported higher values of DOC in NPKF-treated plots than the NPK (Benbi et al., 2015; Li et al., 2020; Mustafa et al., 2020). Approximately 90% of roots in guinea grass are distributed in the top 0–0.40 m depth (Singh, 1996). Therefore, the lowered rhizodeposition and root biomass in sub-surface soil were the reasons for decreased SOC content with increasing depth (Basak et al., 2021a; Anantha et al., 2022). The increased DOC stock in 0.3–0.7 m soil depth was mainly because of the leaching of the soluble soil organic molecules to lower depths through well-drained silty loam soil under the influence of irrigation and/or rainwater (Hussain et al., 2020).

The FYM applications rate was different for the treatment FYM and NPKF. Thus, the annual total C input to soil was greater in FYM than NPKF ($P < 0.05$). However, the content of passive pools was similar for FYM and NPKF treatments. Among the four pools (C_{VL}, C_L, C_{LL}, and C_{NL}), very labile and less-labile pools of SOC were sensitive to NM practices. Therefore, the C_{VL} fraction increased with annual C input. A major part of the sequestered SOC in NPK, FYM, and NPKF was retained in less labile pools (C_{LL}) (Table 3). A greater amount of SOC allocation in C_{LL} with similar treatments was earlier reported for sorghum–wheat and rice–wheat cropping systems (Datta et al., 2018; Singh and Benbi, 2018; Basak et al., 2021a).

Management practices affect soil quality and influence soil functions and crop productivity. The CMI indicating higher stable SOC is used to assess the impact of management options on soil productivity. The FYM and NPKF had greater values

TABLE 6 Annual return of crop residue C (Mg ha^{-1}), carbon sequestration potential indices for soil (0–0.7 m), yield, and sustainable yield index for different nutrient managements in perennial grass–legume system.

Treatment	Root biomass C	Stuble biomass C	Rhizodeposition C	Organic amendment C	Annual C input to the soil	TOC stock (Mg ha^{-1}	% C build-up over control	C build-up rate (Mg C ha^{-1} y^{-1})	C sequestration (Mg C ha^{-1} soil)	Percent SOC stabilization	Dry forage yield (Mg ha^{-1})	Sustainable yield index
Initial						56.5						
Control	1.92^{B}	0.89^{C}	1.06^{B}	–	3.87^{D}	57.36^{B}			0.82^{B}		220.5^{C}	0.53^{C}
NPK	2.23^{A}	1.29^{B}	1.23^{A}	–	4.74^{C}	69.56^{B}	21.28^{B}	1.22^{B}	13.02^{B}		302.2^{AB}	0.56^{B}
FYM	2.25^{A}	1.45^{A}	1.24^{A}	4.00^{A}	8.94^{A}	102.94^{A}	80.14^{A}	4.56^{A}	46.40^{A}	50.9	309.2^{A}	0.63^{A}
NPKF	2.16^{A}	1.33^{AB}	1.19^{A}	3.46^{B}	8.15^{B}	95.65^{A}	66.93^{A}	3.83^{A}	39.11^{A}	43.9	299.4^{B}	0.57^{B}

Values followed by different letters (uppercase for different treatments) within a column differ significantly by Duncan's multiple range test ($P < 0.05$).

of CMI content on imposing these practices. Greater SOC further corroborated this in less labile and non-labile pools under these treatments. The findings of this experiment were in agreement with the CMI values reported for groundnut (Anantha et al., 2022) and pearl millet–wheat systems (Moharana et al., 2012). The screening of MBC, DOC, C_{pp} for DFY and WBOC, and SI_m for SYI highlighted the significance of these parameters as complementary indicators for capturing the effect of nutrient management practices on soil properties and productivity potential of perennial grass–legume systems. Benbi et al. (2015) also identified MBC and DOC as the most sensitive indicator of management-induced changes in the soil.

The FYM and NPKF recorded a higher C build-up. The greater C sequestration in FYM-treated soils compared to NPK was mainly associated with a wider C: N ratio and lignin and polyphenols in FYM. These constituents of FYM form stable complexes with proteins of plant origin to protect them from further microbial attack with the resultant stabilization of SOC in soils (Majumder et al., 2008b). Earlier studies reported approximately 27% stabilization of the applied C in long-term experiments in semi-arid regions of India (Srinivasarao et al., 2012; Anantha et al., 2022). The C–stabilization vary 44–51% in FYM and NPKF treatment in perennial grass–legume cropping. The C stabilization is the outcome of the interaction of the factors *viz.*, C inputs and their biochemical constituents, soil properties, soil perturbation, covering of soil surface, and net "fallowing period" in the calendar year (Schmidt et al., 2011). In this study, large biomass return, the smothering effect of component crops, and minimum soil disturbances protected the applied carbon from oxidation and were responsible for greater SOC stabilization. Lower carbon addition and increased nitrogen availability promoting organic matter decomposition in NPKF were responsible for low SOC stabilization in NPKF compared to FYM.

5. Conclusion

The perennial grass–legume-based cropping system favors the build-up of SOC. Nutrient management through organic manure alone, in combination with NPK fertilizers, had a greater potential to sequester carbon in the soil. Integrated nutrient management and sole FYM partitioned a greater proportion of SOC in the non-labile pool with a 44–51% stabilization of the applied carbon. FYM alone or in combination with NPK also improved the carbon management index and stratification ratio. The dissolved organic and microbial biomass C and SOC in passive pools were identified for predicting the dry fodder yield. Walkley–Black oxidizable organic C and sensitivity indices of SOC pools were good predictors for the sustainable yield index of the perennial grass–legume system. Thus, this study concludes that organics and integrated nutrient management practices can ensure the stay of the SOC in soil for a longer period. Integrating perennial grass–legume-based cropping in the farming system can also provide an opportunity to maintain long-term sustainability and offset the farm enterprises' carbon footprint in similar agro-ecologies.

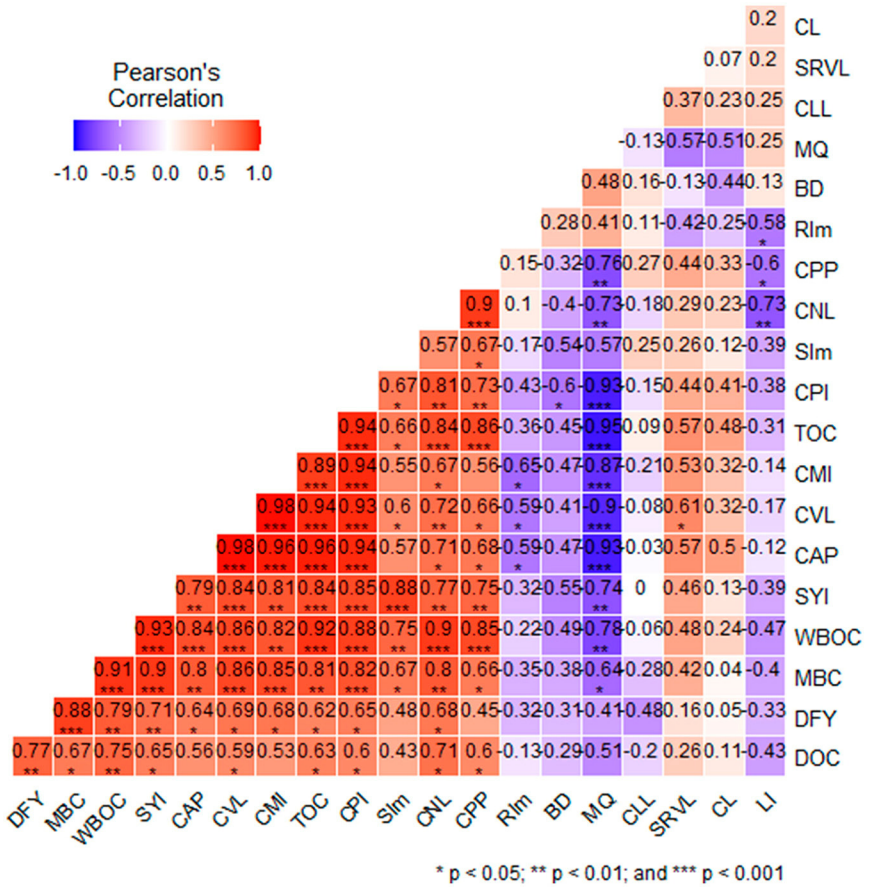

FIGURE 2
Correlation matrix among system yield attributes and SOC pools and their indices; DFY, dry forage yield; SYI, sustainable yield index; MBC, microbial biomass carbon; WBOC, Walkley–Black organic carbon; TOC, total organic carbon; DOC, dissolved organic C; RI_{mean}, mean recalcitrant index; C_{VL}, very labile pool of soil organic carbon; C_L, labile pool of soil organic carbon; C_{LL}, less labile pool of soil organic carbon; C_{AP}, active pool of soil organic carbon; C_{PP}, passive pool of soil organic carbon; RI_m, mean recalcitrant indices; CPI, carbon pool index; CMI, carbon management index; BD, bulk density; MQ, microbial quotient; SR_{VL}, stratification ratio of labile pool of soil organic carbon; SI_{mean}, mean sensitivity index; without *, *, ** and *** represent non-significant, significance at $P < 0.05$, 0.01, and 0.001.

TABLE 7 Regression (backward elimination models) between soil parameters and dependent variables.

Dependent variable	Intercept	Independent variable I	Independent variable II	Independent variable III	R^2	Adjusted R^2
DFY	30.5 ± 19.0	163.3*** ± 24.2(MBC)	341.5*** ± 65.3 (DOC)	−6.15*** ± 1.51(C_{PP})	0.96	0.95
SYI	0.46*** ± 0.02	0.002*** ± 0.003(WBOC)	0.0004** ± 0.0001(SI_m)		0.96	0.95

** and ***represent significance at $P < 0.01$ and 0.001; DFY, dry fodder yield; SYI, sustainable yield index; MBC, microbial biomass C; DOC, dissolved organic C; WBOC, Walkley–Black oxidizable organic C; SI_{mean}, mean sensitivity index.

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

AR: conceptualization, formal analysis, funding acquisition, and original draft preparation and final editing. NB: conceptualization, formal analysis, and original draft preparation and final editing. AD: investigation and methodology. SR and JS: investigation. SD and TK: data curation and investigation. SK: investigation and funding acquisition. PC, PS, and SB: data curation and preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

The reviewer SS declared a past co-authorship with the author SB to the handling editor.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1173986/full#supplementary-material

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EDITED BY
Dilfuza Egamberdieva,
Leibniz Center for Agricultural Landscape Research (ZALF), Germany

REVIEWED BY
Murugan Kumar,
National Bureau of Agriculturally Important Microorganisms (ICAR), India
Vyacheslav Vladimirovich Shurigin,
Chinese Academy of Sciences (CAS), China

*CORRESPONDENCE
Qiujun Lu
✉ luqiujun19@gmail.com
Jianli Yan
✉ yanjianli00@gmail.com

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Effects of SMOF on soil properties, root-zone microbial community structure, metabolites, and maize (*Zea mays* L.) response on a reclaimed barren mountainous land

Xuqing Li[1], Daoze Wang[2], Qiujun Lu[3]*, Zhongling Tian[4] and Jianli Yan[1]*

[1]Institute of Vegetable, Hangzhou Academy of Agricultural Sciences, Hangzhou, China, [2]Hangzhou Service Center for Rural Revitalization, Hangzhou, China, [3]Hangzhou Agricultural and Rural Affairs Guarantee Center, Hangzhou, China, [4]Key Laboratory of Pollution Exposure and Health Intervention of Zhejiang Province, Interdisciplinary Research Academy (IRA), Zhejiang Shuren University, Hangzhou, China

Introduction: Maize is the largest crop produced in China. With the growing population and the rapid development of urbanization and industrialization, maize has been recently cultivated in reclaimed barren mountainous lands in Zhejiang Province, China. However, the soil is usually not suitable for cultivation because of its low pH and poor nutrient conditions. To improve soil quality for crop growth, various fertilizers, including inorganic, organic, and microbial fertilizers, were used in the field. Among them, organic fertilizer-based sheep manure greatly improved the soil quality and has been widely adopted in reclaimed barren mountainous lands. But the mechanism of action was not well clear.

Methods: The field experiment (SMOF, COF, CCF and the control) was carried out on a reclaimed barren mountainous land in Dayang Village, Hangzhou City, Zhejiang Province, China. To systematically evaluate the effect of SMOF on reclaimed barren mountainous lands, soil properties, the root-zone microbial community structure, metabolites, and maize response were investigated.

Results: Compared with the control, SMOF could not significantly affect the soil pH but caused 46.10%, 28.28%, 101.94%, 56.35%, 79.07%, and 76.07% increases in the OMC, total N, available P, available K, MBC, and MBN, respectively. Based on 16S amplicon sequencing of soil bacteria, compared with the control, SMOF caused a 11.06–334.85% increase in the RA of *Ohtaekwangia, Sphingomonas, unclassified_Sphingomonadaceae*, and *Saccharibacteria* and a 11.91–38.60% reduction in the RA of *Spartobacteria, Gemmatimonas, Gp4, Flavisolibacter, Subdivision3, Gp6*, and *unclassified_Betaproteobacteria*, respectively. Moreover, based on ITS amplicon sequencing of soil fungi, SMOF also caused a 42.52–330.86% increase in the RA of *Podospora, Clitopilus, Ascobolus, Mortierella*, and *Sordaria* and a 20.98–64.46% reduction in the RA of *Knufia, Fusarium, Verticillium,* and *Gibberella,* respectively, compared with the control. RDA of microbial communities and soil properties revealed that the main variables of bacterial and fungal communities included available K, OMC, available P, MBN, and available K, pH, and MBC, respectively. In addition, LC-MS analysis indicated that 15 significant DEMs belonged to benzenoids, lipids, organoheterocyclic compounds, organic acids, phenylpropanoids, polyketides, and organic nitrogen compounds in SMOF and the control group, among which four DEMs were significantly correlated with two genera of bacteria and 10 DEMs were significantly correlated with five genera of fungi. The results revealed complicated interactions between microbes and DEMs in the soil of the maize root zone. Furthermore, the results of

field experiments demonstrated that SMOF could cause a significant increase in maize ears and plant biomass.

Conclusions: Overall, the results of this study showed that the application of SMOF not only significantly modified the physical, chemical, and biological properties of reclaimed barren mountainous land but also promoted maize growth. SMOF can be used as a good amendment for maize production in reclaimed barren mountainous lands.

KEYWORDS

SMOF, soil property, microbial community, metabonomics, maize, reclaimed barren mountainous land

1. Introduction

Maize (*Zea mays* L.) originated in the highlands of Mexico ~8,700 years ago (Piperno et al., 2009), is the second most plentiful crop globally (Ort and Long, 2014); it is also the largest crop produced in China (Gong et al., 2015). However, with the growing population and the rapid development of urbanization and industrialization, achieving grain supply security on limited arable land is a major challenge in contemporary times (Gong et al., 2015). In recent years, the barren mountainous areas were reclaimed for agricultural use in Zhejiang Province, China, to meet the demand for cultivated land (Wang et al., 2017). However, in most situations, reclaimed barren mountainous lands were not suitable for cultivation due to their acidic soil and nutrient-poor conditions (Li et al., 2023). Therefore, to develop the mountainous maize industry on reclaimed mountainous lands in Zhejiang Province, China, it is necessary to find effective measures to improve soil quality for the growth of maize.

In the last decades, to meet the requirements of crops, many farmers have resorted to using inorganic fertilizers (including commercial compound fertilizers, CCF). However, prolonged and excessive use of inorganic fertilizers has resulted in soil acidification, hardening, ecosystem degradation, environmental pollution, and so on (Lam et al., 2012; Hu et al., 2014; Roy et al., 2014; Gregorich et al., 2015; Yan et al., 2021). It is well-known that the quality of soil is affected by various factors (including physical, chemical, and biological properties). In the case of newly reclaimed lands, the quality of the soil can be greatly improved by increasing the use of organic fertilizers (such as crop residues, livestock manure, biogas liquid, and so on), which not only modify the physical and chemical properties of the soil but also have a great influence on the microbial communities (Li et al., 2021b, 2022a,b). For example, Li et al. (2021b) indicated that the soil quality was ameliorated by commercial organic fertilizers (COF), which caused a significant increase in pH, organic matter contents (OMC), available phosphorus (P), total nitrogen (N), alkaline hydrolysis N, total bacterial numbers, microbial biomass carbon (MBC), and a greatly increased height and total dry weight of maize seedlings in newly reclaimed lands compared with the control. Li et al. (2022b) showed that two kinds of COF, including PMMR-OF and SM-OF, not only significantly increased the weight of air-dried corn on newly reclaimed lands but also significantly changed the microbiota and chemical properties of corn soil. Li et al. (2022a) discovered that COF, SM, and MR not only significantly changed the pH of the soil but also increased the OMC considerably (in particular SM) and also caused a differential change in the bacterial and fungal community of the newly reclaimed lands.

Sheep manure organic fertilizer (SMOF) has recently been widely used on reclaimed barren mountainous lands in Hangzhou City, Zhejiang Province, China. Based on our previous research, organic fertilizer-based sheep manure had a beneficial effect on the growth of maize by improving the soil quality of newly reclaimed lands (Li et al., 2022b). However, the specific relationships among soil, microbes, and maize on reclaimed barren mountainous lands after applying SMOF were unclear. Thus, the aim of the study was to systematically evaluate the effect of SMOF on maize growth, root-zone soil properties, microbial community structure, and metabolites in reclaimed barren mountainous lands. In addition, we examined the correlation between soil properties, the microbial community structure, the correlation between differentially expressed metabolites (DEMs), and related microbes. This study provided a scientific basis for applying SMOF to promote maize cultivation on reclaimed barren mountainous lands in the future.

2. Materials and methods

2.1. Experimental design

The field experiment was carried out on a reclaimed barren mountainous land in Dayang Village (29°25′36″N; 119°28′52″E; 200 m above sea level), Hangzhou City, Zhejiang Province, China. The soil type was acidic red with gravel, based on the soil classification system of the FAO-UNESCO, and the initial properties of the top 20 cm of soil were as follows: pH 5.94, OMC 1.49%, total N 0.98 g/kg, available P 50.64 mg/kg, and available K 144.85 mg/kg.

Three kinds of fertilizers were used in the field experiment: (1) sheep mature organic fertilizer (SMOF, mostly consisting of sheep manure, NPK ≥ 4%, OMC ≥ 30%) was provided by Hangzhou Nanwuzhuang Soil Fertilizer Co., Ltd. (Hangzhou, China); (2) commercial organic fertilizer (COF, mostly consisting of chicken manure and mushroom residues, NPK ≥ 4%, OMC ≥ 30%) was provided by Jiande Kairong Soil Fertilizer Co., Ltd. (Hangzhou, China); and (3) chemical compound fertilizer (CCF, N-P-K, 16-16-16, NPK = 48%, containing nitrate N and sulfate K) was provided by Shenzhen Batian Ecological Engineering Co., Ltd.

(Shenzhen, China). The dosage of SMOF, COF, and CCF in this study was set according to the local farmers' habits. In detail, through the application of different fertilizers to the reclaimed barren mountainous land, the field experiment consisted of four different treatments: (1) SMOF at 1.50 kg/m^2; (2) COF at 1.50 kg/m^2; (3) CCF at 75 g/m^2; and (4) the treatment without any fertilizer was applied as the control.

The field experiment was randomly designed and conducted from 6 April to 2 July 2022. The area of each plot was 24 m^2 (length 7.5 m and width 3.2 m, respectively), and the planting density of maize was 35 × 50 cm. On 6 April, the top 0–20 cm of the soil of the experimental field was mixed with different fertilizers before planting, and then the seedlings of maize (cultivar "qianjiangtian 1", provided by Hangzhou Academy of Agricultural Sciences, Hangzhou, China) were planted in the above-mentioned field. In detail, the maize seeds were placed into 50-cell seedling trays containing reclaimed barren mountainous land soil on 1 March and then kept at 25°C and 65−75% relative humidity for 5 weeks. Fresh maize ears and plants were harvested and measured after 87 days of planting. Each treatment had three replicates.

2.2. Measure of maize parameters and soil properties

To evaluate the impact of SMOF on the biomass of maize in the reclaimed barren mountainous land, the fresh weight of maize ears and plants was measured using a digital scale (TCS-50, Shanghai Hento Industrial Co., Ltd., Shanghai, China) after 87 days of planting. Growth promotion efficacy (GPE%) was calculated using the following formula: GPE% = (treatment − control)/control × 100%.

Moreover, soil pH, OMC, total N, available P, available K, MBC, and microbial biomass nitrogen (MBN) were detected as previously described (Jackson, 1973; Brookes et al., 1985; Vance et al., 1987; Baran et al., 2019). In detail, when maize ears and plants from each plot were collected, ∼1.0 kg of fresh root-zone soil (5–20 cm) was sampled simultaneously. After drying at room temperature and passing through a 0.45-mm sieve to remove fine roots and debris, the soil properties were measured. In brief, soil pH was measured at a soil/distilled water suspension ratio of 1:5 (g/mL) with a pH meter (FE28, Mettler-Toledo, Zurich, Switzerland); the content of organic matter was determined by the $K_2Cr_2O_7$ oxidation external heating method; total N was determined using an automatic Kjeldahl distillation-titration unit; available P was determined by hydrochloric acid–ammonium fluoride extraction molybdenum–antimony anti–colorimetry; available K was extracted with 1 M ammonium acetate and determined by a flame photometer; MBC and MBN were determined using the chloroform fumigation extraction method. All the treatments had three replicates.

2.3. Genome sequencing

When maize ears and plants were collected on 2 July 2022, each plot sampled 20 g of maize root-zone soil and stored it at −80°C. DNA was extracted from the soil samples using the E.Z.N.A™ Mag–Bind Soil DNA Kit (OMEGA, Norcross, GA, USA) according to the manufacturer's protocols. The DNA quality was assessed using a Qubit® 3.0 fluorometer (ThermoFisher Scientific, USA).

The V3–V4 region of maize root-zone bacterial 16S rRNA genes and the ITS1 region of fungal ITS genes were amplified using the universal primers 341F (5′-CCT ACG GGN GGC WGC AG−3′) and 805R (5′-GAC TAC HVG GGT ATC TAA TCC−3′) (Wu et al., 2015), and ITS1F (5′-CTT GGT CAT TTA GGA AGT AA−3′) and ITS2 (5′-GCT GCG TTC TTC ATC GAT GC−3′) (Adams et al., 2013), respectively. The PCR assay volume was 30 μl, including 15 μl 2 × Hieff® Robust PCR Master Mix, 1 μl (10 μM) of each universal primer, 1 μl of DNA template, and 12 μl of ddH_2O. The PCR thermal protocol generally consisted of an initial denaturation at 94°C for 3 min, 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. After the PCR amplicons were purified with Hieff NGS™ DNA selection beads (Yeasen, Shanghai, China), they were pooled in equal amounts, and pair-end (2× 250 bp) sequencing was accomplished using the Illumina MiSeq™/Hiseq™ system (Sangon Biotechnology Co., Ltd., Shanghai, China).

The bioinformatics analysis of the microbe was conducted following the methodology used in our previous studies (Jiang et al., 2022; Li et al., 2023). In other words, to ensure high data quality, the raw data were preprocessed using PRINSEQ to remove low-quality reads (average quality score < 20) (Schmieder and Edwards, 2011) and were merged using PEAR (v0.9.8) (Zhang et al., 2014). After the primers were trimmed with Cutadapt (v1.18) (Martin, 2011), clean reads were analyzed using Usearch with a 97% similarity cutoff to generate operational taxonomic units (OTUs) (Edgar, 2013, 2016). After selection of the representative read of each OTU using the QIIME package (v2020.06) (Caporaso et al., 2010), all 16S rDNA and ITS representative reads were annotated by blasting against the RDP database using the RDP classifier (Wang et al., 2007; Quast et al., 2013), and the UNITE database using BLAST (Altschul et al., 1997; Urmas et al., 2013), respectively.

2.4. Metabolomics assay

Maize root-zone soil samples were collected on 2 July 2022, and 10 g of soil from each plot was stored at −80°C. After thawing at 4°C, 0.1 g of each soil sample was extracted in a 1 ML extract solution (methanol: H_2O = 3:1, with the isotopically labeled internal standard mixture), homogenized at 35 Hz for 4 min, and sonicated for 5 min in an ice-water bath (repeat three times). Then, the samples were incubated at −40°C for 1 h, centrifuged at 4°C for 15 min (12,000 rpm), and the supernatant was transferred into a new glass vial for further analysis. Indeed, the liquid chromatography-mass spectrometry (LC-MS) analyses were performed using a UHPLC system (Vanquish, Thermo Fisher Scientific), and the conditions were set as follows: chromatographic column: waters ACQUITY UPLC HSS T3 column (1.8 μm, 2.1 mm × 100 mm); mobile phase A: 5 mM ammonium acetate and 5 mM acetic acid in the water; mobile phase B: acetonitrile; column temperature: 4°C, injection volume: 2 μl; gradient program: 0–0.5 min, 95% B; 0.5–7 min, 95% B; 7–8 min, 65−40% B; 8–9 min,

40% B; 9–9.1 min, 40−95% B; 9.1–12 min, 95% B. The ESI source conditions were set as follows: sheath gas flow rate, 50 Arb; aux gas flow rate, 15 Arb; capillary temperature, 320°C; full MS resolution, 60,000; MS/MS resolution, 15,000 collision energy as 10/30/60 in NCE mode; spray voltage, 3.8 kV (positive) or 3.4 kV (negative), respectively. The repeatability and reliability of the analysis process were evaluated by inserting one quality control (QC) sample, which was prepared by mixing an equal aliquot of the supernatants from all samples. All the treatments had six replicates. The Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo) was used to acquire MS/MS spectra, and the obtained data in this study were converted to the mzXML format using ProteoWizard and processed with an in-house program, then an in-house MS2 database (SANGON) was applied for metabolite annotation.

2.5. Statistical analysis

The SPSS software (v16.0, SPSS Inc., Chicago, IL, USA) was used to perform one-way variance analysis (ANOVA). Origin software (v2023, Hampton, MA, USA) was employed for analyzing OTUs and alpha diversity indices (including Chao1, Shannon, and the Simpson index). The principal component analysis (PCA) based on beta diversity metrics from the Bray–Curtis metrics was utilized to observe the structural variation of root-zone soil microbes across samples used in this study (Ramette, 2007). The differences in root-zone soil microbes among different samples were tested using permutational multivariate ANOVA (PERMANOVA), with 999 permutations used to calculate p-values (Dixon, 2003). To further observe the differentially abundant microbes between groups, linear discriminant analysis effect size (LEfSe) was conducted to discover the differential biomarkers and estimate the biomarkers' effect size (Segata et al., 2011). To investigate the impact of different environmental factors (such as pH, OMC, total N, available P, available K, MBC, and MBN) on microbial community structure, redundancy discriminant analysis (RDA) was performed using Origin (v2022, Hampton, MA, USA). To investigate the impact of different fertilizers on microbial co-occurrence patterns, a Sparcc correlation coefficient among maize root-zone soil microbial communities was measured based on the relative abundance (RA) of OTUs at different treatments (SMOF, COF, CCF, and control). The high RA (>1%) and statistically significant correlations ($p < 0.01$, Sparcc's correlation $N > 0.5$ or <-0.5) among OTU levels were built into the network analysis. To observe the topology of the co-occurrence networks, the network graph analysis was performed based on these measurements, including average degree, modularity, nodes, and edges. To investigate the effect of SMOF on the metabolites, orthogonal projections to latent structures-discriminant analysis (OPLS-DA), volcano plots, heat maps, chord plots, and matchstick analyses on four different treatments were conducted with the MetaboAnalyst 4.0 platform. The screening thresholds for DEMs were set as variable importance in the projection (VIP) > 1 and $p < 0.05$. To investigate the association between DEMs and differential microbes in different treatment groups, a Spearman correlation coefficient among the high RA of maize root-zone soil microbes (top 40 bacteria or fungi at the genus level) and significant DEMs (the largest VIP value, $p < 0.05$) was measured by clustering a hot map (Hollander et al., 2008).

3. Results

3.1. Impacts of SMOF on maize production

To evaluate the impacts of SMOF on maize production in the reclaimed barren mountainous land, the fresh weight of maize ears and plants was measured after 87 days of planting when harvested. The results showed that SMOF significantly promoted the growth of maize under field conditions (Table 1). Compared to the control, SMOF caused a 12.29 and 10.53% increase in the fresh weight of maize ears and plants, respectively, while COF caused a 9.72 and 21.57% increase, and CCF resulted in a 0.83% reduction and 15.74% increase, respectively. Furthermore, the fresh weight of maize ears was found to be 1.13-fold higher in the SMOF treatment, while in the COF treatment, it was 1.11-fold higher compared to the CCF treatment. These results suggest that SMOF had a greater effect on promoting the growth of maize ears compared to the other treatments (including COF, CCF, and the control).

3.2. Impacts of SMOF on the pH of the soil, chemical properties, and total biomass

The results indicated that there was no significant difference in the pH of the soil between SMOF and the control, but SMOF significantly increased the OMC, total N, available P, available K, MBC, and MBN (Table 2). In other words, compared with the control, SMOF caused a 0.50% increase, while COF and CCF caused a 1.67 and 7.18% reduction in the soil pH; the soil OMC was significantly increased by 46.10%, 26.24%, and 40.43% by SMOF, COF, and CCF, respectively; SMOF, COF, and CCF caused a significant increase by 28.28%, 20.20%, and 26.26% in total N, respectively; the content of available P and available K was significantly increased by SMOF, COF, and CCF, with a 101.94%, 67.49%, 53.51%, and 56.35%, 20.80%, and 31.80% increase, respectively. In addition, compared to the control, the MBC and MBN values were unaffected by CCF but significantly increased by SMOF and COF, with 79.09%, 66.01% (for MBC), and 76.07%, 63.81% (for MBN) increases, respectively.

3.3. Impacts of SMOF on the microbial community of maize

The original microbial sequence data of all soil samples from four different treatments (SMOF, COF, CCF, and the control) were quality-controlled, and a total of 2,109,642 high-quality 16S rRNA gene sequences were obtained from the high-throughput amplicon sequencing. Among them, the high-quality sequences of each sample range from 154,878 to 193,807. A total of 92,480 OTUs from 13 bacterial phyla were identified, and the distribution of OTUs across all four treatments is shown in Figure 1A. Compared with the control, the number of bacterial OTUs was significantly increased by SMOF, COF, and CCF, with a 11.03%, 4.68%, and

TABLE 1 Impacts of SMOF on maize growth promotion.

Treatments	Weight of maize ears (kg)	GPE%	Weight of maize plants (kg)	GPE%
SMOF (1.50 kg/m^2)	38.52 ± 0.99a	12.29a	47.75 ± 0.88b	10.53b
COF (1.50 kg/m^2)	37.63 ± 2.40a	9.72a	52.52 ± 1.15a	21.57a
CCF (75 g/m^2)	34.02 ± 0.88b	−0.83b	50.00 ± 0.83b	15.74ab
Control	34.30 ± 1.84b	–	43.20 ± 1.65c	–

GPE, growth promotion efficacy; SMOF, sheep mature organic fertilizer; COF, commercial organic fertilizer; CCF, chemical compound fertilizer; control, without any fertilizer.
Means are averages ± standard deviations (SD). Different lowercase letters within the same columns reveal the significance among different treatments ($p < 0.05$).

TABLE 2 Impacts of SMOF on the pH, chemical properties, and total biomass of maize soil.

Treatments	pH	OMC (%)	Total N (g/kg)	Available P (mg/kg)	Available K (mg/kg)	MBC (mg/kg)	MBN (mg/kg)
SMOF (1.50 kg/m^2)	6.02 ± 0.16a	2.06 ± 0.08 a	1.27 ± 0.05a	99.94 ± 7.34a	229.68 ± 7.54a	106.31 ± 4.47a	201.53 ± 2.12a
COF (1.50 kg/m^2)	5.89 ± 0.09a	1.78 ± 0.06ab	1.19 ± 0.04a	82.89 ± 1.62b	177.45 ± 6.79c	98.56 ± 4.28a	187.50 ± 6.44b
CCF (75 g/m^2)	5.56 ± 0.19b	1.98 ± 0.03a	1.25 ± 0.06a	75.97 ± 3.18b	193.62 ± 8.37b	62.77 ± 6.06b	116.46 ± 5.34c
Control	5.99 ± 0.11a	1.41 ± 0.11b	0.99 ± 0.08b	49.49 ± 4.09c	146.90 ± 4.47d	59.37 ± 1.25b	114.46 ± 3.15c

OMC, organic matter contents; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; SMOF, sheep mature organic fertilizer; COF, commercial organic fertilizer; CCF, chemical compound fertilizer; Control, without any fertilizer.
Means are averages ± standard deviations (SD). Different lowercase letters within the same columns reveal the significance among different treatments ($p < 0.05$).

6.50% increase, respectively. Similarly, after the raw data were quality-controlled, a total of 4,417,896 high-quality ITS gene sequences were obtained from all soil samples from the four treatments. Among them, the high-quality sequences of each sample range from 215,206 to 475,487. A total of 14,768 OTUs from six fungal phyla were identified, and the distribution of OTUs across all four treatments is shown in Figure 1B. The fungal OTU number was slightly increased (1.70%, 0.67%, and 8.31%, respectively) by SMOF, COF, and CCF compared with the control. Overall, the average bacterial OTU number was 6.65-, 6.33-, 5.99-, and 6.09-fold greater than that of fungi in SMOF, COF, CCF, and the control, respectively. Compared to the control, SMOF caused a greater ratio of bacterial to fungal OTU distribution.

The RA of the top 10 bacterial classes (Figure 2A) and the whole eight fungal classes (Figure 2C) were observed across all the maize soil samples. Moreover, the top 10 bacterial genera (Figure 2B) and fungal genera (Figure 2D) with an average RA > 1% were highlighted across all the maize soil samples. The results showed that the application of SMOF resulted in significant changes in the microbial community composition of maize root-zone soil in the reclaimed barren mountainous land at the class and genus levels compared to the control. In other words, compared with the control, SMOF significantly increased the RA of *Cytophagia* (153.37%), *Gammaproteobacteria* (39.71%), *Bacilli* (37.97%), *Deltaproteobacteria* (12.47%), *Alphaproteobacteria* (12.20%), and *Saccharibacteria* (11.06%) while also reducing the RA of *Anaerolineae* (66.20%), *Spartobacteria* (35.67%), *Gemmatimonadetes* (30.65%), *Acidobacteria_Gp4* (22.46%), *Subdivision3* (18.35%), and *Acidobacteria_Gp6* (14.27%) at the class level of bacteria. Furthermore, SMOF significantly increased the RA of *Ohtaekwangia* (334.85%), *Sphingomonas* (54.90%), *unclassified_Sphingomonadaceae* (17.76%), and *Saccharibacteria* (11.06%) while reducing the RA of *Spartobacteria* (38.60%), *Gemmatimonas* (30.65%), *Gp4* (27.99%), *Flavisolibacter* (20.17%), *Subdivision3* (18.58%), *Gp6* (14.27%), and *unclassified_Betaproteobacteria* (11.91%) at the genus level of bacteria. Similarly, compared with the control, SMOF significantly increased the RA of *Mortierellomycetes* (70.79%), *Sordariomycetes* (21.02%), *Agaricomycetes* (13.93%), and *Pezizomycetes* (11.19%) while reducing the RA of *Rozellomycotina* (82.16%), *Eurotiomycetes* (63.72%), *Dothideomycetes* (48.33%), and *Tremellomycetes* (15.41%) at the class level of the fungus. Additionally, SMOF significantly increased the RA of *Podospora* (330.86%), *Clitopilus* (86.46%), *Ascobolus* (85.81%), *Mortierella* (74.15%), and *Sordaria* (42.52%) and also reduced the RA of *Knufia* (64.46%), *Fusarium* (42.84%), *Verticillium* (36.22%), and *Gibberella* (20.98%) at the genus level of the fungus. All these results indicated that the changes in the number of specific bacteria and fungi in different treatments might be due to the different nutrients under different fertilizer conditions, and different fertilizers, including SMOF, could affect the abundance of bacteria and fungi in the root-zone soil of maize to regulate the composition of the microbial community.

3.4. Impacts of SMOF on microbial α- and β-diversity of maize

The alpha diversity analysis evaluated microbial species richness and diversity within each maize soil sample based on three indices, including Chao1, Shannon, and Simpson (Figure 1). Compared with the control, the bacterial Chao1 index was significantly increased by SMOF (7.91%) and CCF (5.60%), but no significant change was observed in the Shannon and Simpson index of bacterial community among all four different treatments. Moreover, no significant difference was found in the Chao1, Shannon, and Simpson index of fungal community among all four treatments. The results revealed that the bacterial Chao1 index was 5.60-, 5.83-, 5.77-, and 5.65-fold, and the bacterial Shannon index

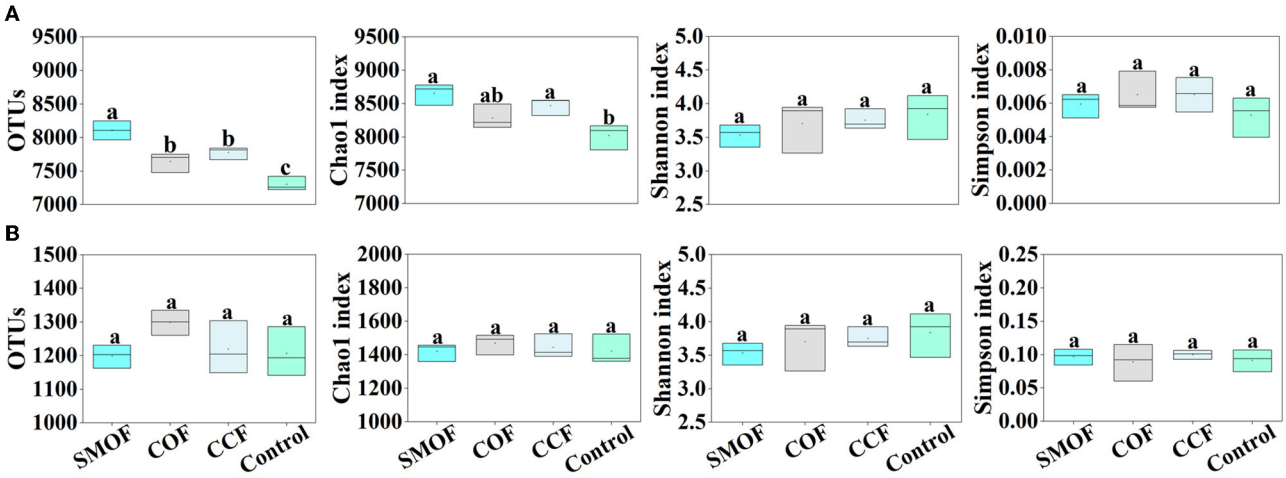

FIGURE 1
Impacts of SMOF on the OTU distribution, Chao1 richness index, Shannon's diversity index, and Simpson's diversity index of microbes in maize root-zone soil. **(A)** Bacteria, **(B)** fungi. Different lowercase letters above columns indicate statistical differences ($p < 0.05$).

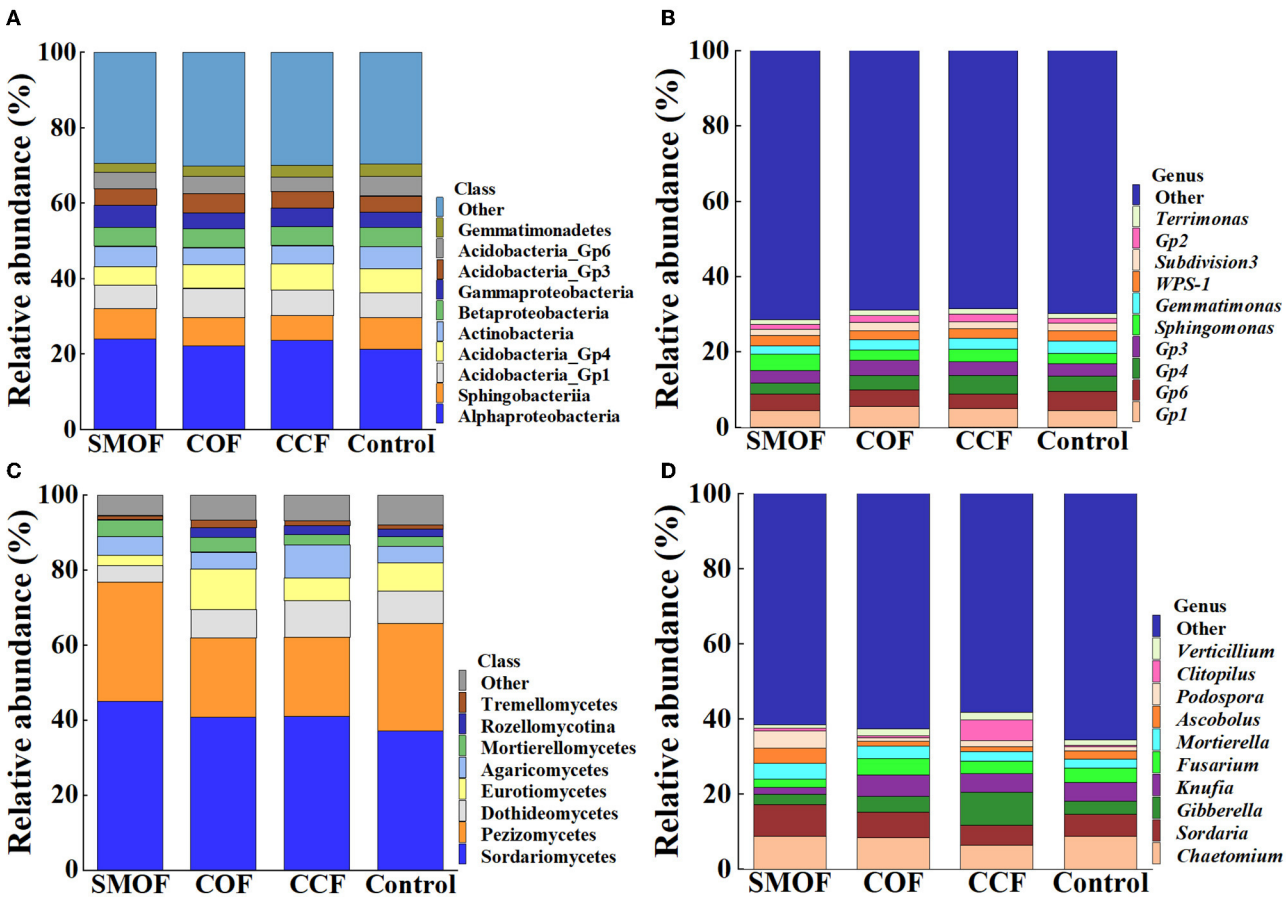

FIGURE 2
Relative abundance (RA) of the top 10 dominant bacterial **(A, B)** and fungal **(C, D)** classes and genera in four different treatments, respectively.

was 1.95-, 1.83-, 1.81-, and 1.78-fold greater than that of fungi in SMOF, COF, CCF, and the control, respectively. The bacterial Simpson index was 0.06-, 0.08-, 0.07-, and 0.05-fold less than fungi in SMOF, COF, CCF, and the control, respectively. Compared to the control, SMOF caused a greater ratio of bacterial and fungal diversity index (Shannon index), but the microbial richness

index (Chao1 index) and diversity index (Simpson index) were differentially affected by different treatments. Overall, SMOF could significantly increase the microbial richness of maize root-zone soil in the reclaimed barren mountainous land.

To further study SMOF on maize root-zone microbial communities, principal component analysis (PCA) at the OTU level based on the Bray–Curtis distance was performed (Figure 3). The PCA analysis revealed that the soil root-zone bacterial communities of SMOF, COF, CCF, and the control formed four different groups, and SMOF was well separated from the other three treatments. The first principal component (PCA1) revealed 15.63% of the variability in the bacterial community, and the second principal component (PCA2) explained 12.11%. A PERMANOVA on samples of all four treatments also showed that different fertilizers explained 19.3% of the variation ($p = 0.026$) (Figure 3A). In addition, the PCA analysis of fungal community structure indicated that all samples from four treatments were divided into four groups. However, there was an overlap among all four treatments (Figure 3B). PCA1 and PCA2 explained 15.29 and 13.01% of the total variation in the fungal community, respectively. The bacterial community structure of maize root-zone soil was significantly changed by SMOF, but no significant change was observed in fungal community structure diversity.

3.5. Impacts of SMOF on the root-zone microbiome and biomarkers

Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify the biomarkers with significant differences between the maize root-zone soil microbial communities under SMOF, COF, CCF, and the control conditions (Figure 4). Results showed that 11 bacterial biomarkers were found in SMOF, CCF, and the control (Figure 4A). In other words, SMOF was enriched with two types of *Sphingomonadales*. CCF was enriched with *Opitutus*, *Opitutaceae*, *Opitutales*, and *Opitutae*. The control was enriched with *Pseudonocardiaceae*, *Microvirga*, *Methylobacteriaceae*, *Methylobacillus*, and *Frateuria*. In addition, a total of 15 fungal biomarkers were obtained in SMOF, COF, CCF, and the control (Figure 4B). Specifically, SMOF was enriched with *Ascobolus* and *Podospora*, COF was enriched with two types of *Cephalotrichum*, two types of *Dipodascus*, *Entoloma*, and two types of *Dendrosporium*, CCF was enriched with *Claroideoglomus*, *Thanatephorus*, *Psedophialophora*, and *Schizothecium*, and the control was enriched with *Penicillium* and *Herpotrichiellaceae*.

Furthermore, the difference in RA composition (class and genus level) of the root-zone microbial community under SMOF, COF, CCF, and the control was visually explained through heat maps (Figure 5). At the class level of bacteria, SMOF was enriched with *Gammaproteobacteria*, *Cytophagia*, *Deltaproteobacteria*, and *Alphaproteobacteria* but was reduced with *Spartobacteria*, *Anaerolineae*, and *Gemmatimonadetes* ($p < 0.05$); COF was enriched with *Acidobacteria_Gp1*, *Acidobacteria_Gp3*, *Saccharibacteria*, but was reduced with *Spartobacteria*, and *Anaerolineae* ($p < 0.05$); CCF was enriched with *Acidobacteria_Gp2*, *Planctomycetia*, and *Bacilli* but was reduced with *Sphingobacteriia* and *Acidobacteria_Gp6* ($p < 0.05$). At the genus level of bacteria, SMOF was enriched with *Sphingomonas*, *Ohtaekwangia*, and *unclassified_Alphaproteobacteria* but was reduced with *Spartobacteria* and *Gemmatimonas* ($p < 0.05$); COF was enriched with *Gp1*, *Gp3*, *unclassified_Rhizobiales*, and *Saccharibacteria* but was reduced with *WPS-1* ($p < 0.05$); CCF was enriched with *Gp2* and *Gp4* but was reduced with *unclassified_Chitinophagaceae*, *Gp6*, and *Flavisolibacter* ($p < 0.05$). Furthermore, at the class level of the fungus, SMOF was enriched with *Sordariomycetes* and *Mortierellomycetes* but was reduced with *Dothideomycetes*, *Eurotiomycetes*, and *Rozellomycotina* ($p < 0.05$); COF was enriched with *Tremellomycetes* but was reduced with *Pezizomycetes* ($p < 0.05$); CCF was enriched with *Agaricomycetes* but was reduced with *Pezizomycetes* ($p < 0.05$). At the genus level of the fungus, SMOF was enriched with *Podospora*, *Cristinia*, *Sordaria*, and *Mortierella* but was reduced with *Leptosphaeria*, *Knufia*, and *Fusarium* ($p < 0.05$); COF was enriched with *Aspergillus* but was reduced with *Pulvinula* and *Sodiomyces* ($p < 0.05$); CCF was enriched with *Clitopilus*, *Gibberella*, and *Trichoderma* but was reduced with *Chaetomium* and *Zopfiella* ($p < 0.05$). The results demonstrated that different fertilizer treatments, such as SMOF, COF, and CCF, had an impact on the presence or absence of specific microbial species, ultimately altering the community structure of the maize root-zone soil.

3.6. Impacts of SMOF on the RDA of soil properties and microbial communities

Redundancy discriminant analysis (RDA) was used to examine the correlation between environmental factors and microbial communities in maize root-zone soil (Figure 6; Table 3). The results showed that soil properties influenced the composition of microbial communities at the genus level. In addition, 53.42 and 47.40% of the cumulative variance of the root-zone microbial community-factor correction occurred at the bacterial (Figure 6A) and fungal (Figure 6B) genus levels, respectively. The contributions of the four main variables, available K, OMC, available P, and MBN, explained 25.64%, 25.36%, 25.15%, and 23.38% of the bacterial community at the genus level, respectively, while the contributions of the three main variables, available K, pH, and MBC, explained as 32.96%, 23.49%, and 22.67% of the fungal community at the genus level, respectively. All those suggested that available K, OMC, available P, MBN, and available K, pH, MBC were the main factors influencing the bacterial and fungal communities, respectively (Table 3). The results also showed a complex relationship between microbial growth and soil nutrient elements because different soil properties significantly affected microbial community compositions in maize root-zone soil.

3.7. Impacts of SMOF on co-occurrence networks of root-zone soil microbes

Co-occurrence networks were constructed to explore the complexity of connections between maize root-zone soil microbial

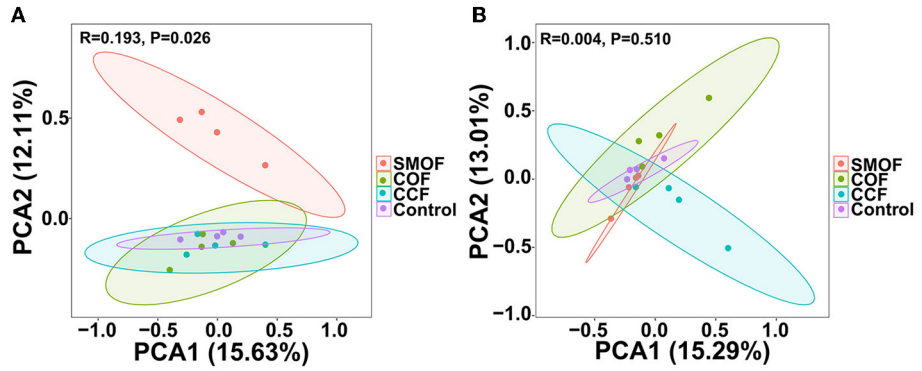

FIGURE 3
Principal component analysis (PCA) of the maize root-zone bacterial **(A)** and fungal **(B)** communities based on OTU abundance. Ellipses have been drawn for each treatment with a confidence limit of 0.95.

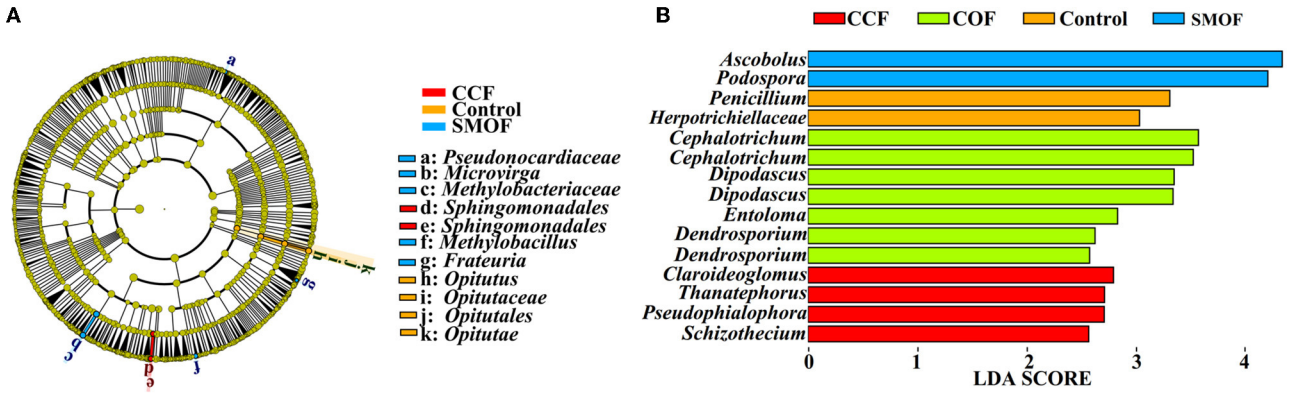

FIGURE 4
Linear discriminant analysis (LDA) effect size (LEfSe) of the bacterial **(A)** and fungal **(B)** taxa, which identifies the most differentially abundant taxa among SMOF, COF, CCF, and the control in different treatments. Only bacterial taxa with LDA values greater than two and fungal taxa with LDA values >2.5 ($p < 0.05$) are shown.

communities in SMOF, COF, CCF and the control. Moreover, the topological properties of the co-occurrence network were calculated to characterize the differences between different treatments (Figure 7). The bacterial network of SMOF consisted of 175 nodes and 205 edges (97 positive edges, 108 negative edges, and an average degree of 2.343), with modularity of −16.046. The COF, CCF, and control were 175 (positive: 71, negative: 104, average degree 2.500), 79 (positive: 79, negative: 0, average degree 1.756), and 225 (positive: 101, negative: 124, average degree 2.885), consisting of edges and 140, 90, and 156 nodes, respectively, and the modularity was −2.578, 0.798, and −6.410, respectively. Moreover, the fungal network of SMOF consisted of 125 nodes and 207 edges (103 positive edges, 104 negative edges, and an average degree of 3.312), with a modularity of −320.674. The COF, CCF, and control were 189 (positive: 90, negative: 99, average degree 2.953), 205 (positive: 113, negative: 92, average degree 2.929), and 217 (positive: 103, negative: 114, average degree 3.417), consisting of edges and 128, 140, and 127 nodes, respectively, and the modularity was −144.934, 3.317, and 1,311.843, respectively.

3.8. Impacts of SMOF on root-zone soil metabolomics

A total of 10,514 peaks were screened in maize root-zone soil from four different treatments, among which 277 metabolites were identified using LC-MS analysis. Furthermore, a score map of metabolites was constructed using OPLS-DA (Figures 8A–C; Table 4). Results showed that the distribution of different treatments could be effectively separated between SMOF, COF, CCF, and the control. Figure 8A presents the sampling distributions of SMOF treatment and the control in the positive and negative areas of t[1]P, respectively, while the model values of SMOF and the control were $R^2X(\text{cum}) = 0.431$, $R^2Y(\text{cum}) = 0.990$, and $Q^2(\text{cum}) = 0.306$. Similarly, Figure 8B presents the sampling distributions of COF treatment and the control in the positive and negative areas of t[1]P, respectively, while the model values of COF and the control were $R^2X(\text{cum}) = 0.364$, $R^2Y(\text{cum}) = 0.987$, and $Q^2(\text{cum}) = 0.269$. Meanwhile, Figure 8C presents the sampling distributions of CCF treatment and the control in the positive and negative areas of t[1]P, respectively, while the model values of CCF

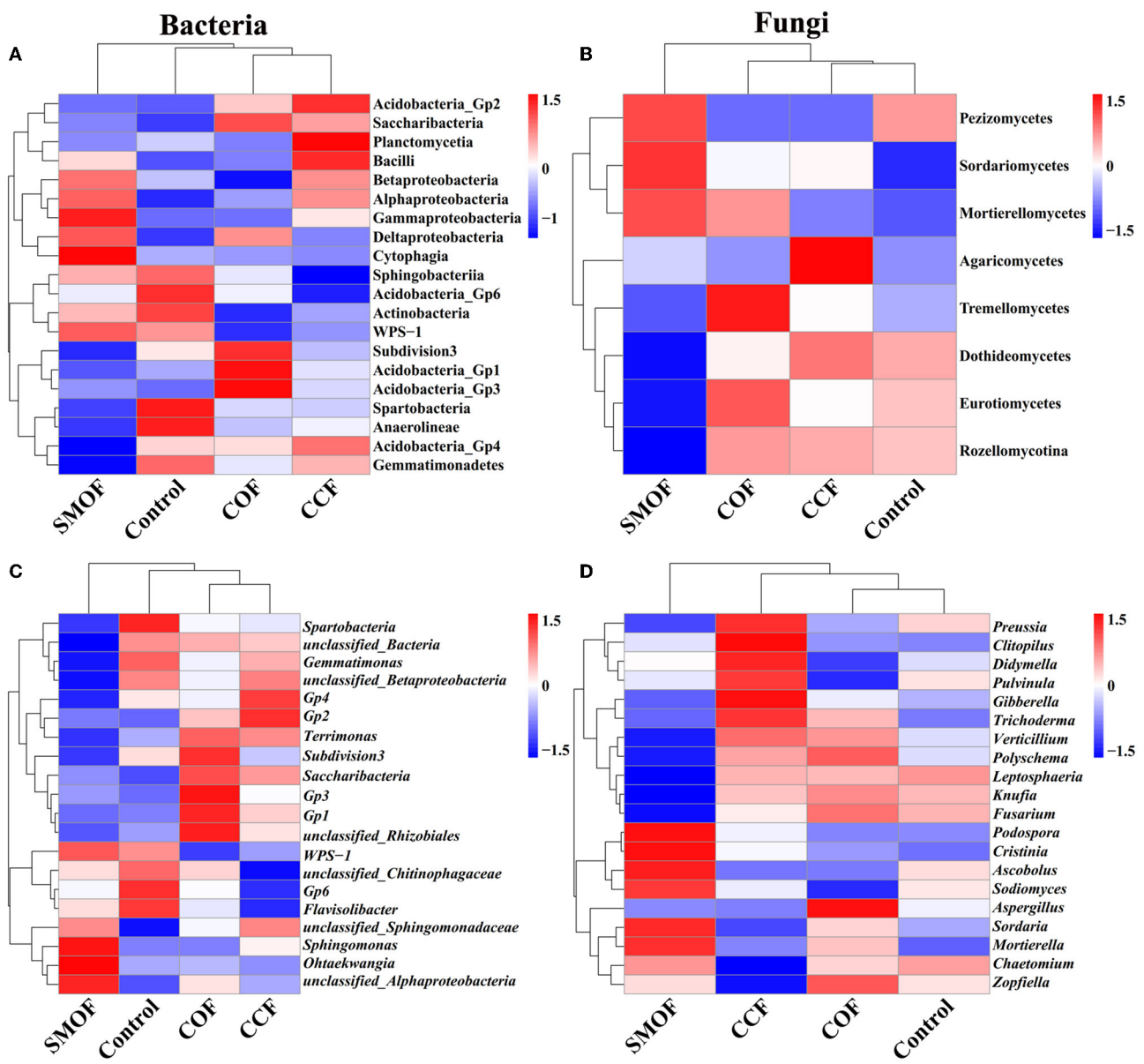

FIGURE 5
Hierarchical clustering analysis and heat maps of dominant bacterial and fungal community abundance at class **(A, B)** and genus **(C, D)** levels, respectively. The tree plot represents a clustering analysis of the dominant bacteria and fungi at class and genus levels according to their Pearson correlation coefficient matrix and relative abundance, and the upper tree plot represents a clustering analysis of soil samples according to the Euclidean distance of the data, respectively.

and the control were $R^2X(\text{cum}) = 0.310$, $R^2Y(\text{cum}) = 0.990$, and $Q^2(\text{cum}) = 0.519$. In view of the obvious separation of samples, it could be inferred that the metabolites in control root-zone soil were significantly changed by the application of the SMOF, COF, and CCF. Furthermore, the volcano plot (based on VIP > 1 and $p < 0.05$) also showed that the metabolites in the SMOF, COF, and CCF treatments were significantly different from the control (Figures 8D–F).

All the 277 metabolites mainly refer to lipids and lipid-like molecules (38.99%), organoheterocyclic compounds (14.44%), benzenoids (13%), organic acids and derivatives (9.75%), organic oxygen compounds (9.39%), organic nitrogen compounds (5.05%), and so on (Figure 9A). The results showed differences between the DEMs' numbers of SMOF, COF, or CCF and the control (Figures 9C–E). Indeed, there were 1,221 DEMs in the groups of SMOF and the control, with 1,107 upregulated and 114 downregulated (Figure 9C); 601 DEMs in the group of COF and the control, with 380 upregulated and 221 downregulated (Figure 9D); and 1,241 DEMs in the group of CCF and the control, with 854 upregulated and 387 downregulated (Figure 9E). Furthermore, to observe the change rules for metabolites, the metabolites with significant differences were normalized and shown in the hierarchical clustering heat map (Figure 10). Indeed, compared with the

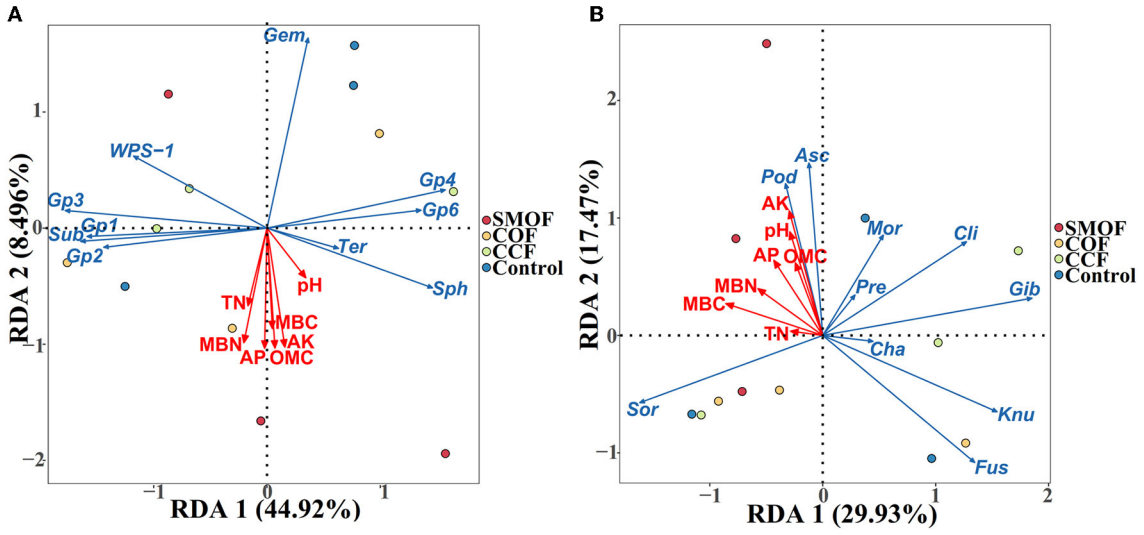

FIGURE 6
Redundancy discriminant analysis (RDA) of the root-zone microbial community compositions at genus levels with soil properties. **(A)** Bacteria. *Gem*, *Gemmatimonas*; *Sph*, *Sphingomonas*; *Sub*, *Subdivision3*; *Ter*, *Terrimonas*. **(B)** Fungi. *Asc*, *Ascobolus*; *Cha*, *Chaetomium*; *Cli*, *Clitopilus*; *Fus*, *Fusarium*; *Gib*, *Gibberella*; *Knu*, *Knufia*; *Mor*, *Mortierella*; *Pod*, *Podospora*; *Pre*, *Preussia*; *Sor*, *Sordaria*. OMC, organic matter contains; TN, total N; AP, available P; AK, available K; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen. Arrows indicate the direction and magnitude of soil properties (pH, OMC, total N, AP, AK, MBC, and MBN) associated with the different bacterial and fungal genera.

TABLE 3 Contribution of the soil environment to bacterial and fungal taxa at the genus level.

Soil environment	Contribution at bacterial genus level (%)	Contribution at fungal genus level (%)
pH	7.29	23.49
OMC	25.36	12.00
Total N	10.87	2.31
Available P	25.15	16.38
Available K	25.64	32.96
MBC	17.76	22.67
MBN	23.38	13.58

control, 34 DEMs (29 increased by 15.43−132.11%, five decreased by 16.91−73.92%), 12 DEMs (six increased by 14.28−61.68%, six decreased by 25.21−65.13%), and 29 DEMs (20 increased by 5.91−160.39%, nine decreased by 25.51−81.17%) were significantly changed by SMOF, COF, and CCF, respectively (Figures 9B, 10; Table 5).

The results also showed that three DEMs [tetramethylpyrazine, (3beta,5beta,8beta,22E,24xi)-Ergosta-6,22-diene-3,5,8-triol, daucol] were common metabolites in all three treatment groups. One DEM (N-Ethylglycine) was a common metabolite in the groups of SMOF or COF and the control; 11 DEMs [(10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,12-dienoic acid, scopolamine, sphingosine, stearidonic acid, (E)-3-(2-Hydroxyphenyl)-2-propenal, N-Ethylacetamide, docosapentaenoic acid (22n-6), (R)-Apiumetin glucoside, 9-HOTE, estriol-16-glucuronide, and 2-(Methylamino)benzoic acid] were common metabolites in the groups of SMOF or CCF and the control; four DEMs (betaine, cholesterol sulfate, PC(20:5(5Z,8Z,11Z,14Z,17Z)/18:4(6Z,9Z,12Z,15Z)), 24-Methylenecholesterol) were common metabolites in the group of COF or CCF and the control. Nineteen DEMs (rotenone, taraxacoside, 8,15-DiHETE, xi-2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, morpholine, Na,Na-Dimethylhistamine, hypogeic acid, 2-Pyrrolidinone, triethylamine, D-lactic acid, cytosine, nortriptyline, prostaglandin E2, edulan I, phenylethylamine, S-Nitrosoglutathione, N-[(4-Hydroxy-3-methoxyphenyl)methyl]octanamide, falcarinone, and [2,2-Bis(2-methylpropoxy)ethyl]benzene) were unique metabolites in the groups of SMOF and the control; four DEMs (3-Hydroxybutyric acid, oleamide, (Â±)-(Z)-2-(5-Tetradecenyl)cyclobutanone, allodesmosine) were unique metabolites in the group of COF and the control; and 11 DEMs (1-Butylamine, 25-Hydroxyvitamin D2, cloversaponin I, 5,7alpha-Dihydro-1,4,4,7a-tetramethyl-4H-indene, histamine, 4-Aminobutyraldehyde, carvyl propionate, PE(P-16:0e/16:0), 5,8,11-Eicosatrienoic acid, 5,7-Dihydro-2-methylthieno[3,4-d]pyrimidine, and annosquamosin B) were unique metabolites in the group of CCF and the control. All those results indicated that these metabolites might play a crucial role in the response of maize to SMOF, COF, or CCF (Figures 9B, 10; Table 5).

Furthermore, chord plot analysis and matchstick analysis were used to visualize the enrichment analysis of these DEMs in three different treatment groups (SMOF, COF, or CCF, and the control) (Figure 11). The results showed that different metabolites were mostly related to each other. Some were positive correlations, whereas some were negative correlations (Figures 11A, C, E). In detail, there were 10 DEMs significantly upregulated and five DEMs significantly downregulated in the groups of SMOF and the control

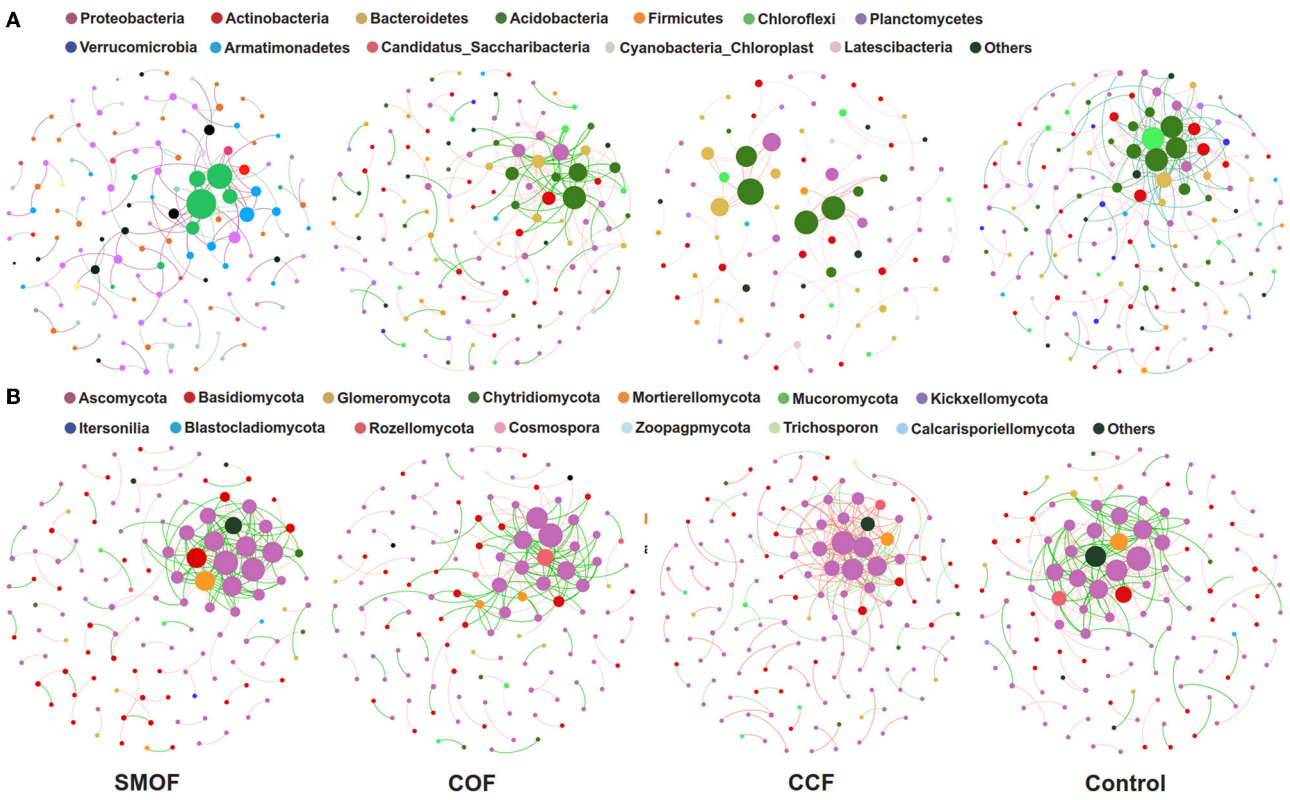

FIGURE 7
Impacts of SMOF on the co-occurrence patterns of soil bacterial **(A)** and fungal **(B)** communities. Networks were constructed at the OTU level. The size of the nodes (OTUs) represented the relative abundance (RA) of the microbe, and the nodes were colored according to phylum. The red and green lines represented positive and negative correlations, respectively.

(Figure 11B); six DEMs significantly upregulated, and the other six DEMs significantly downregulated in the group of COF and the control (Figure 11D); 10 DEMs significantly upregulated and nine DEMs significantly downregulated in the group of CCF and the control (Figure 11F). More attention should be paid to those significant DEMs.

To further analyze correlative relationships between microbes and metabolites, those metabolites with significant differences were normalized, and the clustering heat map was drawn (Figure 12). The results indicated that in the groups of SMOF and the control, four DEMs (N-[(4-Hydroxy-3-methoxyphenyl)methyl]octanamide, tetramethylpyrazine, Dimethylhistamine, and Nitrosoglutathione) were significantly correlated with two genera of bacteria (*Saccharibacteria*, *unclassified_Rhizobiales*); meanwhile, 10 DEMs (edulan I, stearidonic acid, (10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,12-dienoic acid, rotenone, (3beta,5beta,8beta,22E,24xi)-Ergosta-6,22-diene-3,5,8-triol, S-Nitrosoglutathione, 2-Pyrrolidinone, tetramethylpyrazine, [2,2-Bis(2-methylpropoxy)ethyl]benzene, and hypogeic acid) were significantly correlated with five genera of fungi (*Cheilymenia*, *Paraphoma*, *Sodiomyces*, *Dipodascus*, and *Cephalotrichum*). In the group of COF and the control, two DEMs (PC(20:5(5Z,8Z,11Z,14Z,17Z)/18:4(6Z,9Z,12Z,15Z)), and 24-Methylenecholesterol) were significantly correlated with one genus of bacteria (*Rhodopseudomonas*). Meanwhile, 12 DEMs [3-Hydroxybutyric acid, betaine, cholesterol sulfate, (Â±)-(Z)-2-(5-Tetradecenyl)cyclobutanone, Allodesmosine, (3beta,5beta,8beta,22E,24xi)-Ergosta-6,22-diene-3,5,8-triol, oleamide, 24-Methylenecholestero, PC(20:5(5Z,8Z,11Z,14Z,17Z)/18:4(6Z,9Z,12Z,15Z)), tetramethylpyrazine, daucol, and N-Ethylglycine] were significantly correlated with eight genera of fungi (*Gibberella*, *Scutellinia*, *Bisifusarium*, *Didymella*, *Cephalotrichum*, *Dipodascus*, *unclassified_Pyronemataceae*, and *Zopfiella*). In the group of CCF and the control, 13 DEMs [(R)-Apiumetin glucoside, 9-HOTE, stearidonic acid, 25-Hydroxyvitamin D2, (10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,12-dienoic acid, cholesterol sulfate, 5,8,11-eicosatrienoic acid, carvyl propionate, daucol, tetramethylpyrazine, PC(20:5(5Z,8Z,11Z,14Z,17Z)/18:4(6Z,9Z,12Z,15Z)), 24-Methylenecholesterol, and annosquamosin B] were significantly correlated with nine genera of bacteria (*Adhaeribacter*, *Lysobacter, Ohtaekwangia*, *Micromonospora*, *Rhodopseudomonas*, *unclassified_Bacteria*, *Saccharibacteria*, *Subdivision3*, and *Gemmatimonas*); meanwhile, 16 DEMs [25-Hydroxyvitamin D2, PE(P-16:0e/16:0), (10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,12-dienoic acid, cholesterol sulfate, 9-HOTE, 2-(Methylamino)benzoic acid, cloversaponin I, (R)-Apiumetin glucoside, 5,8,11-eicosatrienoic acid, carvyl propionate, betaine, annosquamosin B, tetramethylpyrazine, daucol, estriol-16-Glucuronide, and (3beta,5beta,8beta,22E,24xi)-Ergosta-6,22-diene-3,5,8-triol] were significantly correlated with nine genera of fungi (*Cladorrhinum*,

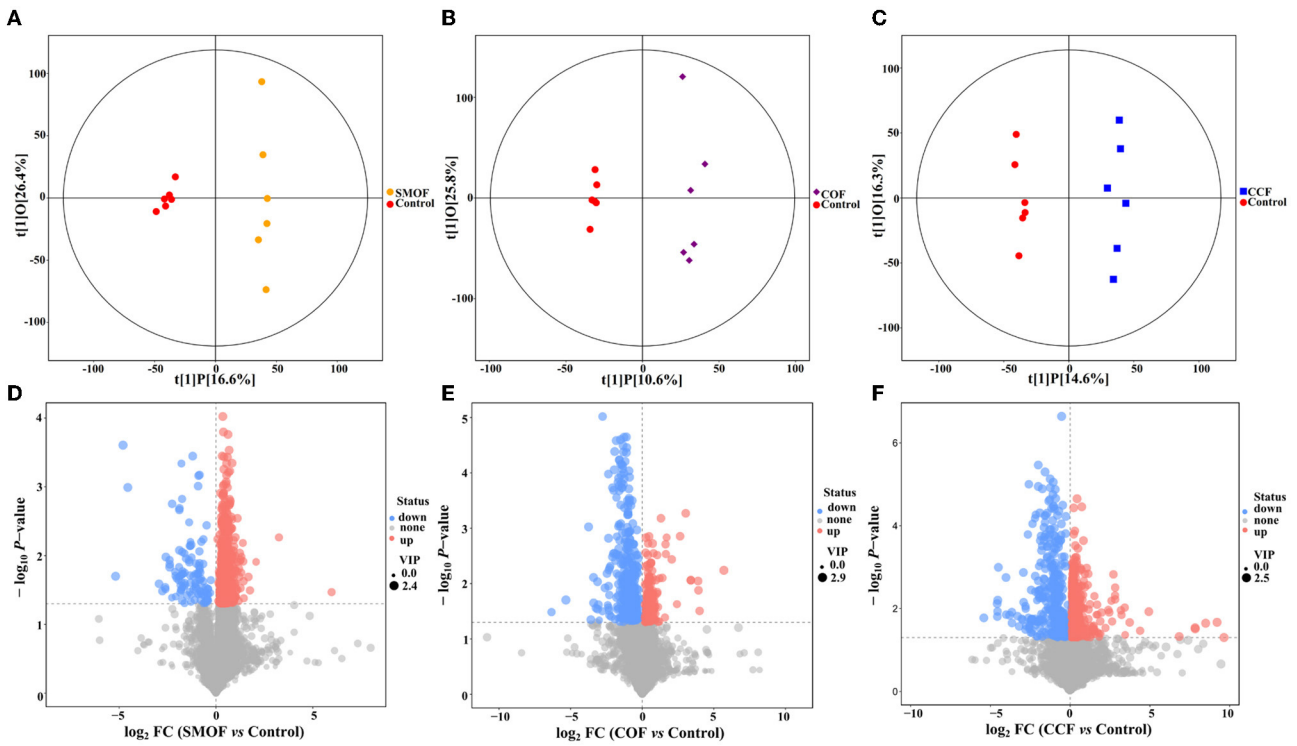

FIGURE 8
Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) score map of maize root-zone soil of the SMOF **(A)**, COF **(B)**, and CCF **(C)** treatment. Volcano plot of differentially accumulated metabolites in SMOF vs. the control **(D)**, COF vs. the control **(E)**, and CCF vs. the control **(F)**. Each point represents a metabolite with VIP > 1 and $p < 0.05$. A blue point indicates metabolites that are downregulated, a red point indicates metabolites that are upregulated, and a gray point indicates non-significant metabolites.

TABLE 4 The model parameters of OPLS-DA in different comparative groups.

OPLS-DA model	R^2X (cum)	R^2Y (cum)	Q^2 (cum)
SMOF vs. the control	0.431	0.990	0.306
COF vs. the control	0.364	0.987	0.269
CCF vs. the control	0.310	0.990	0.519

Paraphoma, *Podospora*, *Cheilymenia*, *Sodiomyces*, *Trichoderma*, *Fusarium*, *Cephalotrichum*, and *Dipodascus*). Taken overall, the DEMs (organic acids, organoheterocyclic compounds, lipids, and secondary metabolites) produced by the application of different fertilizers (including SMOF) in the root-zone soil of maize may enrich the soil microbe and help coordinate the root-zone microbe.

4. Discussion

Cultivated land is the lifeblood and main carrier of food production (Zhou et al., 2021). Cultivated land security is an important guarantee and foundation for food security, social stability, and sustainable development (Zhou et al., 2021). However, with considerable economic development and population growth, various cities have been undergoing rapid urbanization (Zhao, 2016). As the world's most populous country, cultivated lands are occupied on a massive scale in the urban expansion process in China (Tan et al., 2005). Land reclamation is regarded as a viable option to balance occupation and compensation (Li et al., 2023). This practice has been widely adopted worldwide to accommodate urbanization and economic development, especially in developing countries such as China (Hu et al., 2021). However, the soil of reclaimed land usually cannot meet the growth requirements of plants due to poor soil fertility, low soil pH, and high gravel content (Li et al., 2021b).

In most cases, the production capacity of reclaimed land is only 10−30% of the occupied cultivated land in China (Wang et al., 2017), and it often needs 7–10 years of fertilization to reach the level of occupied cultivated land, including acidification adjustment, returning straw to the field, planting green manure, and comprehensive utilization of COF and animal manure (Rendana et al., 2018; Teixeira et al., 2019; Li et al., 2021b). In other words, the content of soil organic matter is highly associated with soil suitability, and the quality of new reclamation land can be effectively improved by increasing the use of organic fertilizers. Indeed, after decades of practice, organic fertilizers have been recognized as valuable soil improvers that could not only improve the physical, chemical, and biological properties of the soil but also increase crop yields (Koocheki and Seyyedi, 2015; Yarami and Sepaskhah, 2015; Qiu et al., 2016; Li et al., 2021b; Yan et al., 2021). In our previous studies, considering the massive consumption in agriculture production, mushroom residue, biogas liquid, vegetable cake, COF, PMMR-OF (organic fertilizer-based pig manure and mushroom residue), and SM-OF

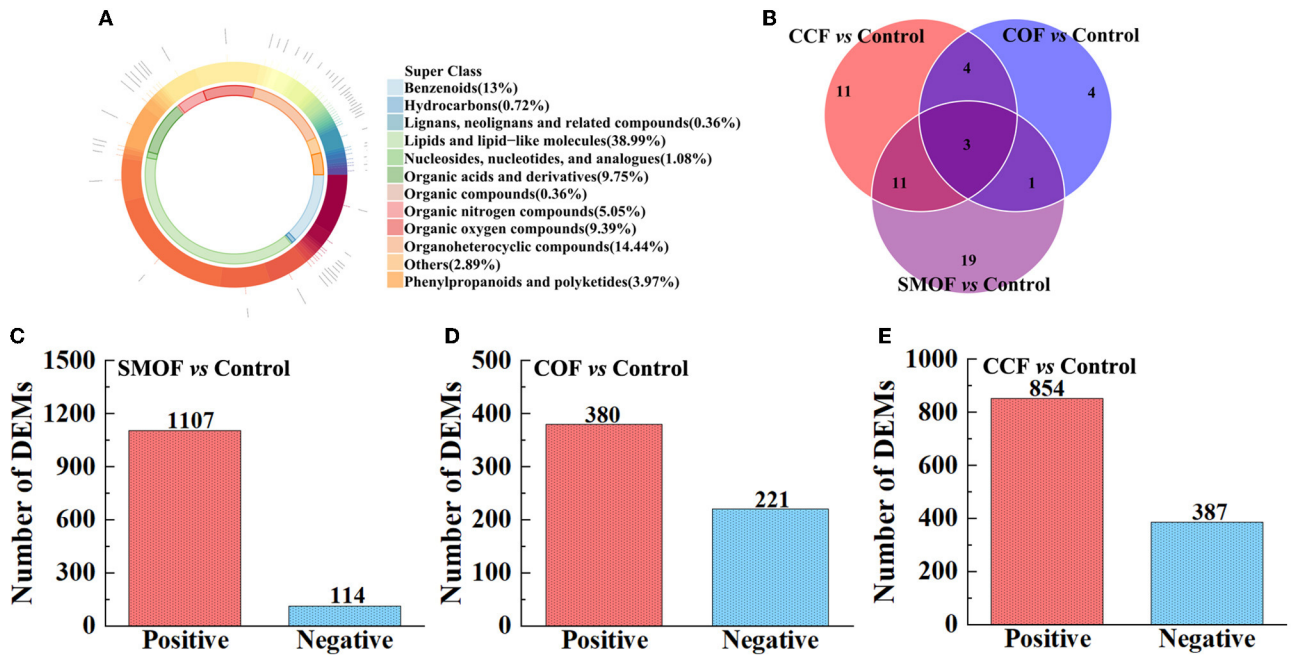

FIGURE 9
Donut plot of metabolite classification and proportion **(A)**, Venn analysis for three groups **(B)**. The number of differentially expressed metabolites (DEMs) in the groups of SMOF and the control **(C)**, COF and the control **(D)**, and CCF and the control **(E)**.

(organic fertilizer-based sheep manure) were selected to be applied to new reclamation land, and the results showed that all these organic fertilizers, especially SM-OF, could not only effectively improve the soil quality of new reclamation land by increasing the soil organic matter but also have a greater effect on sweet potato and corn growth compared to the control (Li et al., 2021b, 2022a,b). According to Therios (1996), animal manures differ in nutrient content. It is common knowledge that sheep manure is referred to as cold manure because of its low nitrogen (N) content, and it is also known as a natural slow-release fertilizer with high phosphorus (P), potassium (K), and other essential elements for plant growth. Recently, sheep manure organic fertilizer (SMOF) has been widely used in the agriculture industry on reclaimed barren mountainous land in Dayang Village, Hangzhou City, Zhejiang Province, China. However, the systematically evaluated SMOF on reclaimed barren mountainous land soil properties, root-zone microbial community structure, metabolites, and maize response is very limited.

In this study, SMOF generally significantly promoted maize growth compared to CCF and the control. Moreover, some previous studies also reported that the use of sheep manure could improve the production of plants. For example, El Gammal and Salama (2016) showed that increasing the sheep manure application rate could induce a progressive enhancement of guava trees' growth and fruit set. Amanullah et al. (2019) revealed that the application of N in the form of 50% urea and 50% organic sources (including sheep manure) could significantly increase total rice biomass. Li et al. (2022a) reported that the application of sheep manure resulted in a significantly positive effect on sweet potato production on the new reclamation land.

The soil properties in maize fields were differentially affected by different treatments (SMOF, COF, CCF, and the control), and the effects differed depending on the soil parameters and types of fertilizer. In detail, the soil pH was slightly increased by SMOF but significantly decreased by CCF. A similar observation was observed by Han et al. (2016), which may be due to the H^+ released into the soil after NH_4^+ used by the plants (Magdoff et al., 1997); the leaching of basic cations such as magnesium (Mg), calcium (Ca), and potassium (K) from the soil (Han et al., 2016); and the proportion of soil microaggregates (<0.25 mm) decreased by the widespread use of CCF (Li et al., 2023). Huang et al. (2009) reported that the soil OMC and total N were widely used as the main parameters for evaluating soil fertility because of their heterogeneous mixtures of organic substances. In our study, the soil OMC and total N were significantly increased by SMOF, COF, and CCF compared with the control. Similar results were obtained by Dong et al. (2012), which may be because the application of organic and chemical fertilizers can promote plant growth and thus return more root residues to the soil (Hyvönen et al., 2008); organic and chemical fertilizers are beneficial to the accumulation of soil organic matter and thus improve the soil's fertility (Dong et al., 2012). The results also showed that available soil P and K contents were significantly affected by different fertilizer treatments (SMOF, COF, and CCF). In particular, SMOF, COF, and CCF led to significantly higher values of soil available P (99.94, 82.89, and 75.97 mg/kg, respectively) and available K (229.68, 177.45, and 193.62 mg/kg, respectively) than the control (49.49, 146.90 mg/kg,

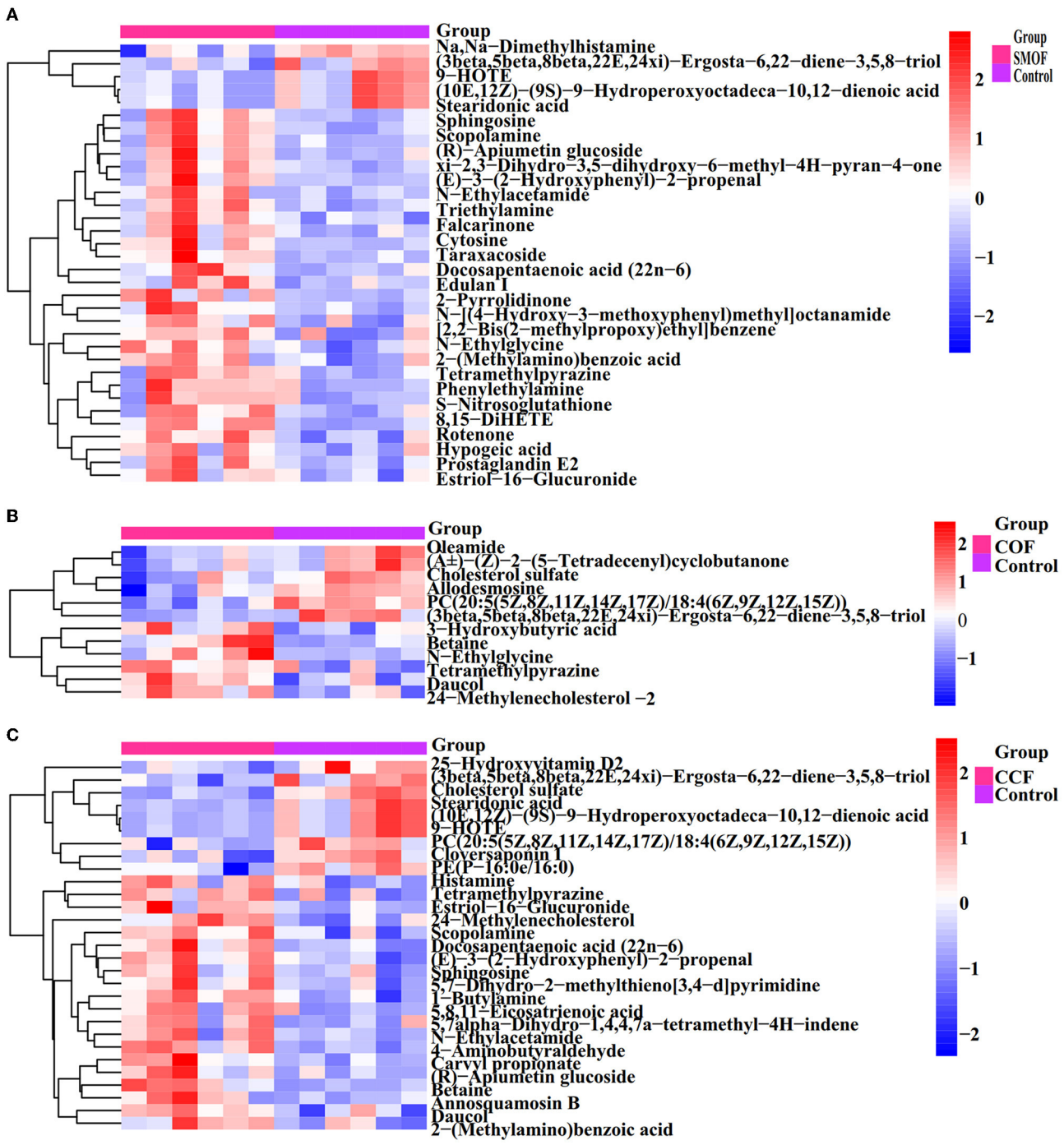

FIGURE 10
Heat map of hierarchical clustering analysis for groups SMOF, COF, or CCF vs. the control. The tree plot represents a clustering analysis of the differentially expressed metabolites (DEMs) significantly changed by SMOF, COF, or CCF according to their Person correlation coefficient matrix and relative abundance.

respectively). Previous studies have also shown that the application of manure could significantly increase available P (Huang et al., 2010), while available K was the highest in NPK treatments (Dong et al., 2012). In our study, available K was higher in the SMOF treatment than in the other treatments (including CCF), which may be due to the higher concentration of K^+ in sheep manure (Zhang et al., 2015; Alhrout et al., 2018). Furthermore, MBC and MBN were significantly increased by SMOF and COF compared with the control, but there was no significant difference between CCF and the control. Soil microbial biomass is widely regarded as an important ecological indicator of changes in soil quality and acts as a source of nutrients available for plant uptake and growth (Haripal

and Sahoo, 2014; Li et al., 2016). Overall, it can be inferred that SMOF is potentially an effective fertilizer to improve the quality of reclaimed barren mountainous land soil.

Microbial communities inhabiting the maize root zone of three fertilizer treatments and the control were examined using 16S rRNA and ITS gene high-throughput sequencing. We used the number of OTUs, the RA of the top 10 classes, and genera to measure the bacterial and fungal abundance and communities under four different treatment conditions. The results showed that the bacterial OTU number was significantly increased by SMOF, followed by CCF and COF, while the fungal OTU number was slightly increased by CCF, SMOF, and COF, as compared with the control. The results also showed that compared with the control, SMOF significantly increased the RA of *Ohtaekwangia*, *Sphingomonas*, *unclassified_Sphingomonadaceae*, and *Saccharibacteria* and reduced the RA of *Spartobacteria*, *Gemmatimonas*, *Gp4*, *Flavisolibacter*, *Subdivision3*, *Gp6*, and *unclassified_Betaproteobacteria* at the bacterial genus level; meanwhile, SMOF also significantly increased the RA of *Podospora*, *Clitopilus*, *Ascobolus*, *Mortierella*, and *Sordaria*, and reduced the RA of *Knufia*, *Fusarium*, *Verticillium*, and *Gibberella* at the fungal genus level. The change in the number of specific microbes in four different treatments may be due to the different nutrients in different fertilizers. Indeed, more attention should be paid to *Ohtaekwangia*, *Sphingomonas*, *Sphingomonadacea*, *Saccharibacteria*, *Podospora*, and *Mortierella*. Previous studies have reported that *Ohtaekwangia* showed a significant negative correlation with *Ralstonia solanacearum* (Deng et al., 2021); *Sphingomonas* possessed multifaceted functions, including remediation of environmental contaminations, producing highly beneficial phytohormones, degradation of organometallic compounds, and improving plant growth during stress conditions (Asaf et al., 2020); *Sphingomonadacea* could decompose organic pollutants (Timoshenko et al., 2021); *Saccharibacteria* was pointed out to be involved in hydrocarbon degradation (Figuroa-Gonzalez et al., 2020); *Podospora* produced a wide variety of carbohydrate-active enzymes, including xylanases, cellulases, and lytic polysaccharide monooxygenases (Silar, 2013; Fanuel et al., 2017); *Mortierella* was reported as a plant growth-promoting fungus (PGPF) by mobilizing P from insoluble forms and producing siderophores and phytoregulators (Srinivasan et al., 2012; Wani et al., 2017; Ceci et al., 2018; Ozimek and Hanaka, 2020). Furthermore, we used alpha diversity (Chao1, Shannon, and the Simpson index) and beta diversity (PCA) to measure the microbial species richness and diversity within each maize root-zone soil sample under four different fertilizer treatment conditions. The results showed that SMOF significantly increased the bacterial Chao1 index, but no significant difference was observed in the Chao1 index of fungi and the Shannon and Simpson index of bacteria and fungi. Meanwhile, the PCA analysis revealed that the bacterial community structure of the maize root-zone soil was significantly changed by SMOF (PERMANOVA, $p < 0.05$), and the different fertilizers explained 19.3% ($p = 0.026$) of the variation, but no significant change was observed in fungal community structure diversity. In other words, the richness of the microbe was increased by SMOF, but the diversity was differentially affected by SMOF. The current results align with those of Li et al. (2023), who reported that different fertilizers altered the composition of the pakchoi rhizosphere soil microbial community. Similar results were also obtained by Li et al. (2022c), who reported that three kinds of microbial fertilizer (CMA, TMF, and SMF) could increase the OTU number and bacterial Chao1 index of corn rhizosphere soil in newly reclaimed land, but no significant difference was observed in the Shannon index. Taken overall, the application of SMOF could favor the diversity of beneficial microbes and reshape the root zone microbial abundance distribution by enriching specific soil microbes in maize in a reclaimed barren mountainous land.

To further elucidate the impact of SMOF on microbial groups, LEfSe (LDA > 2 for bacteria or LDA > 2.5 for fungi, $p < 0.05$) was carried out to explore the role of specific microbes in the fertility improvement of reclaimed barren mountainous land soil. A total of 11 bacterial biomarkers were obtained in SMOF, CCF, and the control. Furthermore, the heat map indicated that SMOF could significantly enrich *Sphingomonas*, *Ohtaekwangia*, and *unclassified_Alphaproteobacteria*. Moreover, a total of 15 fungal biomarkers were collected from four different treatments. The heat map revealed that SMOF could significantly enrich *Podospora*, *Cristinia*, *Sordaria*, and *Mortierella*. It is worth noting that some species of *Sphingomonas*, *Ohtaekwangia*, *Podospora,* and *Mortierella* play an important role in improving plant growth, enhancing resilience to plant pathogens and producing a variety of carbohydrate-active enzymes (Silar, 2013; Asaf et al., 2020; Ozimek and Hanaka, 2020; Deng et al., 2021). In our previous studies, four fungal and five bacterial strains were isolated from new reclamation soil, and those strains had a great ability to improve the soil fertility and promote plant growth by solubilizing P, fixing N, producing siderophores, and indole acetic acid (Li et al., 2021a,c). Those microbes may have greater potential to colonize and affect soil physicochemical properties and microbial communities in reclaimed barren soil.

To test whether some soil physicochemical properties influenced microbial composition dispersion, we used RDA to examine the correlation between microbial communities and environmental factors. The results indicated that a total of 53.42% of the cumulative variance of the root-zone bacterial community-factor correction was at the genus level, and the bacterial communities could be significantly influenced by available K, OMC, available P, and MBN (explained as 25.64%, 25.36%, 25.15%, and 23.38% of the bacterial community, respectively) in all four different treatments. Additionally, a total of 47.40% of the cumulative variance of the root-zone fungal community-factor correction at the genus level, and the fungal communities could be significantly influenced by available K, pH, and MBC (explained as 32.96%, 23.49%, and 22.67% of the fungal community, respectively) in all four different treatments. Previous studies have also revealed that the growth of soil microbes could be affected by a variety of environmental factors. For example, Li et al. (2023) found that the growth of pakchoi soil microbes was affected by available P, AHN, pH, OMC, and total N; Tian et al. (2017) reported that soil organic content exerted the largest effect on the distribution of bacterial communities.

Correlation networks of co-occurring microorganisms allow the visual summary of lots of information, and co-occurrence

TABLE 5 A list of differentially expressed metabolites (DEMs) by relative content, VIP, FC, and a *p*-value.

Compound name	Superclass	Relative content			VIP	Log2 (FC)	*p*-value	Regulated
			Treatment	Control				
(10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,12-dienoic acid	Lipids and lipid-like molecules	SMOF	0.133 ± 0.090b	0.465 ± 0.065a	1.584	−1.809	0.019	Down
Rotenone	Phenylpropanoids and polyketides		0.647 ± 0.009a	0.429 ± 0.038b	1.526	0.593	0.037	Up
Taraxacoside	Organic oxygen compounds		0.021 ± 0.003a	0.017 ± 0.002a	1.640	0.287	0.028	Up
Scopolamine	Organic acids and derivatives		0.091 ± 0.014a	0.072 ± 0.006b	1.790	0.338	0.011	Up
Tetramethylpyrazine	Organoheterocyclic compounds		0.369 ± 0.051a	0.254 ± 0.063b	1.576	0.542	0.044	Up
8,15-DiHETE	Lipids and lipid-like molecules		0.752 ± 0.039a	0.506 ± 0.055b	1.488	0.574	0.033	Up
Sphingosine	Organic nitrogen compounds		0.072 ± 0.012a	0.059 ± 0.003a	1.579	0.285	0.042	Up
xi-2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	Organoheterocyclic compounds		0.204 ± 0.027a	0.174 ± 0.011a	1.594	0.235	0.028	Up
Stearidonic acid	Lipids and lipid-like molecules		0.041 ± 0.011b	0.159 ± 0.023a	1.409	−1.939	0.021	Down
(E)-3-(2-Hydroxyphenyl)-2-propenal	Phenylpropanoids and polyketides		0.066 ± 0.010a	0.055 ± 0.003b	1.582	0.266	0.044	Up
Morpholine	Organoheterocyclic compounds		0.734 ± 0.086a	0.636 ± 0.036b	1.556	0.208	0.027	Up
Na,Na-Dimethylhistamine	Organic nitrogen compounds		0.172 ± 0.027b	0.207 ± 0.008a	1.644	−0.267	0.023	Down
N-Ethylacetamide	Organic acids and derivatives		0.355 ± 0.058a	0.291 ± 0.007b	1.645	0.285	0.043	Up
Hypogeic acid	Lipids and lipid-like molecules		51.341 ± 2.829a	34.086 ± 1.540b	1.494	0.591	0.033	Up
2-Pyrrolidinone	Organoheterocyclic compounds		0.585 ± 0.079a	0.388 ± 0.072b	1.585	0.594	0.030	Up
Triethylamine	Organic nitrogen compounds		0.204 ± 0.034a	0.165 ± 0.010b	1.545	0.304	0.039	Up
D-Lactic acid	Organic acids and derivatives		0.407 ± 0.060a	0.331 ± 0.050a	1.498	0.297	0.039	Up
Cytosine	Organoheterocyclic compounds		0.240 ± 0.038a	0.184 ± 0.027a	1.628	0.386	0.014	Up
Nortriptyline	Benzenoids		0.021 ± 0.003a	0.017 ± 0.003a	1.519	0.307	0.039	Up
Docosapentaenoic acid (22n-6)	Lipids and lipid-like molecules		1.828 ± 0.118a	1.508 ± 0.039b	1.772	0.278	0.029	Up
Prostaglandin E2	Lipids and lipid-like molecules		0.170 ± 0.027a	0.138 ± 0.014a	1.593	0.295	0.030	Up
Edulan I	Organoheterocyclic compounds		0.021 ± 0.005a	0.015 ± 0.002b	1.827	0.516	0.025	Up
(3beta,5beta,8beta,22E,24xi)-Ergosta-6,22-diene-3,5,8-triol	–		0.010 ± 0.005a	0.020 ± 0.008a	1.347	−0.929	0.036	Down
Phenylethylamine	Benzenoids		0.106 ± 0.013a	0.086 ± 0.012b	1.693	0.306	0.018	Up
Daucol	Organoheterocyclic compounds		0.045 ± 0.010a	0.026 ± 0.007b	1.699	0.789	0.010	Up

(Continued)

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TABLE 5 (Continued)

Compound name	Superclass	Relative content			VIP	Log2 (FC)	*p*-value	Regulated
			Treatment	Control				
S-Nitrosoglutathione	Organic acids and derivatives		0.045 ± 0.010a	0.032 ± 0.010b	1.473	0.503	0.042	Up
(R)-Apiumetin glucoside	Phenylpropanoids and polyketides		0.027 ± 0.004a	0.022 ± 0.001b	1.630	0.298	0.031	Up
N-[(4-Hydroxy-3-methoxyphenyl)methyl]octanamide	–		0.031 ± 0.009a	0.018 ± 0.006b	1.432	0.827	0.034	Up
Falcarinone	Organic oxygen compounds		0.016 ± 0.003a	0.012 ± 0.001b	1.764	0.431	0.011	Up
9-HOTE	Lipids and lipid-like molecules		0.094 ± 0.016b	0.306 ± 0.069a	1.575	−1.697	0.018	Down
Estriol-16-Glucuronide	Lipids and lipid-like molecules		0.022 ± 0.002a	0.017 ± 0.001b	2.135	0.407	0.001	Up
N-Ethylglycine	Organic acids and derivatives		0.059 ± 0.007a	0.051 ± 0.002b	1.610	0.207	0.041	Up
2-(Methylamino)benzoic acid	Benzenoids		0.027 ± 0.003a	0.022 ± 0.004b	1.571	0.303	0.036	Up
[2,2-Bis(2-methylpropoxy)ethyl]benzene	Benzenoids		0.064 ± 0.011a	0.028 ± 0.006b	1.880	1.215	0.020	Up
Betaine	Organic acids and derivatives	COF	4.246 ± 0.617a	3.450 ± 0.226b	2.027	0.279	0.030	Up
Tetramethylpyrazine	Organoheterocyclic compounds		0.372 ± 0.063a	0.254 ± 0.063b	1.919	0.552	0.048	Up
Cholesterol sulfate	Lipids and lipid-like molecules		0.194 ± 0.030b	0.299 ± 0.051a	2.117	−0.626	0.021	Down
3-Hydroxybutyric acid	Organic acids and derivatives		0.011 ± 0.001a	0.009 ± 0.001a	1.874	0.265	0.030	Up
Oleamide	Lipids and lipid-like molecules		6.911 ± 0.365b	9.241 ± 0.804a	1.942	−0.419	0.036	Down
PC(20:5(5Z,8Z,11Z,14Z,17Z)/18:4(6Z,9Z,12Z,15Z))	Lipids and lipid-like molecules		0.013 ± 0.011	0.038 ± 0.008	2.241	−1.520	0.001	Down
(Â ±)-(Z)-2-(5-Tetradecenyl)cyclobutanone	–		0.083 ± 0.008a	0.112 ± 0.011b	1.891	−0.435	0.045	Down
(3beta,5beta,8beta,22E,24xi)-Ergosta-6,22-diene-3,5,8-triol	–		0.008 ± 0.002b	0.020 ± 0.008a	2.334	−1.266	0.015	Down
Daucol	Organoheterocyclic compounds		0.042 ± 0.008a	0.026 ± 0.007b	1.919	0.693	0.016	Up
24-Methylenecholesterol	Lipids and lipid-like molecules		0.012 ± 0.001a	0.011 ± 0.001b	2.125	0.193	0.010	Up
Allodesmosine	Carboxylic acids and derivatives		0.011 ± 0.003b	0.014 ± 0.001a	2.121	−0.436	0.025	Down
N-Ethylglycine	Carboxylic acids and derivatives		0.069 ± 0.016a	0.051 ± 0.002b	1.968	0.429	0.041	Up
1-Butylamine	Organic nitrogen compounds	CCF	0.574 ± 0.023a	0.514 ± 0.028a	2.059	0.158	0.002	Up
25-Hydroxyvitamin D2	Lipids and lipid-like molecules		0.020 ± 0.004b	0.038 ± 0.007a	1.393	−0.965	0.036	Down
(10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,12-dienoic acid	Lipids and lipid-like molecules		0.091 ± 0.023b	0.465 ± 0.065a	2.069	−2.348	0.018	Down
Betaine	Organic acids and derivatives		4.891 ± 0.790a	3.500 ± 0.226b	2.123	0.483	0.006	Up

(Continued)

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TABLE 5 (Continued)

Compound name	Superclass	Relative content			VIP	Log2 (FC)	*p*-value	Regulated
			Treatment	Control				
Scopolamine	Organic acids and derivatives		0.080 ± 0.004a	0.072 ± 0.006b	1.727	0.153	0.017	Up
Tetramethylpyrazine	Organoheterocyclic compounds		0.394 ± 0.077a	0.254 ± 0.063b	1.631	0.636	0.030	Up
Sphingosine	Organic nitrogen compounds		0.064 ± 0.004a	0.059 ± 0.003b	1.525	0.112	0.047	Up
Cholesterol sulfate	Lipids and lipid-like molecules		0.159 ± 0.035b	0.299 ± 0.051a	2.283	−0.912	0.000	Down
Stearidonic acid	Lipids and lipid-like molecules		0.030 ± 0.008a	0.159 ± 0.023b	2.050	−2.409	0.020	Down
(E)-3-(2-Hydroxyphenyl)-2-propenal	Phenylpropanoids and polyketides		0.061 ± 0.003a	0.055 ± 0.003b	1.944	0.160	0.005	Up
N-Ethylacetamide	Organic acids and derivatives		0.320 ± 0.023a	0.291 ± 0.007b	1.820	0.135	0.029	Up
Cloversaponin I	Lipids and lipid-like molecules		0.025 ± 0.002b	0.051 ± 0.012a	1.577	−1.047	0.016	Down
PC(20:5(5Z,8Z,11Z,14Z,17Z)/18:4(6Z,9Z,12Z,15Z))	Lipids and lipid-like molecules		0.024 ± 0.005b	0.038 ± 0.008a	1.665	−0.675	0.018	Down
Docosapentaenoic acid (22n-6)	Lipids and lipid-like molecules		1.635 ± 0.077a	1.508 ± 0.003b	2.031	0.117	0.005	Up
5,7alpha-Dihydro-1,4,4,7a-tetramethyl-4H-indene	Hydrocarbons		1.087 ± 0.046a	1.027 ± 0.036a	1.724	0.083	0.028	Up
Histamine	Organic nitrogen compounds		0.126 ± 0.007a	0.117 ± 0.006b	1.711	0.115	0.024	Up
4-Aminobutyraldehyde	Organic oxygen compounds		0.566 ± 0.026a	0.512 ± 0.011b	2.206	0.147	0.001	Up
(3beta,5beta,8beta,22E,24xi)-Ergosta-6,22-diene-3,5,8-triol	–		0.009 ± 0.005b	0.020 ± 0.008a	1.210	−1.175	0.016	Down
Carvyl propionate	Lipids and lipid-like molecules		0.380 ± 0.064a	0.170 ± 0.025b	1.951	1.158	0.011	Up
Daucol	Organoheterocyclic compounds		0.040 ± 0.005a	0.026 ± 0.007b	1.566	0.604	0.019	Up
(R)-Apiumetin glucoside	Phenylpropanoids and polyketides		0.025 ± 0.002a	0.022 ± 0.001a	1.750	0.180	0.027	Up
PE(P-16:0e/16:0)	Lipids and lipid-like molecules		0.027 ± 0.007a	0.036 ± 0.004b	1.594	−0.425	0.015	Down
5,8,11-Eicosatrienoic acid	Lipids and lipid-like molecules		1.406 ± 0.392a	0.913 ± 0.108b	1.537	0.622	0.039	Up
24-Methylenecholesterol	Lipids and lipid-like molecules		0.012 ± 0.001a	0.011 ± 0.001b	1.824	0.214	0.009	Up
5,7-Dihydro-2-methylthieno[3,4-d]pyrimidine	Organoheterocyclic compounds		2.054 ± 0.128a	1.872 ± 0.088b	1.799	0.133	0.017	Up
9-HOTE	Lipids and lipid-like molecules		0.067 ± 0.020b	0.306 ± 0.069a	2.048	−2.199	0.018	Down
Estriol-16-Glucuronide	Lipids and lipid-like molecules		0.020 ± 0.003a	0.017 ± 0.001a	1.804	0.257	0.016	Up
Annosquamosin B	Lipids and lipid-like molecules		0.313 ± 0.073a	0.120 ± 0.048b	1.583	1.381	0.036	Up
2-(Methylamino)benzoic acid	Benzenoids		0.027 ± 0.004a	0.022 ± 0.004b	1.594	0.314	0.041	Up

Different lowercase letters within the same rows reveal the significance between different treatments (SMOF, COF, or CCF) and the control ($p < 0.05$).

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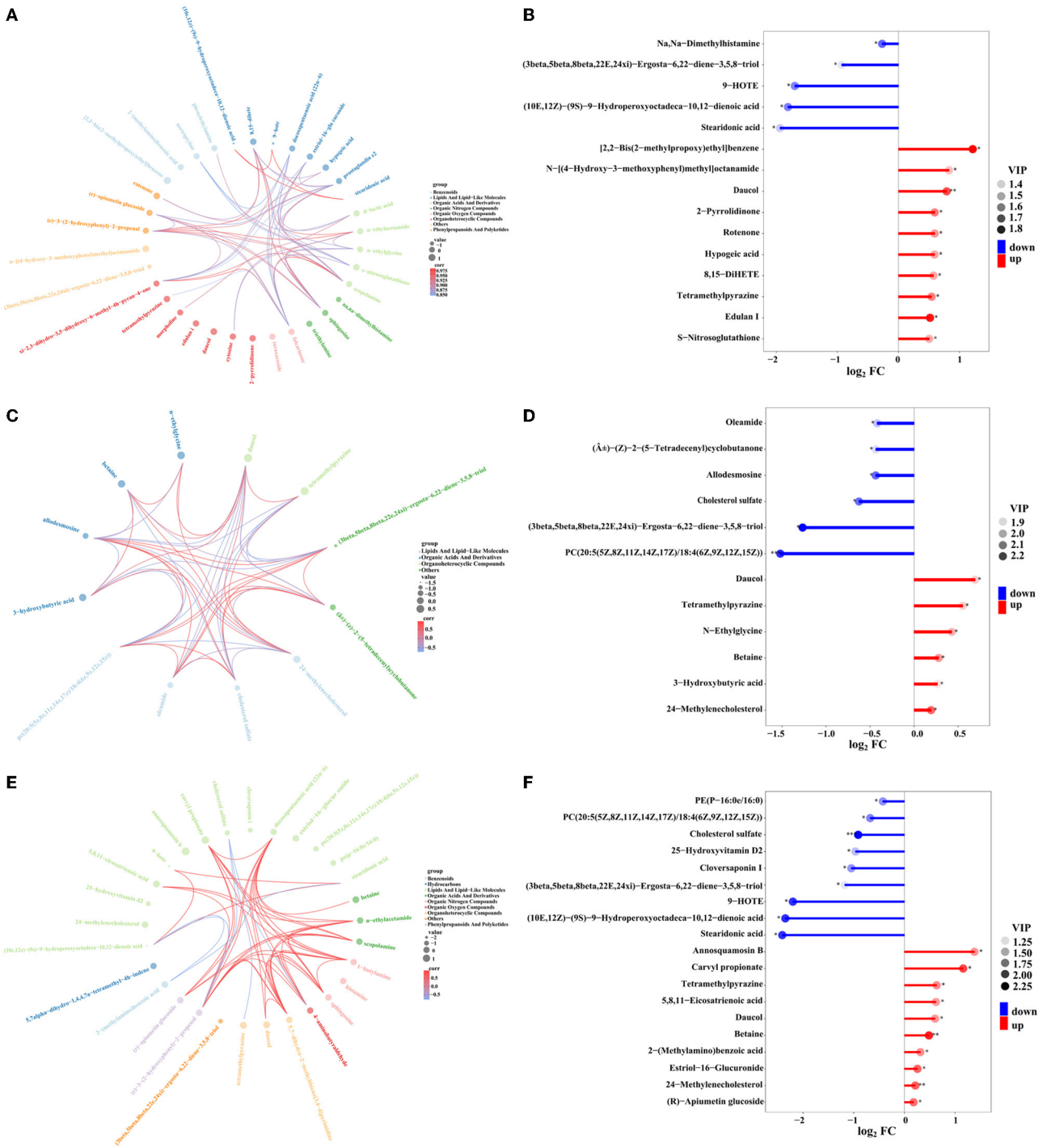

FIGURE 11
Chord plot analysis for the groups of SMOF and the control **(A)**, COF and the control **(C)**, CCF and the control **(E)**, respectively. Nodes represent variables; text color is associated with different metabolites, and chords represent correlations. Matchstick analysis for the groups of SMOF and the control **(B)**, COF and the control **(D)**, and CCF and the control **(F)**, respectively. The color of the dot represents the size of the VIP value, *represents $0.01 < p < 0.05$, **represents $0.001 < p < 0.01$, and ***represents $p < 0.001$.

networks offer new insights into microbial interaction analysis (Chaffron et al., 2010; Jiang et al., 2022). In this study, we applied correlation-based network results to analyze the interaction between OTUs of different treatments (Sparcc's correlation $N > 0.5$ or < -0.5, $p < 0.01$). It is well-known that more nodes and edges and higher community diversity represent a complex and stable network structure (Hernandez et al., 2021), and high modularity is also an indicator of the network's structural stability (Ma et al., 2021). In our study, four different treatments had different microbial co-occurrence network structures. However, compared with the control, in the SMOF treatment, the number of bacterial network nodes increased, but the edges, the average

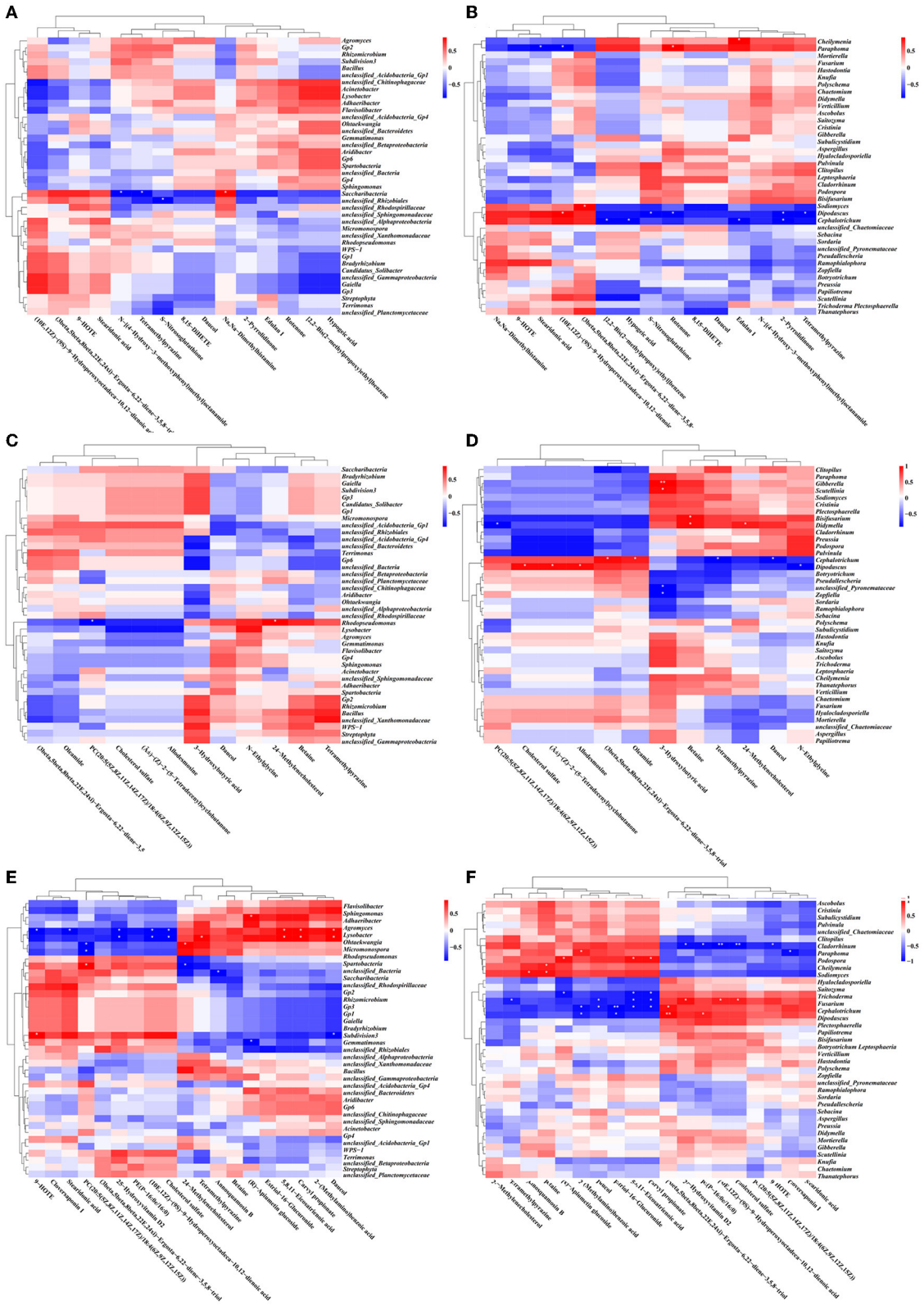

FIGURE 12
Correlation heat map between DEMs and the related microbe in different treatment groups. Based on the top 40 genera of bacteria: SMOF vs. the control **(A)**, COF vs. the control **(C)**, and CCF vs. the control **(E)**; based on the top 40 genera of fungi: SMOF vs. the control **(B)**, COF vs. the control **(D)**, and CCF vs. the control **(F)**. *Indicated a significant correlation at $p < 0.05$, **indicated a significant correlation at $p < 0.01$.

degree, and the modularity decreased; meanwhile, the number of fungal network nodes, edges, and the average degree decreased, but the modularity increased. Strikingly, most of the relationships between the bacterial and fungal communities in the SMOF treatment were positive, which indicated that most bacteria and fungi had similar guilds or niches and were mutually beneficial rather than competitive (Deng et al., 2016).

A total of 1,054 peaks were screened from maize root-zone soil in four different treatments. The results of OPLS-DA revealed that the metabolites in control root-zone soil were significantly changed by SMOF, COF, and CCF, which was also verified by the volcano plot. A total of 277 metabolites were identified by LC-MS analysis, which mainly belongs to lipids and lipid-like molecules, organoheterocyclic compounds, benzenoids, organic acids and derivatives, organic oxygen compounds, and so on. In the SMOF and the control groups, there were 1,221 DEMs screened, with 1,107 upregulated and 114 downregulated. Compared to the control, 34 DEMs were significantly changed by SMOF, among which 10 DEMs (S-Nitrosoglutathione, edulan I, tetramethylpyrazine, 8,15-DiHETE, hypogeic acid, rotenone, 2-Pyrrolidinone, daucol, N-[(4-Hydroxy-3-methoxyphenyl)methyl]octanamide, [2,2-Bis(2-methylpropoxy)ethyl]benzene) were significantly upregulated, and five DEMs (stearidonic acid, (10E,12Z)-(9S)-9-Hydroperoxyoctadeca-10,12-dienoic acid, 9-HOTE, (3beta,5beta,8beta,22E,24xi)-Ergosta-6,22-diene-3,5,8-triol, Na,Na-Dimethylhistamine) were significantly downregulated. As we know, benzenoids, lipids, organoheterocyclic compounds, organic acids, phenylpropanoids, polyketides, and other secondary metabolites are essential to life, playing a vital role in the metabolism of all living cells. Previous reports have shown that a number of different benzenoid compounds are rapidly produced in plants or microbes in response to insects, pathogens, or stress (Keen and Taylor, 1975; Herrmann, 1995); lipids are essential for the integrity of cells and organelles by acting as a hydrophobic barrier for the membrane (Kim, 2020); organic acids play significant and varied roles in rhizosphere acidification and mineral weathering, contributing protons and serving as ligands for complex metals; they can promote redox reactions with electron-deficient metals (a rhizosphere-promoted process considered in the next section on redox cycling) (Daniel et al., 2007). To further explore the correlation between microbes and DEMs, a clustering heat map was drawn. The results showed that in the groups of SMOF and the control, there were four DEMs significantly correlated with two genera of bacteria, among which *Saccharibacteria* was significantly negatively correlated with two DEMs and positively correlated with one DEM, and *unclassified_Rhizobiales* was significantly negatively correlated with one DEM; meanwhile, 10 DEMs significantly correlated with five genera of fungi, among which *Cheilymenia* was significantly positively correlated with one DEM, *Paraphoma* was significantly negatively correlated with two DEMs, and positively correlated with one DEM, *Sodiomyces* was significantly positively correlated with one DEM, *Dipodascus* was significantly positively correlated with one DEM, and negatively correlated with four DEMs, and *Cephalotrichum* was significantly negatively correlated with four DEMs. The results revealed complicated interactions between microbes and DEMs in maize root zone soil.

5. Conclusions

In conclusion, our results showed that SMOF not only significantly improved the soil quality (including the soil's physical, chemical, and biological properties) but also promoted maize growth. This suggests that SMOF can be a good amendment for maize production in a reclaimed barren mountainous land. Compared with the control, SMOF caused a differential change in the microbial community of reclaimed barren mountainous land. Some specific beneficial microbes, such as *Ohtaekwangia*, *Sphingomonas*, *Sphingomonadacea*, *Saccharibacteria*, *Podospora,* and *Mortierella*, have been found to be closely related to the soil improvement by SMOF, indicating the relationships among microbes, fertilizer, and soil. Furthermore, RDA results showed that the composition of bacterial and fungal communities in maize root-zone soil was significantly affected by available K, OMC, P, MBN, and available K, pH, and MBC, respectively. In SMOF treatment, most microbes had similar guilds or niches and were mutually beneficial rather than competitive. In addition, SMOF resulted in a significant change in the kinds and relative contents of metabolites in the root-zone soil. The correlation heat map showed a significant correlation at the genus level between related microbial groups and DEMs of maize root-zone soil in SMOF. All these results revealed that SMOF could influence the interaction among soil properties, microbial communities, and secondary metabolites and improve maize growth. We believe that these may play an important role in improving soil quality and promoting maize production in the reclaimed barren mountainous land.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repositories are NCBI and CNGBdb, with accession number PRJNA951893, PRJNA951350 and CNP0004202, respectively.

Author contributions

Conceptualization: XL, DW, QL, ZT, and JY. Methodology and investigation: XL, DW, and JY. Software: XL. Validation: XL, QL, ZT, and JY. Formal analysis, resources, and visualization: XL, QL, and JY. Data curation, supervision, project administration, and funding acquisition: QL and JY. Writing—original draft preparation and writing—review and editing: XL and JY. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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EDITED BY
Priyanka Chandra,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Dharmendra Kumar,
Central Potato Research Institute (ICAR), India
Jegan Sekar,
M S Swaminathan Research Foundation, India

*CORRESPONDENCE
Muhammad Usama Marghoob
✉ usamamarghoob91@yahoo.com
Fathia Mubeen
✉ mufathia@yahoo.com

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Assessment of halotolerant bacterial and fungal consortia for augmentation of wheat in saline soils

Muhammad Usama Marghoob[1]*, Aniqa Nawaz[1], Muhammad Ahmad[1], Muhammad Qandeel Waheed[2], Muhammad Hassaan Khan[3], Muhammad Imtiaz[1], Ejaz ul Islam[1], Asma Imran[1] and Fathia Mubeen[1]*

[1]Soil and Environmental Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Constituent College of Pakistan Institute of Engineering and Applied Sciences (NIBGE-C, PIEAS), Islamabad, Pakistan, [2]Plant Breeding and Genetic Division, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan, [3]Department of Bioinformatics and Biotechnology, Government College University Faisalabad (GCUF), Faisalabad, Pakistan

Adaptations of green technologies to counter abiotic stress, including salinity for crops like wheat by using halotolerant microbes, is a promising approach. The current study investigated 17 salt-affected agroecological zones from the Punjab and Sindh provinces of Pakistan to explore the potential of indigenous microbial flora, with their multiple biochemical characteristics in addition to plant growth promoting (PGP) traits, for enhanced wheat production in saline areas. Initially, 297 isolated pure bacterial colonies were screened for salt tolerance, biochemical, and PGP traits. Three bacterial strains belonging to *Pantoea* spp. and *Erwinia rhaphontici* with possession of multiple characteristics were selected for the development of the halotolerant bacterial consortium. Inoculation of two local wheat varieties, Faisalabad 2008 and Galaxy 2013, with the consortium for *in vitro* seed germination assay and sand microcosm experiments exhibited significant improvement of selected plant growth parameters like germination percentage and root structure. Two previously reported PGP fungal strains of *Trichoderma harzianum* and *T. viridae* were also used as fungal consortium separately for pot experiments and field trials. The pot experiments exhibited a positive correlation of consortia with metabolic *viz.* catalase, peroxidase, and proline and agronomical parameters including shoot length, dry weight, number of spikes, spike length, and 100 grain weight. To evaluate their performance under natural environmental conditions, field trials were conducted at three salt-affected sites. Agronomical attributes including days of flowering and maturity, flag leaf weight, length and width, shoot length, number of spikes, spike length, spike weight, number of seeds spike^{-1}, 1,000 grain weight, and plot yield indicated the efficiency of these microbes to enhance wheat growth. Concisely, the bacterial consortium showed better performance and Faisalabad 2008 was a more resistant variety as compared to Galaxy 2013. Initial promising results indicate that further extensive research on indigenous microbes might lead to the development of Pakistan's first saline-specific biofertilizers and sustainable eco-friendly agriculture practices.

KEYWORDS

abiotic stress, salinity, halotolerant PGPR, plant-microbe interaction, root structure architecture

1. Introduction

With the rapidly growing and evolving world, new problems, especially those linked to climate change, are emerging. For instance, the key requirement for increasing world population is the availability of sufficient food. However, anthropogenic activities along with other environmental issues are driving factors associated with food security. Collectively, these problems are not only deteriorating the planet but also pose a serious threat to agriculture (Nawaz A. et al., 2020).

Expanding salinization of agricultural lands, which is a global issue in general and serious threat to countries like Pakistan, needs special attention (Shah et al., 2021). Wheat (*Triticum aestivum*) is Pakistan's main staple food and is considered the gold crop of the country (Nawaz A. et al., 2020; Azmat et al., 2021). In addition to many other major crops including rice, maize, etc., wheat also belongs to the group of plants known as "glycophytes" as it is susceptible to saline conditions (Uçarlı, 2020). Therefore, special emphasis is given to exploring options for mitigating the adverse effects of salinity on wheat. Currently, different approaches, e.g., physical and chemical applications, plant breeding, and molecular biology techniques, are being practiced and investigated to tackle this problem, but the outcomes are not satisfactory (Nouri et al., 2017; Ben-Laouane et al., 2020; Perez-Jimenez and Perez-Tornero, 2020; Rani et al., 2022). However, the use of microorganisms is considered to be a more efficient and ecofriendly approach for sustainable agriculture (Mukhtar et al., 2020; Nawaz A. et al., 2020; Marghoob et al., 2022).

Microbes are helping mankind even before their discovery. The microbial world plays a key role in mitigation of major problems. Exploiting the microbial flora, with plant growth promoting (PGP) characteristics, for improve plant growth and combating biotic and abiotic stresses is a well-known phenomenon (Nawaz A. et al., 2020; Cochard et al., 2022). These tiny creatures harbor genes that facilitate plant growth through activities including N_2-fixation, P-solubilization, K-solubilization, siderophore and other phytohormones production, antimicrobial compounds, and induced systemic resistance against biotic and abiotic agents (Islam et al., 2016; Nawaz et al., 2021; Fatima et al., 2022).

In current era, exploiting the halotolerant indigenous microbial flora, for crop improvement in a saline environment, is a promising approach to address the adverse effects of salinity (Li et al., 2022). In addition to other PGP traits, some indigenous microbes from harsh environments also contain ACC-deaminase enzyme that positively regulates ethylene, produced excessively in plants under stress environments and these elevated levels are dangerous for plant health (Bouffaud et al., 2018; Ben-Laouane et al., 2020). Production of osmoprotectants like glycine betaine, proline, and ectoine, etc. also help both plants and microbes to adapt the harsh saline conditions (Chen et al., 2021). Production of some secondary metabolites like exopolysaccharides (EPS) and other enzymatic activities also leverage these microbes for better adaptation to harsh environments and to facilitate crop resilience against abiotic factors (Mishra et al., 2016; Batool et al., 2017; Ahmad M. et al., 2022). Production of EPS not only helps these microbes in adaptations but also improves soil properties and helps in Na^+ scavenging (Egamberdieva et al., 2019). In addition to other PGP activities, these microbes also have the potential to facilitate plant growth under saline environment by enhancing plant metabolic activities like Catalase, Peroxidase, superoxide dismutase and production of osmoprotectants (Sekar et al., 2019; Nawaz M. S. et al., 2020).

Interestingly, PGP-related research has mainly focused on bacteria and mycorrhiza previously; however, recent findings unleashed the hidden PGP potential of some fungal strains, particularly from the genus *Trichoderma*, in addition to their role as a biocontrol agent against phytopathogens (Martinez-Medina et al., 2014; Faraz et al., 2020; Sánchez-Montesinos et al., 2020). Strains belonging to *Trichoderma* are not only important to improving plant growth by their involvement in nutrient uptake, improved soil physiochemical properties, phytohormone production, and combating phytopathogens, but they also have a promising role in alleviating biotic stresses, including salinity (Sánchez-Montesinos et al., 2020).

Similarly, the application of multiple strains possessing a diverse set of PGP characteristics, in the form of a consortium, under field conditions is considered a practical approach to minimizing the adverse effects of environmental and seasonal variations on the survival and functional potential of individual microbes (Etesami and Maheshwari, 2018). Various studies showed that the efficacy of microbes used in this form of consortium perform better than inoculation with individual strains (Sekar et al., 2016; Devi et al., 2022; Kaur et al., 2023).

The current study was designed to focus on the saline agricultural zones of the Indus Basin region from the provinces of Punjab and Sindh, Pakistan (the breadbasket of the country), where low wheat production is directly linked to the expansion of salinity in association with other factors (Syed et al., 2020). Moreover, most of the areas in these vicinities have remained unexplored in terms of microbial flora and their PGP traits. The present study hypothesized that isolation and characterization of bacterial strains from these soils would facilitate the finding of the indigenous microbial flora with PGP traits that could be used in the future for the formulation of saline-specific biofertilizers, and outcomes of the study would not only help in wheat growth improvement under saline conditions but would also lead to less dependency on chemical fertilizers.

2. Materials and methods

2.1. Isolation and characterization of PGP bacterial strains

2.1.1. Soil samples collection

For the isolation of efficient plant growth-promoting halotolerant bacterial strains, rhizospheric and bulk soil samples were collected from 17 salt-affected agricultural lands located in the Punjab and Sindh provinces of the Indus Basin region, Pakistan. Wheat is cultivated in these areas during winter with other rotational summer crops. A list of sample sites is provided in Supplementary File 1, Table 1. Three soil samples were collected from a depth of 4–6 inches using a sterile soil probe from each location, and they were then homogenized and carefully transferred to the soil physiology lab, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, in sterile bags and stored at 4°C. Basic chemical properties, electrical conductivity (EC) and pH of soil, were determined for each sample in order to estimate the salinity level to evaluate these samples for isolation of potential halotolerant PGP microbes.

2.1.2. Selection of halotolerant bacterial isolates

For bacterial strain isolations, serial dilution method was opted. Briefly, 1 g of homogenized soil sample from each site was added to 9 mL normal saline (NS) solution (0.9% NaCl) and mixed thoroughly to make a 10^{-1} dilution. For 10^{-2} dilution, 1 mL from 10^{-1} was added to 9 ml NS and was mixed properly. Serial dilutions were prepared up to 10^{-5} using this method, and 10^{-3} and 10^{-5} dilutions were used for spreading on LB agar and LB agar plates supplemented with 1% NaCl in triplicates. Plates were incubated at 28 ± 2°C for 24–48 h.

Plates were observed for bacterial growth, and 297 bacterial isolates were selected to determine their minimal inhibitory concentration (MIC) for NaCl. LB agar plates supplemented with NaCl in a gradually increasing order (0.5, 1, 5, 7, and 10%) were used for this purpose. Plates were incubated at 28 ± 2°C for 24–48 h.

2.1.3. Screening for plant growth promoting characteristics

Only those bacterial strains that were able to withstand MIC (7% and/ or 10%) were selected for assessment of PGP characteristics like P-, Zn- and K-solubilization, IAA and siderophore production, and ACC deaminase activity, etc. Briefly, P- solubilization activity was checked using Pikovskaya's medium by methods described earlier (Pikovskaya, 1948) both qualitatively and quantitatively. IAA production was evaluated by following the protocol of Gordon and Weber (1951) on tryptophan-supplemented media. K- solubilization characteristics were determined by observing hollow zones on Alexandrov's medium (Boubekri et al., 2021). Siderophore production was detected by using the standard procedure described by Schwyn and Neilands (1987). ACC deaminase activity was assessed as described by Kumar et al. (2021).

2.2. Detection of selected biochemical pathways

Different biochemical pathways and metabolites especially linked with the survival of microorganisms under challenging environmental conditions (e.g., exopolysaccharides, amylase, hydrogen cyanide, catalase, and peroxidase production) were also investigated. Briefly, the ability of exopolysaccharide (EPS) production for the selected halotolerant strains was evaluated by the method described by Batool et al. (2017). A starch hydrolysis test (Peltier and Beckord, 1945) was carried out for the detection of α-amylase enzyme production. Hydrogen Cyanide (HCN) production, an efficient strategy of microbes against potential pathogens, was detected by the method described earlier in the literature (Nandi et al., 2017). Catalysis activity for toxic hydrogen peroxide (H_2O_2) conversion into water and Oxygen by bacteria using catalase enzymes was determined by the previously described method (Attia et al., 2022).

2.3. Molecular identification of selected strains

Overnight-grown bacterial cultures on LB broth were used for bacterial DNA isolation and amplification through PCR. Bacterial genomic DNA was extracted by using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Carlsbad, CA, United States of America) as per the manufacturer's instruction. DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, United States of America). Universal primer set fD1 and rD1 and the optimized PCR profile (Supplementary File 1, Table 2) were used for bacterial 16S rRNA gene amplification (Fatima et al., 2022). PCR products were confirmed on 1% agarose gel before shipping to Macrogen, Inc. South Korea, for sequencing.

The 16S rRNA gene partial sequences of UM16, UM 58, and UM 83 were compared with available sequences in the GenBank database using the NCBI Basic Local Alignment Search Tool (BLAST) and were submitted to the NCBI gene bank repository, and accession numbers were obtained.

A neighbor-joining phylogenetic tree using MEGA software v.11.0 with 10,000 bootstrap values, where bootstrap values are shown in figure, was constructed.

2.4. Strains compatibility assessment

The cross-streak method on LB media was used to check the strains compatibility in the possible consortium (Fatima et al., 2022).

2.5. *In vitro* assessment for improvement in wheat growth parameters under saline stress

To evaluate the response of wheat germination in saline environments at different NaCl concentrations in the presence of PGP bacteria, three selected bacterial strains, UM 16, UM 58, and UM 83, were used in the form of the consortium to conduct plate assay for seed germination.

2.5.1. Plate assay for seed germination

Seeds of two wheat varieties, Galaxy 2013 (V1) and Faisalabad 2008 (V2), were collected from Ayub Agriculture Research Institute (AARI), Faisalabad, and were used for the experimentation.

Healthy seeds of both varieties were surface sterilized using sodium hypochlorite (2%) and autoclaved water (Cheng et al., 1997). Seed priming of glycophytes with a saline solution of lower concentrations (halopriming) might also improve seed germination (Uçarlı, 2020); therefore, seeds soaked in sterilized water (T1) and 0.2 mM NaCl solution (T2) were used as controls separately. For seed biopriming, seeds were soaked for 30–40 min in 24-h old LB-broth culture with an adjusted OD of 0.1 at 600 nm of consortium for bacterial treatment (T3).

Seeds were transferred to Petri dishes containing 1% water agar [and 0, 50, 100, and 150 mM of NaCl for each treatment, where; 10 mM ~ 1dSm^{-1} (Rahnama et al., 2011)] aseptically in such a manner that each plate carried 6–7 seeds for each treatment in triplicates. Plates were incubated at 24 ± 4°C for 7 days in a growth chamber and were observed daily for seed germination.

The germination percentage (GP) was calculated by using the following formula (Manmathan and Lapitan, 2013):

$$GP = \text{germinated seeds} / \text{total seeds} \times 100$$

2.6. Plant root structure studies

In order to understand the halotropism response, seeds of two wheat varieties, Faisalabad 2008 and Galaxy 2013, were used to visualize the effect of inoculation on root growth and architecture under NaCl stress. Seeds were sterilized and grown on 1% agar plates as described in the previous section. Seeds were transferred to pots containing sterilized sand after 7 days of germination and were transferred to the greenhouse. Sterilized tap water, NaCl solutions of selected concentrations (0, 20, 40, 60, and 80 mM), and Hoagland solution (Hoagland and Arnon, 1950; Spehia et al., 2018) with 80 and 100% concentrations were used for control and treatments as per requirement in the following manner; T1: C100 (Hoagland 100% conc.), T2: C80 (Hoagland 80% conc.), and T3: TB (bacterial consortium and Hoagland 80% conc.).

Plants were harvested after 18–20 days and roots were carefully collected, washed, and properly labeled. Three plants from each treatment were used for root structure parameters studies. A Rhizoscanner (Epson photo scanner V700) installed with WinRHIZO (Regent Int. Co., Ltd.) was used to study selected parameters including root length (cm), surface area (cm^2), the average diameter (mm), root volume (cm^3), and root tips.

2.7. Revival of PGP fungal strains

Two previously reported PGP fungal strains, *Trichoderma harzianum* and *T. viridae* (Faraz et al., 2020), were collected from the National Bioresource Collection Center (NBRC), NIBGE, Pakistan, and revived on PDA media. Spores were placed on PDA plates and incubated at 28 ± 2 °C for 5–7 days.

2.8. Preparation of bacterial and fungal bioformulations

Three selected bacterial strains, UM 16, UM 58, and UM 83, and fungal strains *Trichoderma harzianum* and *T. viridae* were used for the development of bacterial and fungal consortium separately for pot experiments and field trials. Overnight LB broth cultures of bacteria were mixed equally after calculating the cell density, and the final concentration of the consortium was 10^8 CFU ml^{-1}. Fungal cultures of 5–7 days, grown in PD broth, were used, and the final concentration of spores was adjusted to 10^8 ml^{-1}. Previously characterized sterilized filter mud was used as carrier material (Suleman et al., 2018).

2.9. Pot experiment under control and saline conditions

In order to evaluate the wheat growth response to fungal and bacterial consortia at different NaCl concentrations under natural environmental conditions, a pot experiment was conducted (Dec 2017-April 2018). Earthen pots containing 11–12 Kg homogenized sterile soil were used and three different salinity levels (2, 6, and 10 dSm^{-1}) were attained by using NaCl solutions of different concentrations and monitoring of soil periodically with an EC meter to maintain maximum homogeneity. Two controls, T1: 100% farmer recommended dose (FRD) of fertilizer and T2: 80% FRD were used, and bacterial (T3) and fungal (T4) consortia with 80% FRD were used separately to evaluate their effects on wheat at different salinity levels. The recommended dose of chemical fertilizers applied in this region is N:P; 15:100 Kg ha^{-1}. The required FRD of fertilizers in the form of Urea and DAP was applied at the time of sowing and first and second irrigation. The wheat varieties Faisalabad 2008 and Galaxy 2013 were used, and a randomized complete block design (RCBD) with triplicates for each treatment was opted for. In each pot, 10–12 seeds were planted.

2.9.1. Agronomical and biochemical parameters

At the thinning stage, five/six plants were uprooted from each pot, and three plants were selected randomly for the detection of selected metabolic/enzymatic activities associated with salinity stress (catalase, peroxidase, and free proline content) using previously optimized protocols (Nawaz A. et al., 2020). At the maturity stage, selected agronomical parameters *viz.* shoot length, shoot dry weight, number of spikes per plant, spike length, and 100 grain weight were calculated for three randomly selected plants from each replicate.

2.10. Field trials

In order to evaluate the response of selected PGP bacterial and fungal consortia under natural saline conditions on wheat cultivation, trials were conducted at three different sites *viz.* the Soil Salinity Research Institute (SSRI), Pindi Bhattiyan (2018–2019) (S1); a salt-affected farmer's field in Mouza sheikhana, Jhang (2020–2021) (S2); and Biosaline Research Station (BSRS II), Pakka Anna (2018–2019) (S3).

2.10.1. Soil chemical properties

The soil's basic chemical properties were determined for each site to estimate the salinity level and other nutritional factors before experimentation. Standard procedures were followed to determine electrical conductivity (EC), pH, soil organic matter (SOM), total nitrogen (N), available phosphorous (P), and ionic concentrations (Cl^-, Na^+, K^+; Walkley and Black, 1934; Olsen, 1954; Richard, 1954; Imran et al., 2021).

2.10.2. Experimental design

Briefly, two local wheat varieties, Faisalabad 2008 and Galaxy 2013, were selected, and healthy untreated seeds were used for two controls, one with 100% FRD of fertilizer (T1) and the second with 80% FRD (T2). Seeds, bio-primed with bacterial (T3) and fungal (T4) consortia separately as bioinoculants, were used for treatments in these trials, and a randomized complete block design (RCBD) with plot size (2×5 m^2) in triplicates for each location was implemented.

2.10.3. Plant growth parameters

Different agronomical parameters like days of flowering, days of maturity, flag leaf width, flag leaf length, flag leaf weight, plant height, number of spikes per plant, spike length with awns, spike weight, number of grains per spike, 1,000 grain weight, and total yield per plot were calculated to estimate the effects of formulations.

2.11. Statistical analyses

For the data analysis of the pot experiment, the parameters were assessed as mean ± S.E (standard error) followed by Dunnett's tests and Tukey's HSD tests for comparison with control (i.e., FDR 100%) individually and for intra-dosage comparison, respectively. All the statistical analyses were made using JMP v.11 (SAS, Cary, NC). The graphs were designed using Microsoft Excel v.2019.0. While for the field data, all the analyses were made in R. software v.4.2.1. for two-way ANOVA and combined correlation using tidyverse, AICcmodavg, and corrplot R packages. Boxplots and correlation matrixes were visualized using ggplot2 and ggthemes packages.

3. Results

3.1. Basic chemical properties of soil samples

Data for the ECs and pHs of all 17 samples is provided in Supplementary File 1, Table 1. Briefly, all soils were saline, ranging from moderately (4–8 dSm^{-1}) to very strongly saline (>16) as per criteria described by Abrol et al. (1988). The lowest EC (6.08 ± 0.56) was recorded for samples collected from Choa-Saidan Shah-Kallar Kahar Road, Punjab (30.4319, 71.9958), and the highest (35.34 ± 1.02) was for samples from Bhago Bhutto, Dhairki, Sindh (28.0403, 69.6561). Soil pH for samples ranged from neutral to alkaline, and the lowest pH (7.23 ± 0.04) was observed for samples from Talaganag-Kallar Kahar Road, Punjab (32.7863, 72.6968) and the highest (9.02 ± 0.082) for Choa-Saidan Shah- Kallar Kahar Road, Punjab (32.7485, 72.7574).

3.2. Isolation and MIC determination for salt tolerance:

A total of 297 salt-tolerant strains were isolated from 17 sites in Punjab and Sindh (Supplementary File 2, Table 1). Their MIC (minimal inhibitory concentration) was determined, and 141 bacterial isolates that were able to withstand 7% and/ or 10% NaCl concentration were screened to assess plant growth-promoting characteristics (Supplementary File 2, Table 2).

3.3. Screening and selection of bacterial strains for bacterial consortia based on PGP activities and biochemical characterization

Details of selected PGP and biochemical characteristics of 141 halotolerant bacteria are provided in Supplementary File 2, Table 3. Many strains showed possession of multiple characteristics, and three strains UM 16, UM 58, and UM 83 were shortlisted to evaluate their performance on wheat growth under saline conditions. Briefly, these strains were selected on the basis of their overall PGP and biochemical properties. All three strains were able to solubilize the P, K, and Zn. None of the isolates were able to produce IAA as no color change was observed after the addition of Salkowski reagent. All three strains were able to produce siderophore, and average activity was recorded on CAS agar plates. Bacterial strains UM 16, UM 58, and UM 83 were catalase positive and oxidase negative. All strains were positive for EPS production and Amylase activity. No color change in media indicated the absence of HCN production for all three strains. ACC deaminase activity was detected only for UM 16. A summary of PGP traits including solubilization indices for P, K, and Zn, and selected biochemical characterization of three selected strains is provided in Table 1.

3.4. Molecular identification

Bacterial strain UM 16 showed 99.76% similarity with the *Erwinia rhapontici* strain, strain UM 58 was identified as *Pantoea agglomerans* based on 99.61% similarity with the *P. agglomerans* strain submitted in the gene bank, while strain UM 83 was classified as *Pantoea* sp. on the basis of 97.01% similarity. The phylogenetic tree (Figure 1) indicated their origin, and the accession numbers obtained for UM 16, UM 58, and UM 83 are OQ318271, OQ318275, and OQ318278, respectively.

3.5. Strains compatibility assessment

No strain hindered the growth of other strains, and hence they were all considered compatible with each other for the formulation of consortium.

3.6. Plate assay for seed germination

The effects of seed priming with water, 0.2 mM NaCl solution, and bacterial treatment on seed germination of both wheat varieties at different NaCl concentration is illustrated in Figure 2; Table 2. Overall, the germination percentage was better for Faisalabad 2008 under saline conditions as compared to Galaxy 2013, and the bacterial consortium improved the germination rate under saline conditions for both varieties. Seeds treated with 0.2 mM NaCl also showed relatively better performances than the control (T1). No seed germination was observed at 100 and 150 mM NaCl concentration in the case of Galaxy 2013, while only 4.7% seed germination was observed for the Faisalabad 2008 variety in the case of 0.2 mM NaCl pretreated seeds at 150 mM NaCl concentration and 14.2% for the bacterial consortium.

3.7. Rhizoscanning for plant root architecture studies

Root rhizoscanning was conducted for two wheat varieties, Faisalabad 2008 and Galaxy 2013, at different salinity levels to observe the impact of bacterial treatment on various root parameters, including length and morphology. The plants treated with bacteria in both varieties exhibited significantly greater root length, surface area, and average root diameter compared to the non-treated plants across all salinity levels. The highest values for root length, surface area, and average root diameter were observed at 20 and 40 mM NaCl

TABLE 1 Assessment of plant growth promoting and other selected biochemical characteristics of three bacterial strains used in consortium for evaluation of wheat growth under saline environments.

	Solubilization Index			Production				Other metabolic/ enzymatic activities			
	P	Zn	K	IAA	EPS	HCN	Siderophore	ACC deaminase	Catalase	Oxidase	Amylase
UM 16	3.33	3.42	4	–	+	–	+	+	+	–	+
UM 58	2.30	3.75	4.75	–	+	–	+	–	+	–	+
UM 83	1.28	2.33	4.75	–	+	–	+	–	+	–	+

Where, P = Phosphorus, Zn = Zinc, K = Potassium, IAA = Indole acetic acid, EPS = Exopolysaccharides, HCN = Hydrogen cyanide and "+" is an indication of presence while "–" indicates absence or no activity.

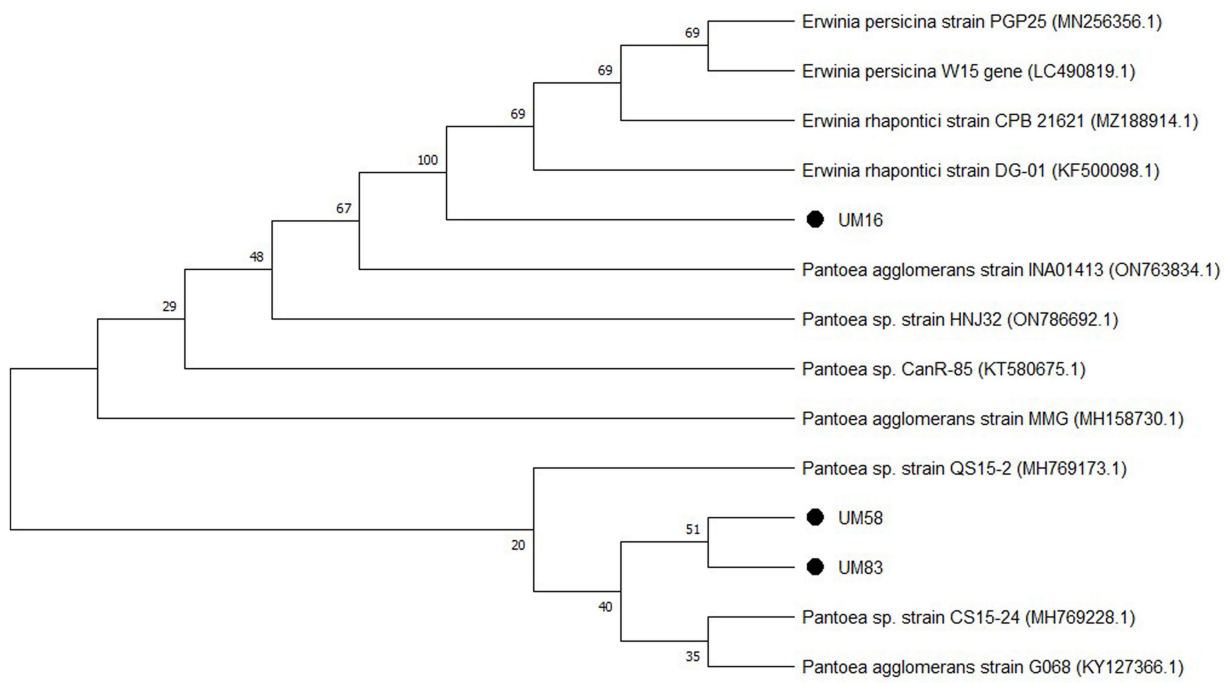

FIGURE 1
Phylogenetic tree of bacterial strains UM 16, UM 58, and UM 83, constructed using the neighbour-joining (NJ) method where >20% bootstrap values (10000) are shown at the nodes.

concentrations, ranging from 122 to 176 cm in length, 12 to 27cm^2 in surface area, and 0.37 to 0.44 mm in root diameter. The maximum root volume and number of root tips were recorded at 0 and 20 mM NaCl concentrations, measuring 0.117 to 0.116cm^3 and 165 to 334, respectively, as shown in Figures 3–5; Supplementary File 1, Tables 3, 4. Overall, all the seedlings treated with bacteria displayed superior performance compared to the control group (C2) that received 80% strength of Hoagland solution.

3.8. Pot experiment under control and saline conditions

The pot experiment was carried out to assess the five agronomical (shoot length and dry weight, number and length of spike, and 100 grain weight) and metabolic (catalases, peroxidases, and proline) parameters using two wheat varieties at three NaCl salinity levels. For the statistical significance, the trial was assessed using Dunnett's test ($p < 0.05$) which correspondences to the control (FRD 100%) at each salinity level per treatment while the significance across the test salinity levels was assessed using ANOVA followed by Tukey's HSD test ($p < 0.05$).

For agronomical parameters, in Galaxy 2013, the statistical significance varied with salinity level; however, it was observed that the plants tested with FRD 80% and fungal consortium have shown significantly varied statutes compared to the control and bacterial consortium. During the experiment, it was observed that fungal inoculation improved the agronomical parameters compared to FRD 80% at the vegetative stage. However, no significant effect was observed on plants with respect to agronomical parameters, when studied at maturity levels (Supplementary Data File 1, Tables 5–8). Meanwhile, when plants were subjected to the highest tested NaCl salinity level, i.e., 10 dSm^{-1}, a few parameters did exhibit elevated significance for bacterial consortium. A similar trend was observed for Faisalabad 2008, but it was observed that Faisalabad 2008 (Figures 6, 7) was more responsive to tested parameters compared to

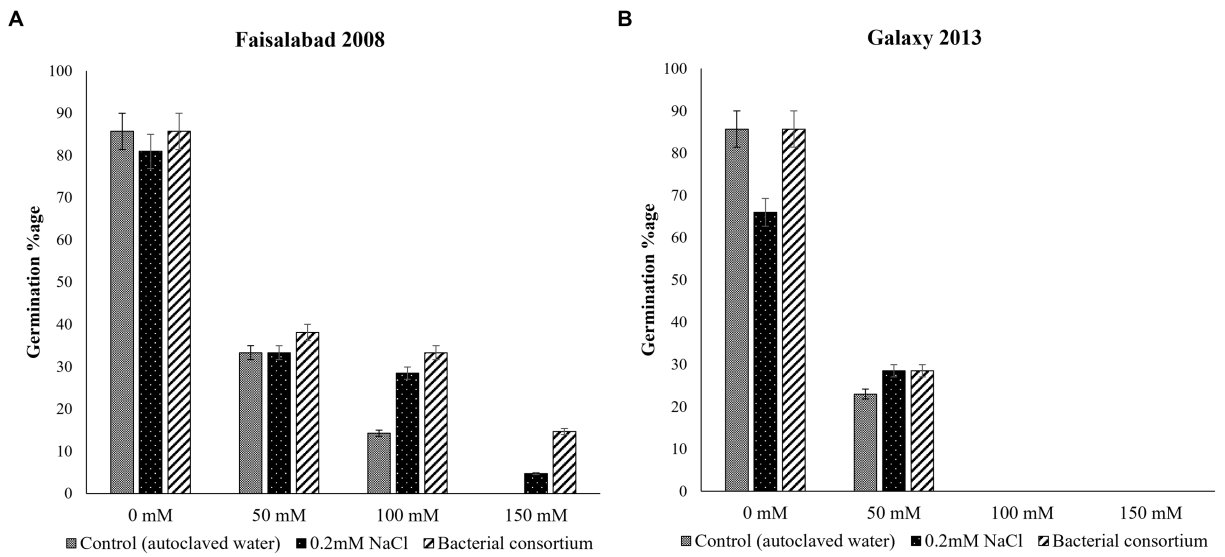

FIGURE 2
Effects of seed priming with autoclaved water, 0.2mM NaCl solution, and bacterial consortium on seed germination percentage of wheat varieties **(A)** Faisalabad 2008 and **(B)** Galaxy 2013 at 0, 50, 100, and 150, mM concentrations of NaCl.

TABLE 2 Seed germination percentage of wheat varieties; Faisalabad 2008 and Galaxy 2013 at four different concentrations of NaCl, primed with autoclaved water, 0.2mM NaCl solution, and bacterial consortium.

NaCl concentration	Seed germination percentage (%)					
	Faisalabad 2008			Galaxy 2013		
	Control (autoclaved water)	0.2mM NaCl	Bacterial consortium	Control (autoclaved water)	0.2mM NaCl	Bacterial consortium
0 mM	85.7	80.95	85.7	85.70	66.00	85.7
50 mM	33.33	33.33	38.1	23	28.5	28.5
100 mM	14.28	28.5	33.33	0	0	0
150 mM	0	4.7	14.7	0	0	0

Galaxy 2013 (Figures 6, 7). A general trend to infer the agronomical statutes is; bacterial consortium ~ FRD 100% > fungal consortium ~ FRD 80% and 2 dSm^{-1} > 6 dSm^{-1} > 10 dSm^{-1}, suggesting the low grain yield was directly affected due to the other reducing agronomical parameters with the increase of salinity levels. For metabolic parameters, treating plants with bacterial consortium leads to significantly elevated differences compared to all the other plants for both the varieties. However, with the increase of the NaCl dosage, metabolic parameter decline was observed in all the tested plants. Generally, the significant differences were observed for both varieties at moderate saline conditions (i.e., 6 dSm^{-1}) in the following pattern: bacterial consortium > FRD 100% > fungal consortia > FRD 80%, while lower at non-saline conditions (i.e., 2 dSm^{-1}) and strongest at high saline conditions (i.e., 10 dSm^{-1}) (Figure 8).

3.9. Field trials

3.9.1. Soil physicochemical properties

A summary of soil standard parameters like texture and selected chemical parameters is given in Table 3. Briefly, pHs for soils at three different sites ranging from mildly alkaline for Mouza Sheikhana, Jhang (7.56 ± 0.12) and Pindi Bhattiyan (7.73 ± 0.03) to moderately alkaline for Pakka Anna (8.21 ± 0.09). Soil ECs, at all three different sites, fall under the moderately saline (EC: 4–8) category with Mouza Sheikhana, Jhang having the highest (7.92 ± 0.14) followed by Pakka Anna (7.18 ± 0.09) and Pindi Bhattiyan (6.3 ± 10.27). Typical properties of saline soils (high ionic concentrations and lower OM and N values) were present at these sites. The highest concentration of chlorides (meq L^{-1}) was observed for Mouza Sheikhana Jhang (251 ± 4.87) followed by Pakka Anna (247 ± 6) and Pindi Bhattiyan (187 ± 1.69). Water-soluble Na^{+} (g L^{-1}) was also highest at Mouza Sheikhana Jhang (31.4 ± 0.97) followed by Pindi Bhattiyan (18 ± 0.28) and Pakka Anna (15.01 ± 4.08). The available P met the recommended normal values for these regions (Figures 9, 10).

3.9.2. Soil agronomical parameters

Overall, bacterial treatment (T3) performed better compared to fungal treatment (T4) and control with reduced fertilizer (T2) at all three sites, and Faisalabad 2008 showed better outcomes compared to Galaxy 2013 (Figures 9, 10; Supplementary File 1, Tables 9–11). For the studied agronomical parameters, the response to bacterial (T3)

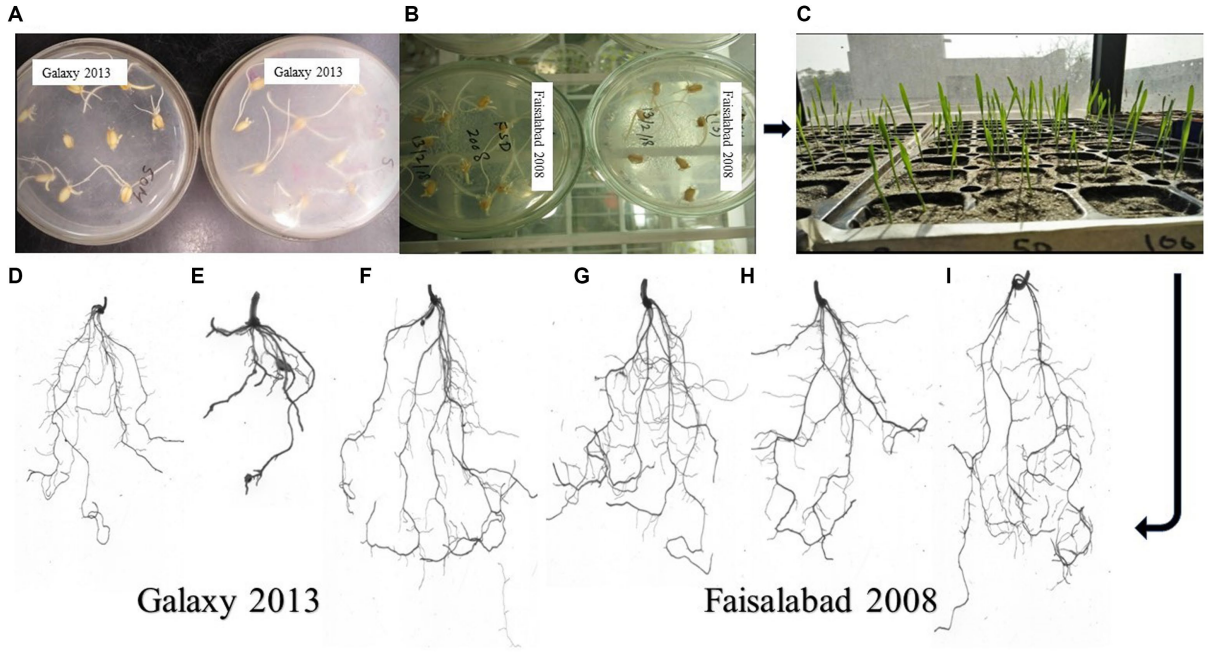

FIGURE 3
Root Architecture studies of two wheat varieties using a plate germination assay, where **(A)** Galaxy 2013 and **(B)** Faisalabad 2008 showed seed germination on 1% agar plates with 40 and 60mM NaCl, respectively, and **(C)** plant growth in sterilized sand pots at different NaCl concentrations. Differences in root structure for Galaxy 2013 at 40mM **(D)** 100% Hoagland concentration, **(E)** 80% Hoagland **(F)** Bacterial consortium, and for Faisalabad 2008 at 60mM **(G)** 100% Hoagland concentration **(H)** 80% Hoagland **(I)** Bacterial consortium.

and fungal (T4) inoculations in the form of percentage (%) increase as compared to control with 20% reduced fertilizer (T2) at all three sites was calculated. Briefly, a maximum % increase for 1,000 grain weight was observed for bacterial treatment of both varieties, 21.8% for Faisalabad 2008 and 15.9% for Galaxy 2013 at Pakka Anna. Similarly, a maximum increase in plot yield was observed for Faisalabad 2008 at Pakka Anna (10.07%) and Jhang (13.5%), while an increase of 21.7% was observed for Galaxy 2013 at Jhang. Details for all studied agronomical parameters are provided in Supplementary File 1, Table 12.

Statistical analysis using a two-way ANOVA at each location indicated that both treatments and wheat genotypes individually have a significant relationship with almost all parameters considered in this study except yield per plot (Supplementary File 1, Tables 13–15). Combined correlation graphs while considering both wheat varieties separately and all replicates of all treatments collectively at three sites simultaneously showed that the mutual effect of salinity and reduced fertilizer (T2, T3, and T4) is more drastic compared to salinity solely (T1).

Surprisingly, most parameters were significantly or less significantly positively correlated with each other but were, however, also negatively correlated with yield per plot for each variety. For example, Plant height for T1 was positively correlated with flag leaf length (0.775), flag leaf weight (0.779), and number of grains per spike (0.700) for Galaxy 2013 but a negative correlation (−0.395) for 1,000 grain weight and yield per plot (−0.389) was observed. However, for T2, plant height showed positive correlation with number of grains (0.765). Similarly, for T3, plant height showed a positive correlation with flag leaf length (0.847), flag leaf weight (0.0.941), and number of grains per spike (0.781).

In the case of Faisalabad 2008, in addition to flag leaf length (0.750), flag leaf weight (0.0.910), and number of grains per spike (0.710), plant height was also positively correlated with number of spikes per plant (0.774) for T1. Similarly, yield per plot was only negatively correlated with spike weight (−0.035). For T3. All parameters were positively correlated with plant height except for1000 grain weight (−0.227) and spike length with awns (−0.191). The fungal consortium (T4) showed a higher negative correlation with all parameters (−0.909 to −0.735) for spike length with awns except 1,000 grain weight (−0.094), yield per plot (−0.227), and plant height (−0.519). Details of correlation studies among different agronomical parameters and treatments for each variety are provided in Figures 11, 12.

4. Discussion

Despite all the recent advancements and application of the latest technologies for sustainable agriculture, the threat to food security due to continual climate change is still persistent. The expansion of salinity around the globe generally, and in Pakistan particularly, needs special attention (Nawaz A. et al., 2020). Scientists in countries facing the challenges of agricultural lands deterioration due to salinity are giving special emphasis to the exploration of the indigenous microbial flora for the reclamation of saline soils and to exploit the hidden potential of these microbes for crops augmentation in addition to finding the solution through classical and modern approaches like breeding, gene editing, and development of transgenic crops (Perez-Jimenez and Perez-Tornero, 2020; Marghoob et al., 2022). The present study was conducted to explore the indigenous microbial flora of the Indus basin region of Pakistan and its efficacy to overcome the problems associated with low

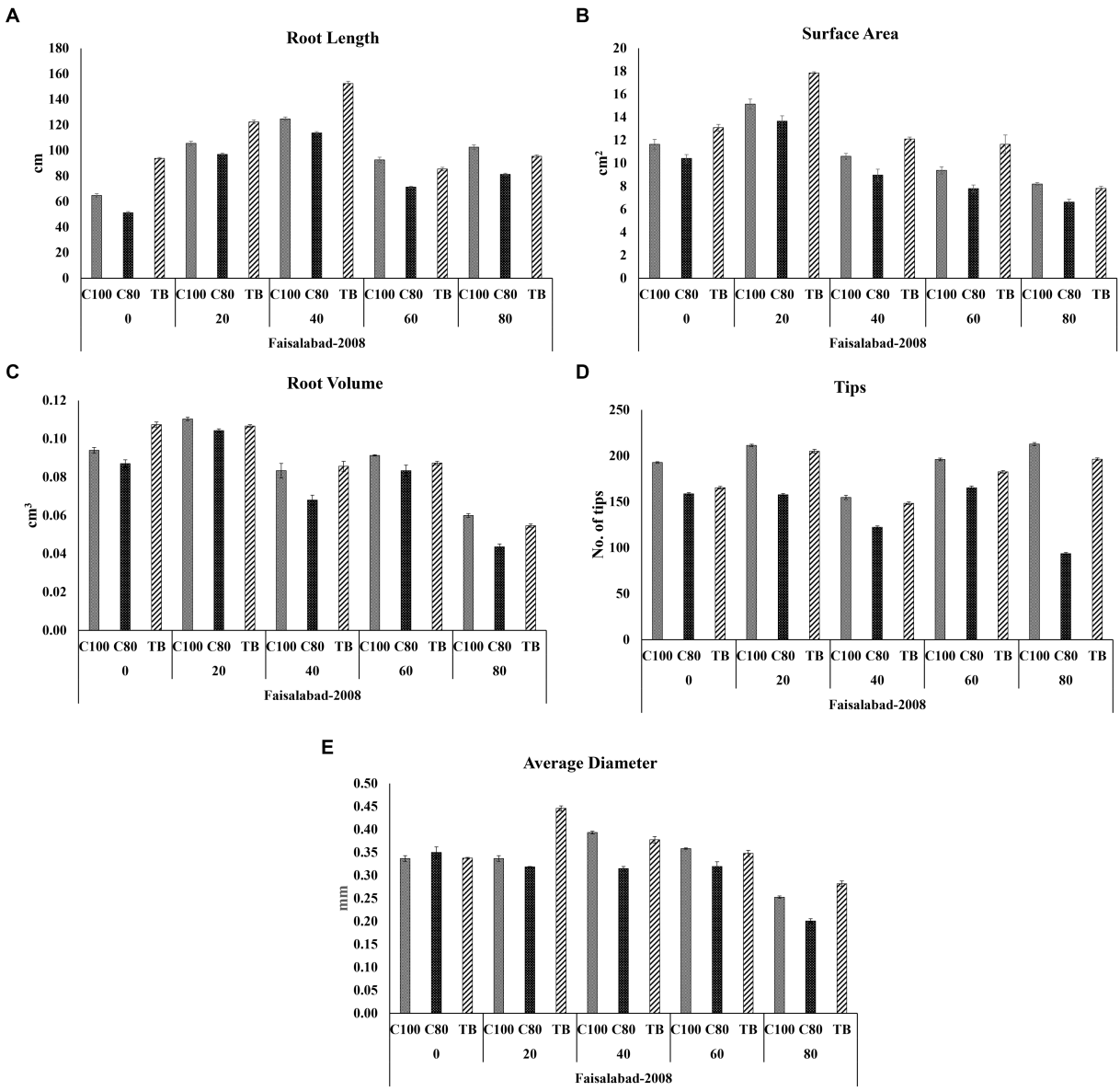

FIGURE 4
Effects of different concentrations of Hoagland solution C100 = 100%; C80 = 80% and BT = (bacterial consortium and 80% Hoagland solution) on selected root structure traits **(A)** Root length; **(B)** Surface area; **(C)** Root volume; **(D)** Tips **(E)** Average diameter, of wheat variety Faisalabad 2008 at 0, 20, 40, 60, and 80 mM NaCl concentrations where the bar indicates the standard error when n = 3.

wheat production by mitigating the adverse effect of salinity, and initial promising results indicated the potential of selected strains for salinity stress alleviation in addition to less dependency on chemical fertilizers.

In the current scenario, salt-affected agroecological zones present in the Indus basin region of Pakistan provide an ideal location, as the agricultural sector mainly relies on this region to meet the wheat requirements and needs special attention to achieve the desired yield for the ever-increasing population. Recent but limited culture-dependent and independent studies revealed that relatively less explored indigenous flora might possess multiple PGP traits in addition to being able to withstand hypersaline conditions (Mukhtar et al., 2020; Nawaz A. et al., 2020; Marghoob et al., 2022). Therefore, soil samples were collected from agricultural fields in the Punjab and Sindh provinces from 17 sites (Supplementary File 1, Table 1), where wheat is grown in the winter season and these areas were affected by various levels of salinity. Soil EC values (Supplementary File 1, Table 1) indicated that all these soils fall under the category of saline soils, ranging from moderately saline (4–8) to very strongly saline (>16) according to Abrol et al. (1988). These soil samples were used for the isolation of halotolerant bacteria and their characterization.

Assessment of microbial flora for possession of salt tolerance and PGP traits are a primary prerequisite before their application for salinity stress mitigation in plants (Li et al., 2022). In order to evaluate the MICs for salt tolerance and PGP and other biochemical traits, the 297 initially isolated pure colonies were screened on salt-supplemented media, and only isolates with the capacity to withstand 7% and/ or 10% NaCl concentration (Supplementary File 2, Table 2) were selected for further evaluation. Out of 141 halotolerant selected strains, a

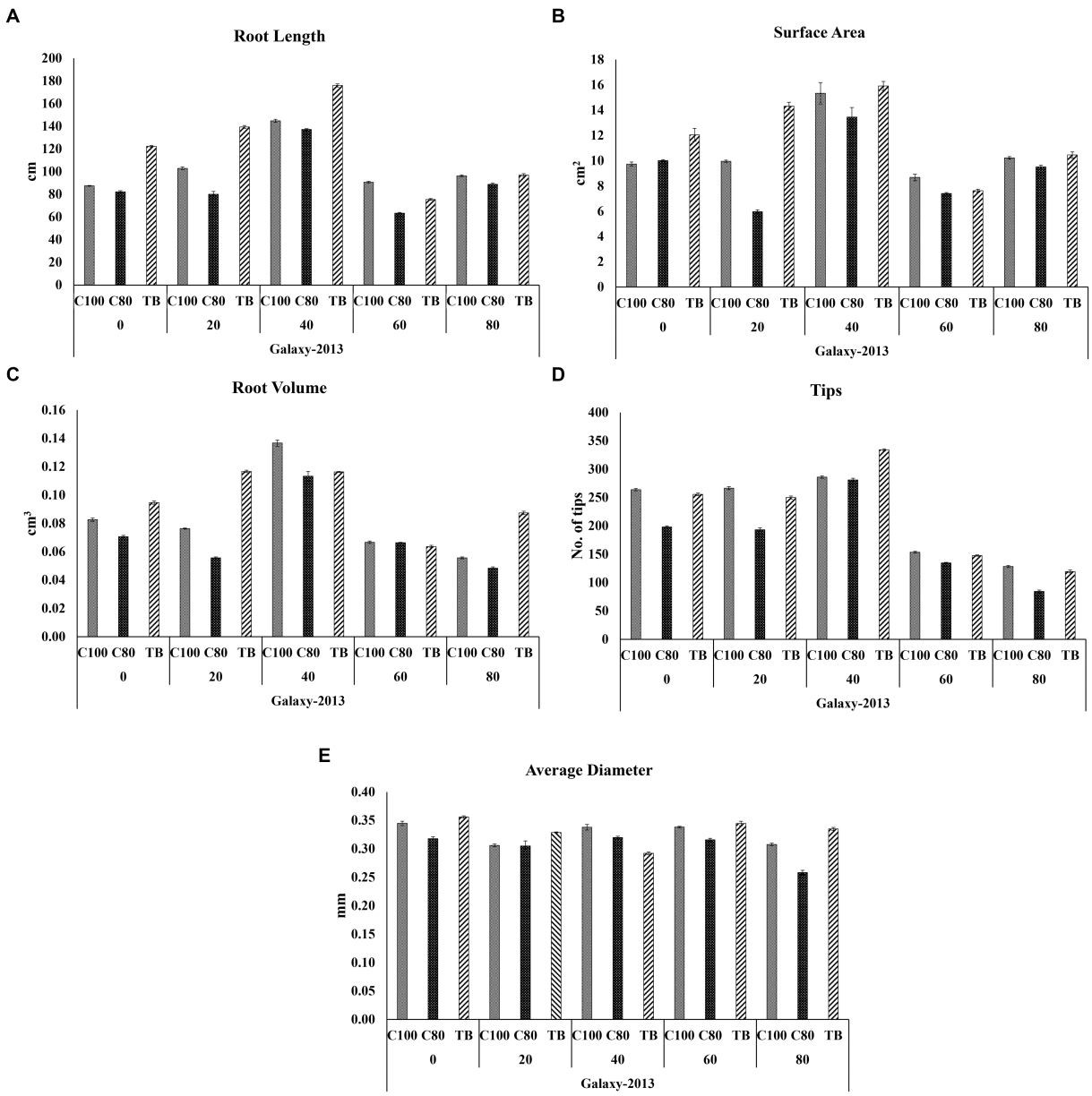

FIGURE 5
Effects of different concentrations of Hoagland solution C100 = 100%; C80 = 80% and BT = (bacterial consortium and 80% Hoagland solution) on selected root structure traits **(A)** Root length; **(B)** Surface area; **(C)** Root volume; **(D)** Tips **(E)** Average diameter, of the wheat variety Galaxy 2013 at 0, 20, 40, 60, and 80 mM NaCl concentrations where the bar indicates a standard error when n = 3.

majority number showed possession of one or multiple characteristics (Supplementary File 2, Table 3) that can help in plant growth promotion directly or indirectly. Interestingly, only a selected set of isolates was able to produce IAA. This could be explained by a recent study, based on a metagenomic survey of salt-affected soils in the Indus Basin region, that proposed the presence of microbes with a tryptophan-independent indole-3-glycerol phosphate synthase (IGPS) mechanism in these soils and hypothesized that the activity of IGPS might be directly linked with salinity (Marghoob et al., 2022).

On the basis of promising PGP and other biochemical characteristics (Table 1), three bacterial strains, UM 16, UM 58, and UM83, were selected to further investigate their role in stress alleviation and crop improvement. Briefly, all three strains were able to withstand NaCl concentrations up to 10%. All three halotolerant strains were able to solubilize inorganic phosphorous, potassium, and zinc. In addition, these strains have the capability to produce siderophores and EPS and possess amylase enzymes, as these characteristics help microbes to survive and facilitate plant growth in harsh conditions (Batool et al., 2017; Mukhtar et al., 2019). No hemolytic activity was observed on the blood agar medium during initial screening, which indicated their nonpathogenic nature (Payment et al., 1994). 16S rRNA gene sequencing and BLAST results revealed that these strains belong to *Erwinia rhapontici, Pantoea agglomerans*, and *Pantoea* sp., respectively.

The role of PGP strains belonging to the genus *Pantoea* in plant growth improvement has been reported previously, and their ability

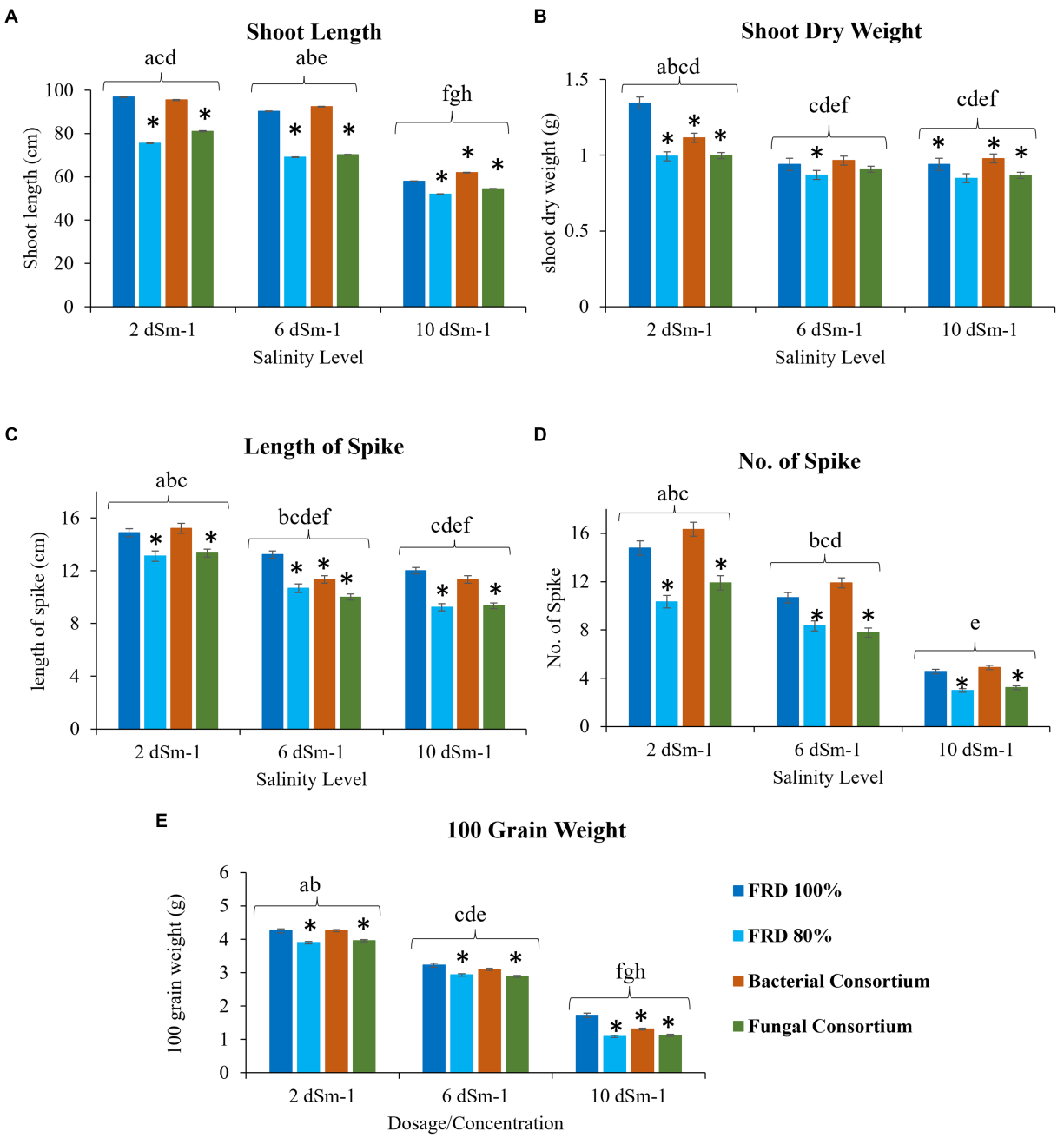

FIGURE 6
Effect of reduced fertilizer: 80% FRD and bacterial and fungal inoculation on agronomical parameters **(A)** Shoot length **(B)** shoot dry weight **(C)** Length of spike **(D)** No. of Spike **(E)** 100 grain weight; at different salinity levels for the wheat variety Faisalabad 2008 in pot experiment where bars indicate the Mean ± Standard Error of N = 3, n = 9; *$p < 0.05$, Dunnett's test relative to control; FRD 100%. Different letters indicate $p < 0.05$, ANOVA followed by Tukey's HSD test across the test dosage.

to display multiple PGP characteristics, including a promising role under biotic and abiotic stresses, is also well established (Mishra et al., 2016; Chakdar et al., 2018; Luziatelli et al., 2020; Lorenzi et al., 2022). Mishra et al. (2016) reported that strains belonging to *Pantoea agglomeran* produce EPS that in addition to helping in osmoregulation under abiotic stresses also improve soil texture through formation of microaggregates and enhance water holding capacity.

Interestingly, similar to many other bacterial families, bacterial strains belonging to the genus *Erwinia* also showcase diverse characteristics from pathogenicity to PGP and stress alleviation (Kang et al., 2014; Saldierna Guzmán et al., 2021; Cochard et al., 2022). Bacterial strains belonging to *E. rhopontici* have been associated with pathogenicity previously; however, a recent study by Kaur et al. (2023) indicated their PGP traits and a consortium containing *E. rhopontici* improved the growth of finger millet even at a reduced dose of chemical fertilizer. The *Erwinia* strain used in this study exhibited no potential threat to the crop as well as to human health. However, further research is recommended to evaluate the pathogenic role of these strains to eliminate the risk of disease on other rotational crops grown in this region.

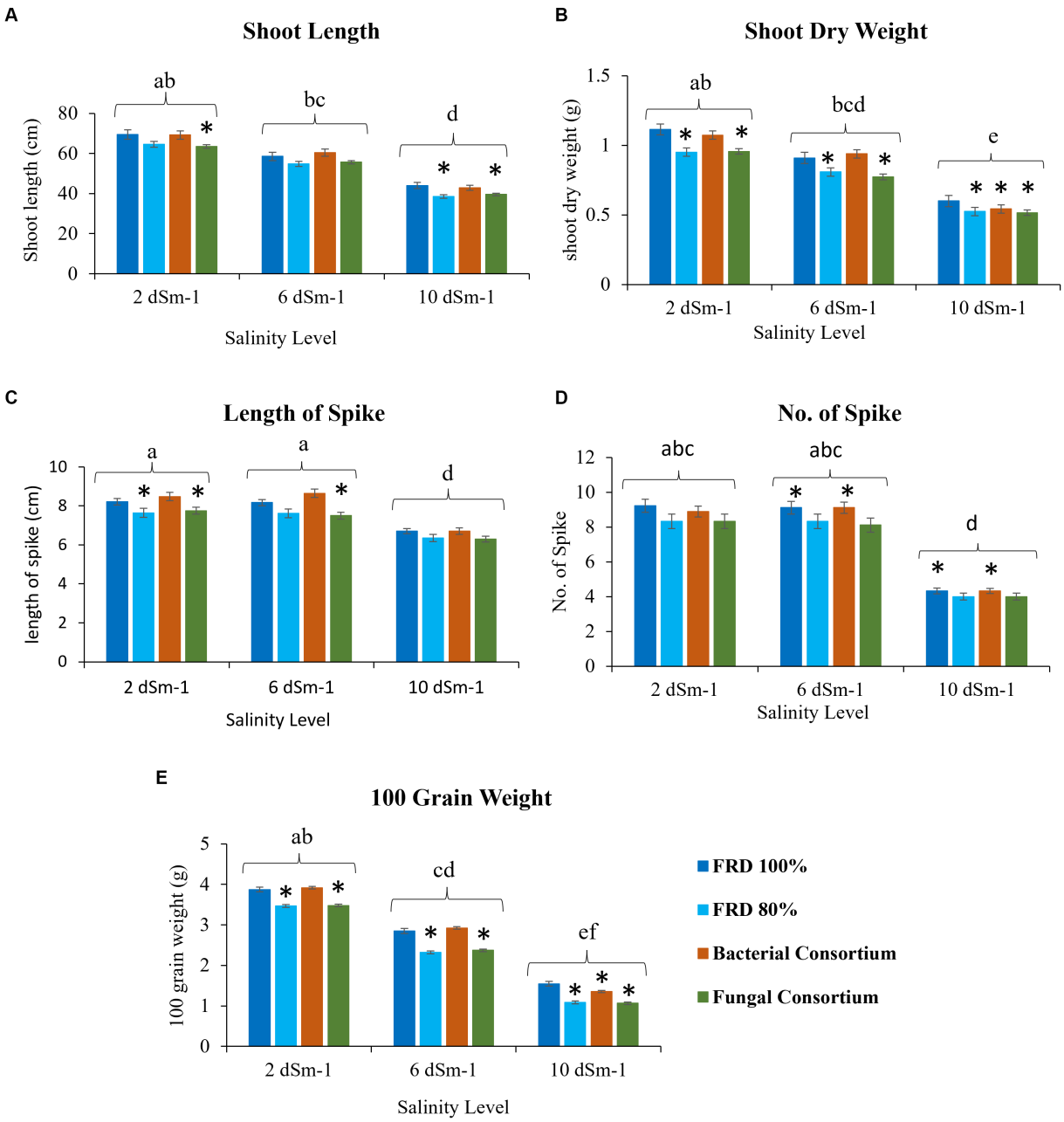

FIGURE 7
Effect of reduced fertilizer: 80% FRD and bacterial and fungal inoculation on agronomical parameters **(A)** Shoot length **(B)** shoot dry weight **(C)** Length of spike **(D)** No. of Spike **(E)** 100 grain weight; at different salinity levels for the wheat variety Galaxy 2013 in pot experiment where bars indicate the Mean ± Standard Error of N = 3, n = 9; *$p < 0.05$, Dunnett's test relative to control; FRD 100%. Different letters indicate $p < 0.05$, ANOVA followed by Tukey's HSD test across the test dosage.

In addition, the *E. rhopontici* strain used in this study also exhibited ACC deaminase activity. This enzyme is of crucial importance for plant growth in stressed environments including that of salinity as it regulates the excessive production of ethylene by breaking the precursor for ethylene, 1-aminocyclopropane-1-carboxylate (ACC), into ammonia and α-ketobutyrate (Bouffaud et al., 2018; Ben-Laouane et al., 2020).

Studies regarding the understanding of mycorrhizal and fungal communities to improve plant overall health and as biocontrol agents suggested the promising role of these creatures for sustainable agriculture (Faraz et al., 2020; Comite et al., 2021). However, research related to exploring the potential of individual fungal strains as plant growth promoters is limited due to the complicated metabolic mechanisms they possess, making it difficult to gain a conclusive idea of how they are involved in the improved growth of plants. In general, indirect effects on overall plant health and growth parameters are associated with the inoculated fungal strains (Martinez-Medina et al., 2014; Sánchez-Montesinos et al., 2020). Literature survey has shown strains of the genus *Trichoderma* might play a vital role in plant growth and stress alleviation for wheat under abiotic stresses like salinity (Sánchez-Montesinos et al., 2020). Hence, two previously isolated and characterized strains of *Trichoderma*, *T. harzianum*, and

TABLE 3 Physicochemical properties of three salt-affected sites selected for field trials.

Location	coordinates	Soil texture	pHs	ECe	Organic matter	Available P	Chlorides	Water soluble K^+	Water soluble Na^+	Nitrogen
				($dS\,m^{-1}$)	(%)	($mg\,kg^{-1}$ soil)	($meq\,L^{-1}$)	($g\,L^{-1}$)	($g\,L^{-1}$)	(%)
SSRI, Pindi Bhattiyan	31°53′45″N, 73°16′34″E	Sandy clay	7.73 ± 0.036	6.31 ± 0.27	1.19 ± 0.117	27.43 ± 1.82	187 ± 1.69	0.43 ± 0.026	18 ± 0.28	0.052 ± 0.004
BSRS II, Pakka Anna, Faisalabad	31°15′00.2″N 72°49′25.0″E	Sandy loam	8.21 ± 0.098	7.18 ± 0.09	0.78 ± 0.098	32.61 ± 0.67	247 ± 6	0.23 ± 0.071	15.01 ± 4.08	0.065 ± 0.007
Farmer's field, Mouza Sheikhana, Jhang	31°21′52″N, 72°22′31″E	Sandy loam	7.56 ± 0.121	7.92 ± 0.14	1.08 ± 0.21	24.52 ± 2.32	251 ± 4.87	0.29 ± 0.038	31.4 ± 0.97	0.071 ± 0.004

EC: Electrical conductivity; P: Phosphorous; K^+: Potassium ion and Na^+: Sodium ion. Values: average ± standard deviation ($n = 3$).

T. viridae (Faraz et al., 2020) were selected to evaluate the response of wheat crop in the presence of saline conditions.

Similarly, application of microbial strains in the form of a consortium, in comparison to the use of individual strains, for field experiments is considered a more promising approach (Devi et al., 2022). Survival and adaptation chances of microbial strains applied in the form of consortia are higher as compared to individual strains (Sekar et al., 2016; Kaur et al., 2023). However, one strain might hinder the growth of other, and cross streak method is therefore considered a robust and convenient approach to check the compatibility of different strains used in a consortium (Semenov et al., 2007). Additionally, as none of the strains displayed antagonistic activity against each other, the consortia of three selected bacterial strains and both fungal strains were developed separately to examine their role in plant growth under saline conditions.

Salinity may hamper the growth of plants at different stages (Etesami and Maheshwari, 2018; Perez-Jimenez and Perez-Tornero, 2020; Hajiabadi et al., 2021); a set of experiments was therefore used in this study to deeply examine the response of two local wheat cultivars, Faisalabad 2008 and Galaxy 2013 (differed based on salt tolerance), in the presence of these inoculums and at different salinity levels *in vitro* as well as under semi-controlled and field conditions according to protocols described in the literature previously for different crops (Mukhtar et al., 2020; Nawaz A. et al., 2020; Perez-Jimenez and Perez-Tornero, 2020).

Initially, a plate assay for seed germination was performed for both wheat varieties at four different salinity levels (0, 50, 100, and 150 mM NaCl), and results indicated that seed germination was better in the case of Faisalabad 2008 compared to Galaxy 2013. However, the germination rate was inversely proportional to increasing salt concentration. Seeds primed with bacterial inoculum performed better than controls. The results were according to expectations as Faisalabad 2008 is considered somewhat resistant to mild saline conditions while Galaxy 2013 is more susceptible (Kanwal et al., 2011; Nawaz et al., 2013; Ahmad A. et al., 2022), and the literature survey indicated the potential of PGP strains including non-pathogenic *Pantoea* and *Erwinia* spp. (Cochard et al., 2022; Lorenzi et al., 2022) for salinity stress alleviation in crops through different metabolic pathways that confer resistance through direct and indirect mechanisms (Nawaz M. S. et al., 2020; Yang et al., 2020).

Additionally, roots are tissues of the plant more directly exposed to abiotic stresses like salinity compared to other parts (Byrt et al., 2018), and root system architecture studies were therefore an important aspect of understanding the response of roots to salinity in the presence of bioformulation. Different root architecture parameters analyzed in this study (Figures 3–5; Supplementary File 1, Tables 3, 4) indicated that in general, the presence of bioformulations positively correlates with these parameters and helps in suppressing the negative effects of salinity and nutrient deficiency. Interestingly, in both contrasting genotypes, effects of low nutrients and bacterial formulation combinations were surprising at 40 mM for Galaxy 2013 and at 60 mM for Faisalabad 2008, as parameter values deviated from the general trend observed in the study.

Generally, studies related to the comparative effects of salinity or abiotic stress, nutrient variation, and bioformulation on root parameters of wheat are scarce (Rahnama et al., 2011; Robin et al., 2016, 2021). However, limited available data indicate that nutrients and bioformulation affect root growth in a complicated way and a

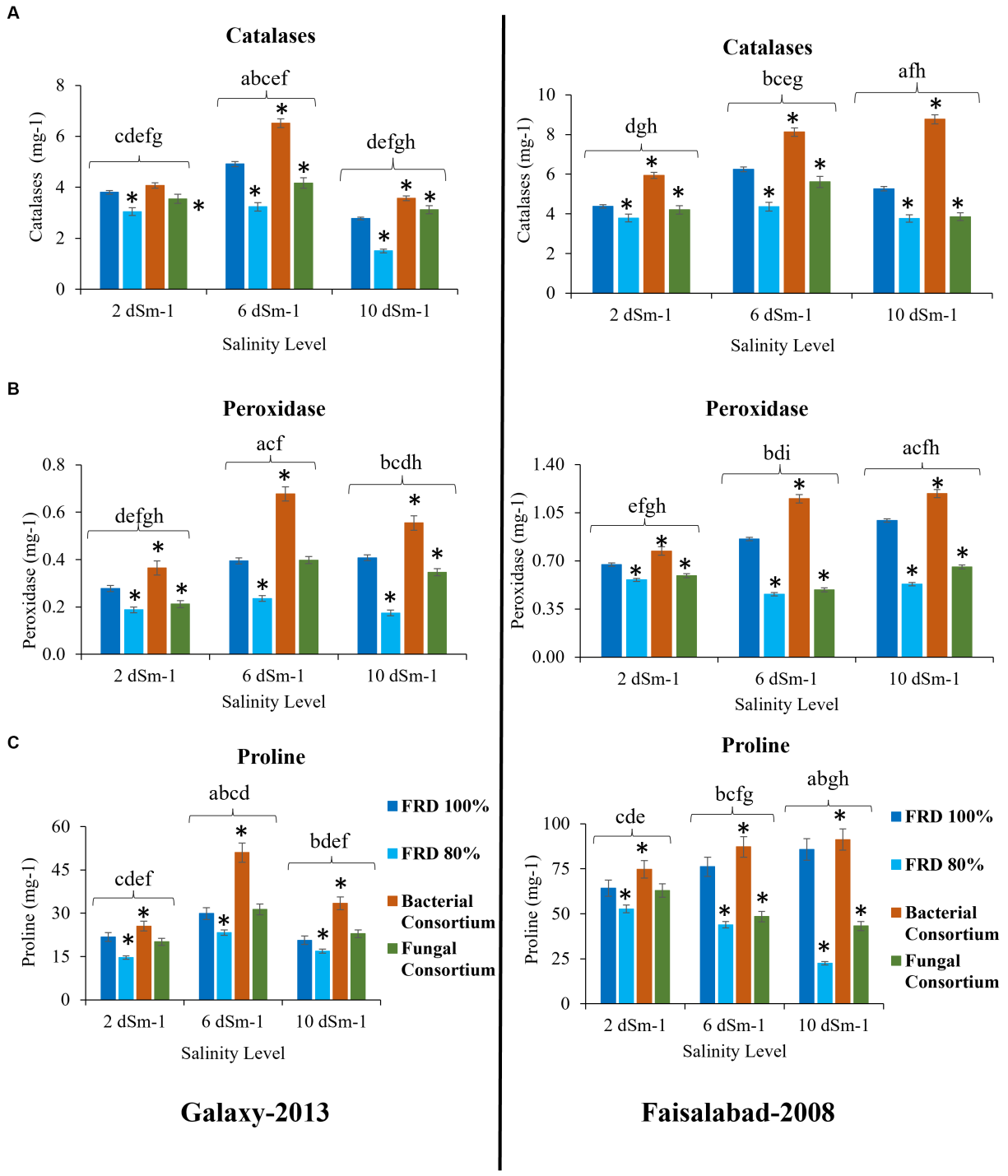

FIGURE 8
Effect of reduced fertilizer: 80% FRD and bacterial and fungal inoculation on biochemical parameters **(A)** Catalase **(B)** Peroxidase **(C)** free Proline content; at different salinity levels for wheat varieties Galaxy 2013 and Faisalabad 2008 in pot experiment where bars indicate the Mean ± Standard Error of N = 3, n = 9; *$p < 0.05$, Dunnett's test relative to control; FRD 100%. Different letters indicate $p < 0.05$, ANOVA followed by Tukey's HSD test across the test dosage.

combination of root architecture parameters, biochemical compounds exchange, and the nutrient combination might provide a better understanding of how different parameters affect root structure formation and function of plants when counted collectively (Buwalda et al., 1988; Li et al., 2014; Arif et al., 2019; Guo et al., 2019; de Souza Campos et al., 2021). Also, these parameters were studied in controlled conditions due to limitations of exploring roots architecture from the soil, and as the soil environment is different to the control conditions, studies incorporating root architecture analysis from soil samples are recommended in the future. Advanced technologies like "X-ray computed tomography (CT), Growth and Luminescence

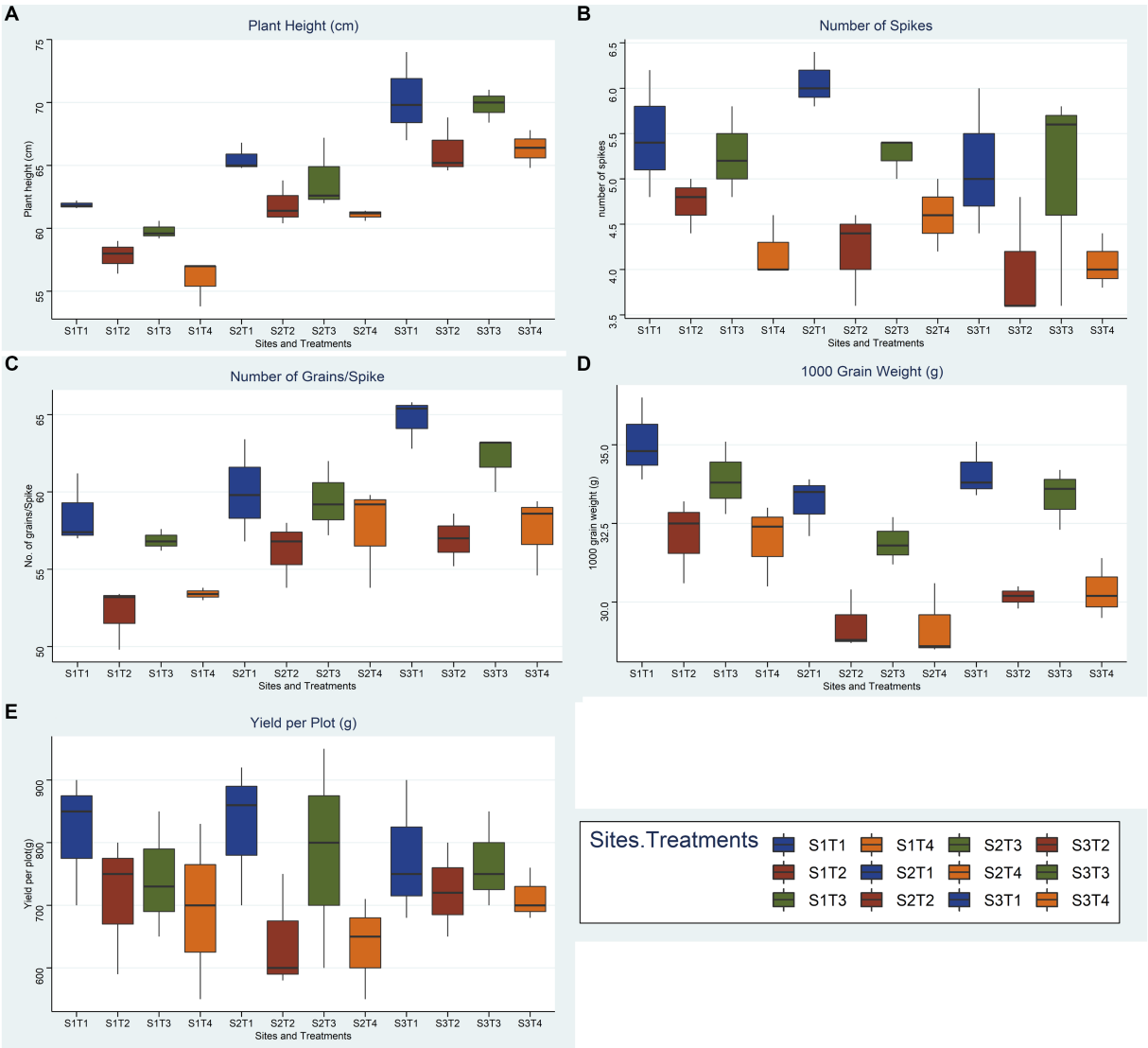

FIGURE 9
Effect of reduced fertilizer: 80% FRD and bacterial and fungal inoculation compared to 100% FRD, on agronomical parameters **(A)** plant height **(B)** number of spikes **(C)** No. of grains per spike **(D)** 1000 grain weight **(E)** yield per plot; of wheat variety Galaxy 2013 for field trials at three site, where, S1 = Pindi Bhattiyan, S2 = Jhang, S3 = Pakka Anna, T1 = Control with 100% FRD, T2 = Control with 80% FRD, T3 = Bacterial consortium and 80% FRD and T4 = fungal consortium and 80% FRD, where N = 3 and n = 15.

Observatory for Roots (GLO-Roots), Soil Rhizotron Phenotyping and computational analysis approach" etc. would facilitate our understanding of the root responses to salinity under natural environmental conditions (Zou et al., 2022).

Concisely, improved grain yield is the ultimate goal of the scientific community, and, based on the available literature, this concept was established that improvement in different agronomical parameters like plant height, number of spikelets, etc., is directly linked with the enhanced grain yield (Tshikunde et al., 2019). However, salinity levels have detrimental effects on different stages of plant growth from seedlings to crop maturity, and they dramatically alter the metabolic pathways (Khaliq et al., 2015; EL Sabagh et al., 2021). On the other hand, seed bio-priming of wheat with PGP microbes improves plant growth and helps increase resistance against biotic and abiotic stresses (Mashabela et al., 2022). To estimate the role of developed PGP fungal and bacterial bioformulations on wheat biochemical and agronomical parameters, pot experiments with three salinity levels and field trials at three different agro-ecological sites were conducted.

Results of the pot experiment indicated that both fungal and bacterial consortia significantly increased the studied biochemical activities (catalase, peroxidase, and free proline content), associated with alleviation of salinity stress, of plants as compared the control at the vegetative stage. Previous studies also showed that the application of bacterial strains helps to induce systemic resistance in plants including wheat against biotic and abiotic stresses through the upregulation of different enzymatic activities especially peroxidase (Khaliq et al., 2015; Byrt et al., 2018; Minaeva et al., 2018). Surprisingly, outcomes of fungal consortium application on overall agronomical parameters and yield at the maturity stage were non-significant. Future studies that investigate the possible reasons are strongly recommended.

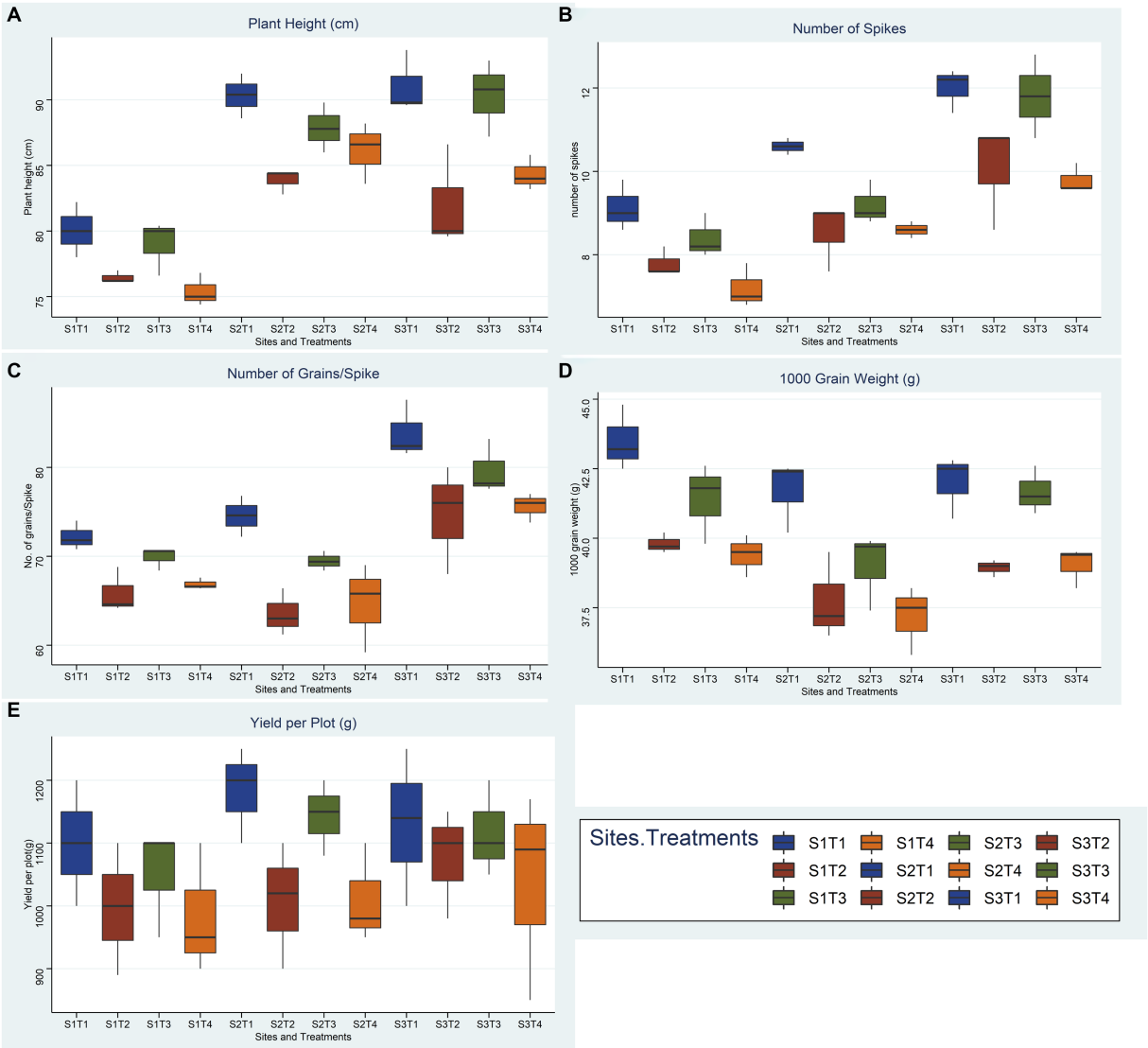

FIGURE 10
Effect of reduced fertilizer: 80% FRD and bacterial and fungal inoculation compared to 100% FRD, on agronomical parameters **(A)** plant height **(B)** number of spikes **(C)** No. of grains per spike **(D)** 1000 grain weight **(E)** yield per plot; of wheat variety Faisaabad 2008 for field trials at three site, where, S1 = Pindi Bhattiyan, S2 = Jhang, S3 = Pakka Anna, T1 = Control with 100% FRD, T2 = Control with 80% FRD, T3 = Bacterial consortium and 80% FRD and T4 = fungal consortium and 80% FRD, where N = 3 and n = 15.

Similarly, bacterial inoculation significantly improved the growth and yield of both wheat varieties compared to the control with reduced fertilizer (80% FRD) at all salinity levels and for field trials at three sites. Faisalabad 2008 performed better than Galaxy 2013, which was not surprising as Faisalabad 2008 is considered more salt tolerant compared to Galaxy 2013 (Kanwal et al., 2011; Anwar et al., 2013), and outcomes of the present study further validated its potential for better performance under abiotic conditions, particularly salinity.

However, the combined correlation analysis showed that although the other parameters were significantly correlated with each other, they were negatively or less significantly related to crucial parameters like grain weight and yield per plot for all treatments at three sites in general, which indicates that these genotypes are not suitable for cultivation in saline soils as salinity severely affected these crucial parameters and the response of both genotypes to saline conditions at multiple sites was not uniform. Studying multiple parameters simultaneously is considered an important technique to evaluate genotypes for resistance development against stresses like salinity, as salinity may or may not have an effect on different parameters at different stages, and a genotype with attributes like higher 1,000 grain weight, spike weight, and yield per plot is considered a good candidate for resistant variety (El-Hendawy et al., 2005; Uzair et al., 2022).

Surprisingly, 1,000 grain weight and yield per plot were not positively correlated. A possible explanation for this phenomenon might be the presence of saline patches. Salinity levels changes even within a field in this region and therefore make is difficult to correlate per plot yield with other parameters (Ijaz et al., 2020). However, overall, plants bioprimed with bacterial treatment still performed better compared to the control with 20% reduced fertilizer and fungal treatment.

On the other hand, the presence of salinity already depletes the nutrient availability (Syed et al., 2020) and our use of the reduced dose

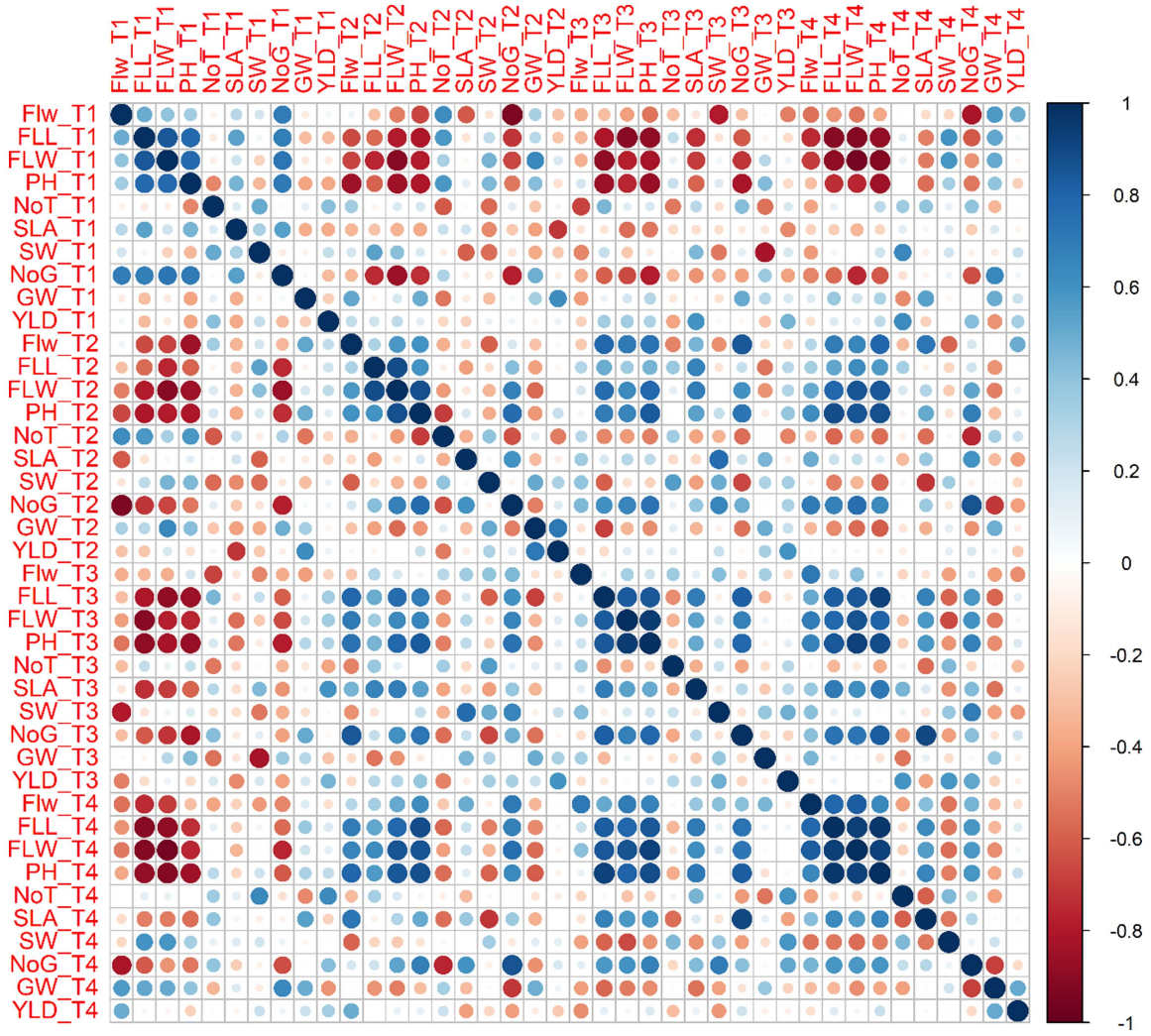

FIGURE 11
Combined correlation of collective average values for agronomical parameters at three sites for all treatment *viz.* T1=Control with 100% FRD, T2=Control with 80% FRD, T3=Bacterial treatment, and T4=Fungal treatment for Galaxy 2013 where Flw=Flag leaf width, FLL=flag leaf length, FLW=flag leaf weight, PH=plant height, NoT=Number of tillers, SLA=Spike length with awns, SW=Spike weight, NoG=Number of grains spike^{-1}, GW=1,000 grain weight, and YLD=Yield per plot. The blue and red circles show the positive and negative correlation, respectively. The circle size and the intensity of the color are proportional to the correlation coefficients.

of chemical fertilizer might also be the reasons for low grain yield and an overall reduction in yield, as nutrient availability is a critical factor for plant overall growth and yield parameters in particular, and a negative correlation to plant height and wheat grain yield is also reported in literature (Islam et al., 2013).

In addition, although the EC levels in T2 (~6dSm^{-1}) of the pot experiment were similar to the field conditions of three different sites, differences in multiple agronomical parameters were observed (Supplementary data). This can be explained by the discussion in previous literature where researchers found differences in pot and field trials outcomes and suggested the possible reasons for these obvious differences might be competition for space, light, nutrients, plant interactions with plants, soil, and environmental conditions and these factors have both advantages and disadvantages for overall plant growth (Kawaletz et al., 2014; Poorter et al., 2016; He et al., 2017).

These preliminary investigations indicated that the use of these indigenous microbial florae not only has the potential to augment wheat production under moderately saline conditions but also pave the way to future investigation for a better understanding of plant–microbe interactions under saline conditions. Development and assessment of a mixed consortium of PGP fungal and bacterial strains after further in-depth studies in the future can also be carried out to evaluate their synergistic effect as initial reports of mixed inoculum are promising (Diedhiou et al., 2016; Comite et al., 2021).

5. Conclusion

Initial screening and evaluation of halotolerant microbial flora from salt-affected agricultural lands in the Indus basin region indicated the potential of these microbes to enhance wheat production by enhancing multiple growth/agronomical parameters. The outcomes of these preliminary studies would leverage the path for the development of saline-specific bio-fertilizer using indigenous

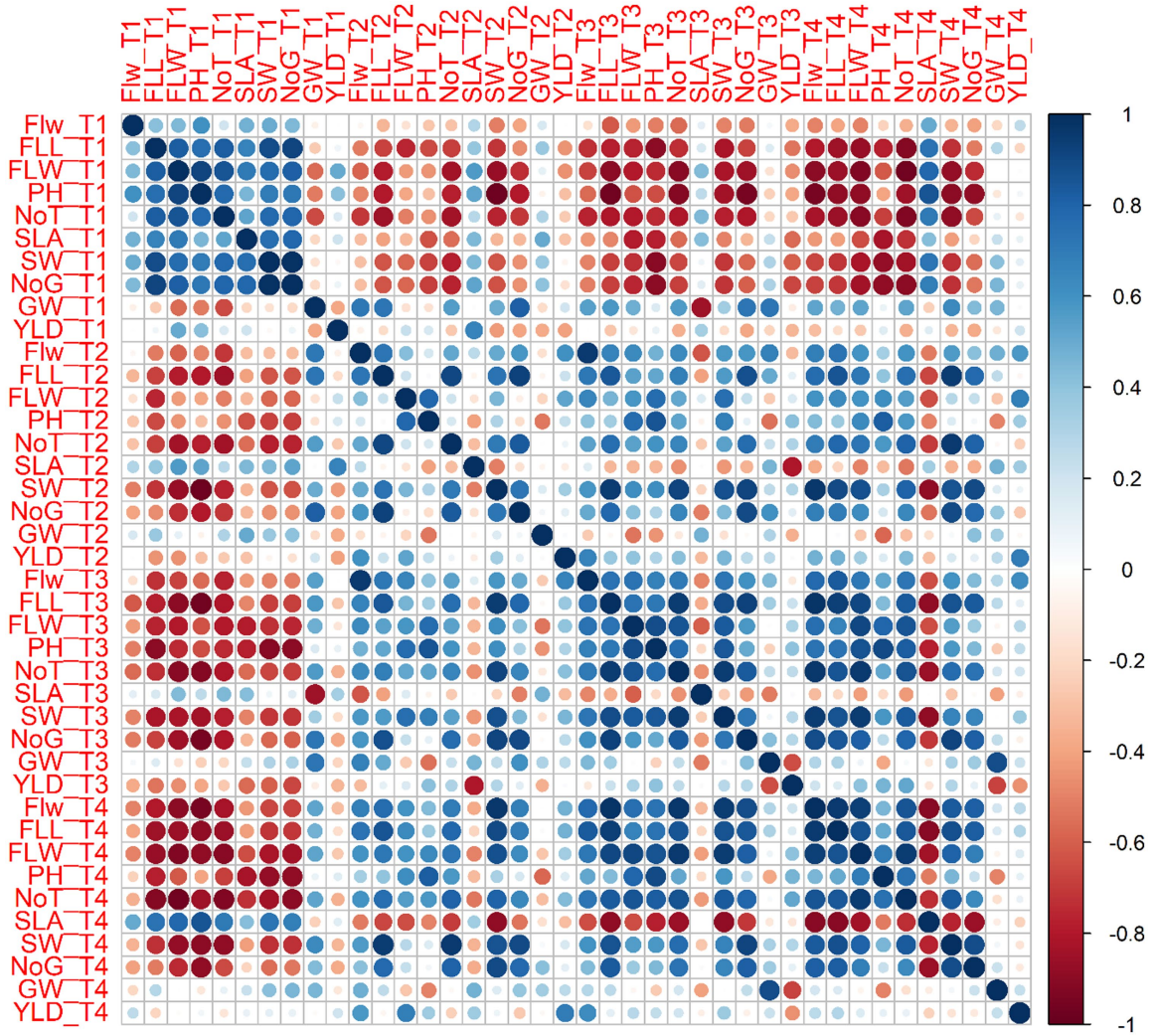

FIGURE 12
Combined correlation of collective average values for agronomical parameters at three sites for all treatment *viz.* T1=Control with 100% FRD, T2=Control with 80% FRD, T3=Bacterial treatment, T4=Fungal treatment for Faisalabad 2008. Where, Flw=Flag leaf width, FLL=flag leaf length, FLW=flag leaf weight, PH=plant height, NoT=Number of tillers, SLA=Spike length with awns, SW=Spike weight, NoG=Number of grains spike^{-1}, GW=1,000 grain weight, and YLD=Yield per plot. The blue and red circles showed the positive and negative correlation, respectively. The circles size and the intensity of the color are proportional to the correlation coefficients.

microbial flora to not only combat abiotic stresses like salinity but also to reduce the usage of chemical fertilizers.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, OQ318271; https://www.ncbi.nlm.nih.gov/genbank/, OQ318275; https://www.ncbi.nlm.nih.gov/genbank/, OQ318278.

Author contributions

MM: conceptualization, sampling, experimentation, field trials, data curation, formal analysis, investigation, writing-original draft, and review and editing. AN: sampling, experimentation, field trials, and review and editing. MA: data analysis, writing-original draft, and review and editing. MW and MK: data analysis and review and editing. EI: Rhizo-scanning. MI: sampling, field trials, and review and editing. AI: conceptualization, supervision, and review and editing. FM: conceptualization, funding acquisition, project administration, supervision, validation, and review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1207784/full#supplementary-material

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EDITED BY
Jeanette M. Norton,
Utah State University, United States

REVIEWED BY
Samadhan Bagul,
ICAR-Directorate of Medicinal and Aromatic Plants Research, India
Savitha Santosh,
Central Research Institute for Dryland Agriculture (ICAR), India
Parul Sundha,
Central Soil Salinity Research Institute (ICAR), India

*CORRESPONDENCE
Latha P. Chandran
✉ lathapc@gmail.com

†These authors have contributed equally to this work and share first authorship

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Silicate solubilizing and plant growth promoting bacteria interact with biogenic silica to impart heat stress tolerance in rice by modulating physiology and gene expression

Chandrakala Chaganti†, Amol Sarjerao Phule†, Latha P. Chandran*†, Bandeppa Sonth†, Venkat Prasad Babu Kavuru, Rajani Govindannagari and Raman Meenakshi Sundaram

ICAR-Indian Institute of Rice Research, Hyderabad, Telangana, India

Heat stress caused due to increasing warming climate has become a severe threat to global food production including rice. Silicon plays a major role in improving growth and productivity of rice by aiding in alleviating heat stress in rice. Soil silicon is only sparingly available to the crops can be made available by silicate solubilizing and plant-growth-promoting bacteria that possess the capacity to solubilize insoluble silicates can increase the availability of soluble silicates in the soil. In addition, plant growth promoting bacteria are known to enhance the tolerance to abiotic stresses of plants, by affecting the biochemical and physiological characteristics of plants. The present study is intended to understand the role of beneficial bacteria viz. *Rhizobium* sp. IIRR N1 a silicate solublizer and *Gluconacetobacter diazotrophicus*, a plant growth promoting bacteria and their interaction with insoluble silicate sources on morpho-physiological and molecular attributes of rice (*Oryza sativa* L.) seedlings after exposure to heat stress in a controlled hydroponic system. Joint inoculation of silicates and both the bacteria increased silicon content in rice tissue, root and shoot biomass, significantly increased the antioxidant enzyme activities (viz. superoxidase dismutase, catalase and ascorbate peroxidase) compared to other treatments with sole application of either silicon or bacteria. The physiological traits (viz. chlorophyll content, relative water content) were also found to be significantly enhanced in presence of silicates and both the bacteria after exposure to heat stress conditions. Expression profiling of shoot and root tissues of rice seedlings revealed that seedlings grown in the presence of silicates and both the bacteria exhibited higher expression of heat shock proteins (HSPs viz., *OsHsp90*, OsHsp100 and *60 kDa chaperonin*), hormone-related genes (*OsIAA6*) and silicon transporters (*OsLsi1* and *OsLsi2*) as compared to seedlings treated with either silicates or with the bacteria alone. The results thus reveal the interactive effect of combined application of silicates along with bacteria *Rhizobium* sp. IIRR N1, *G. diazotrophicus* inoculation not only led to augmented silicon uptake by rice seedlings but also influenced the plant biomass and elicited higher expression of HSPs, hormone-related and silicon transporter genes leading to improved tolerance of seedling to heat stress.

KEYWORDS

heat stress, silicon, plant growth promoting bacteria, *Gluconacetobacter diazotrophicus*, *Rhizobium* sp. IIRR N1, gene expression, qRT-PCR, rice

Introduction

Global warming is predicted to become a serious threat to the food security of many countries (Xu et al., 2021) as the resultant heat stress is a major constraint to crop productivity worldwide (Janni et al., 2020). Heat stress is an increase in temperature above a threshold level for a certain period that causes an irreversible effect on the growth and development of plants at all stages, i.e., from germination to harvesting (Govindaraj et al., 2018; Ding et al., 2021; Ali et al., 2022; Ejaz et al., 2022). Every 1°C increase in global mean temperature has been predicted to decrease global yields of rice by 3.2%, maize by 7.4%, wheat by 6.0%, and soybean by 3.1% (Zhao et al., 2017).

Rice (*Oryza sativa L.*) is a major food crop for more than half of the world's population. The adverse effects of heat stress are estimated to reduce rice production by 41% by the end of the 21st century (Shi et al., 2017). Rice crop maintains normal growth at temperatures ranging from 27 to 32°C without any significant reduction in yield, but temperatures above 32°C negatively affect rice plant development at all growth stages (Aghamolki et al., 2014). The early growth stage is one of the most vital stages of growth, playing an important role in stand establishment that is crucial for realizing rice yield potential (Finch-Savage and Bassel, 2016). This stage of rice is highly susceptible as seedlings experience more heat stress due to their smaller sizes and high radiative energy from the soil and water interface (Jagadish et al., 2021). The optimum growth temperature of rice at the seedling stage is 25–28°C. Heat stress (42–45°C) at the seedling stage, results in increased water loss, withered and yellow leaves, impaired seedling and root growth, and even death of seedlings (Xu et al., 2021).

Heat stress deleteriously alters the morphological (growth and plant biomass), anatomical, and physio-biochemical characteristics (stability of cell membranes and proteins, photosynthetic apparatus, and carbohydrate metabolism) of plants (Ul Hassan et al., 2021; Xu et al., 2021) which can, in case of rice, result in a significant qualitative and quantitative reduction in grain and straw yield. At a molecular level, following plants' sensing of heat stress, several signal cascades are induced, activating transcriptional responses relating to reactive oxygen species (ROS) regulation, plant hormones (Devireddy et al., 2021) and other molecular components like heat shock proteins. ROS, as key signaling molecules, play a pivotal role in enabling plant cells to respond rapidly to heat stress (Mittler et al., 2022). Heat stress disrupts redox homeostasis, which exacerbates ROS toxicity by inactivating antioxidant enzymes, including ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), and glutathione S-transferase (GST) and decreasing the synthesis of non-enzymatic antioxidants such as ascorbic acid, glutathione and phenolic compounds (Sharma et al., 2019; Hasanuzzaman et al., 2020). Phytohormones such as ethylene, auxins, cytokinins, abscisic acid, salicylic acid, jasmonates, brassinosteroids and strigalactones are another group of signaling molecules which are endogenous and help in mediating plant response to stresses, including heat stress (Li et al., 2021). Another molecular response is the expression of heat stress and other related proteins that act as molecular chaperones protecting plants from proteotoxic stress caused by protein misfolding and denaturation (Xu et al., 2021). Tolerance to heat stress is conferred by maintaining plant functions and efficient scavenging of ROS (Lei et al., 2018) and stabilizing the function and structure of protein and enzymes by heat shock proteins (HSPs) in plants (Maestri et al., 2002). An integrated approach using multiple techniques like crop (genetic improvement) and soil management (agronomic) strategies is necessary (Zafar et al., 2018) for imparting thermotolerance in crops. From an agronomic perspective, silicon (Kumar et al., 2022) and beneficial microbe based technologies (Shekhawat et al., 2022) are currently receiving considerable interest as they can be harnessed to provide eco-friendly and cost effective stress mitigating solutions for sustainable heat tolerant crop production systems.

Silicon (Si) is a quasi-essential and beneficial nutrient element involved in the growth and development of crops (Epstein, 1999; Ma et al., 2001; Liang et al., 2005; Hamayun et al., 2010; Kim et al., 2011), particularly under various stresses. Under abiotic stress conditions (Farooq and Dietz, 2015; Muneer et al., 2017; Rahman et al., 2017; Hussain et al., 2019; Zargar et al., 2019; Younis et al., 2020), Si acts as a stress alleviator by enhancing membrane integrity and antioxidant defense system through alterations in cellular and biochemical mechanisms. The role of Si in inducing heat stress tolerance in plants (Tripathi et al., 2017; Frew et al., 2018; Saha et al., 2021; Kumar et al., 2022) including agriculturally important crops like rice is well known (Jinger et al., 2020). In rice, tissue Si plays a major role in the growth and productivity of rice through increases in the strength of stems, erectness of leaves, and photosynthetic rate (Berahim et al., 2021) alleviating injurious effect of stresses in rice (Etesami and Jeong, 2018; Mostofa et al., 2021). In rice, Si is absorbed by rice roots in the form of silicic acid through Si influx (Lsi1) and efflux transporters (Lsi2) (Ma et al., 2011; Yamaji et al., 2015). Further, Si in soil solution taken up by rice roots is translocated into shoots through the transpiration stream of the xylem and unloaded by Lsi6 transporter (Yamaji et al., 2015) where it ultimately accumulates under the cuticle and intracellular spaces (Heckman, 2013) and precipitates in plant cells as phytoliths (Cooke and Leishman, 2011; Rao and Susmitha, 2017).

Since Si plays a prophylactic role under stress conditions, root and shoot tissues, especially in monocots like rice, must possess a minimum concentration of Si to combat the negative effect of stresses (Debona et al., 2017). Additionally, soluble Si only when taken up through the root system and transported from roots via the xylem alone, can activate the metabolic processes that induce beneficial changes in plants (Zellener et al., 2021). Hence the benefits of Si are realizable only when Si, present abundantly in soil and parent mineral rocks as insoluble silicates (Haynes, 2017), is transformed into plant-available soluble Si. Geochemical weathering of silicates, during which dissolved Si in the form of plant-available silicic acid (H_4SiO_4) is released, is inadequate to replenish soluble Si to meet the crop needs as it is an inconsistent process occurring over extended periods (Rao et al., 2019). Soluble Si release from parent rocks also declines with soil age in countries with mature soils (Madhuri, 2018) like India due to desilication which occurs continuously over time. Nutrient stripping when growing silicon-accumulating crops like rice, where the average content of Si is usually more than 40 mg/g of dry biomass (Hughes et al., 2020), also aggravates Si depletion in soil. Si-containing fertilizers can mitigate this loss by increasing the bioavailability of soil Si and fulfilling the Si demand for crops. However, though different commercially produced Si fertilizers are available (Tayade et al., 2022), their use is often constrained by the costs, efficacy and ease of handling and application, besides encountering conflicts in identifying the appropriate source of fertilizer (Haneklaus et al., 2018). For improving soil bioavailable Si concentrations, the newer

and smarter silicon fertilization strategies that are currently under reckoning due to their environment-friendly, agronomically efficient, and renewable nature are (i) the use of biogenic siliceous material as Si source and (ii) soil inoculation with silicate solubilizing microorganisms (Meena et al., 2014; Constantinescu-Aruxandei et al., 2020; Hughes et al., 2020; Raturi et al., 2021). An optimal combination of biogenic silica along with silicate solubilizing bacteria can lead to additive effects by the release of soluble Si from amorphous insoluble Si present in rice straw and diatomaceous earth. However, there are very few studies related to phytogenic/biogenic Si solubilization by microorganisms (Constantinescu-Aruxandei et al., 2020).

Rhizospheric and endophytic plant-growth promoting microorganisms (PGPM) can colonize plant roots (rhizosphere) and increase plant growth (shoot and root growth) and yield through various mechanisms like biosynthesis of phytohormones, exopolysaccharides, and volatile organic compounds (Adhikari et al., 2020; Kim et al., 2020) in addition to stimulating enzymatic activity, production of antioxidants and osmoprotectants (Wang et al., 2018). PGPMs enhance plant tolerance to abiotic stresses, by affecting the biochemical and physiological characteristics of plants (Etesami and Maheshwari, 2018; Asghari et al., 2020). There is increasing evidence that heat stress responses and recovery of plants also depends on their interaction with different microorganisms (Chandrakala et al., 2019a; Dastogeer et al., 2022). While plant growth promotion and stress-alleviating traits of rhizospheric microorganisms have been widely recognized, investigations are still ongoing to understand the endophytic micro-organisms and their role as stress controllers in plants (Mahapatra, 2017; Lata et al., 2018). Navarro-Torre et al. (2023), have put forward the theory that colonization by endophytic bacteria with the ability for vertical transmission across plant generations, can make plants constitutively thermotolerant, thus becoming a potent tool in the management of heat stress in plants. Beneficial microbe-mediated plant thermotolerance is an ecologically sustainable strategy to mitigate heat stress in plants (Shekhawat et al., 2022) but only a few studies have documented interactions between plants and endophytic microbes in the control of heat stress (Shaffique et al., 2022).

Co-application of insoluble silicates along with multiple plant growth promoting bacteria is expected to benefit heat-stressed plants (Al-Garni et al., 2019; Chandrakala et al., 2019b; Vishwakarma et al., 2020), however, research on the synergistic effects due to combined application of siliceous material along with microbes for heat stress alleviation has been scarce. With this background, the present study aimed to understand the interactive effect of the combined application of silicate solubilizing bacteria (*Rhizobium* sp. IIRR N1), plant growth promoting endophytic bacteria (*G. diazotrophicus*), and insoluble silicate sources (diatomaceous earth and rice straw), on morpho-physiological and molecular effects on rice (*Oryza sativa L.*) seedlings exposed to heat stress under a controlled hydroponic system.

Materials and methods

Plant genotype, siliceous material, and bacteria

Surface sterilized and germinated rice seedlings (cv. Swarna) a heat susceptible genotype (Chandel et al., 2013; Umesh et al., 2016) were grown aseptically in a hydroponics assembly. The assembly consisted of phyta jars containing 150 mL Yoshida medium (Yoshida et al., 1972) and perforated glass stand inserts on which germinated seedlings (6 seedlings) were supported such that the roots were immersed in the liquid medium (Chandrakala et al., 2019a). The Yoshida medium was amended with biogenic insoluble silicates sources [diatomaceous earth – 0.01 gm (Himedia RM 3380), which is equivalent to 75 kg/ha and rice straw – 0.33 gm equivalent to 150 kg/ha] and inoculated with bacterial cultures (1×10^8 CFU/mL) of *Rhizobium* sp. IIRR N1 (Accession No. KY348774) and *G. diazotrophicus* PAL 5 (MTCC 1224 acquired from CSIR – Institute of Microbial Technology, Chandigarh). Four such phyta jars with seedlings were placed in a transparent autoclavable bag with a 0.02 µm gas exchange filter patch (Sunbags, Sigma-Aldrich, Cat. no. B7026) that enabled sterile air exchange into the assembly and sealed. The rice seedlings were grown for 30 days under 16 h light and 8 h dark cycles at ambient temperature (28 ± 2°C). After 30 days of co-culturing seedlings in the presence of bacteria and insoluble silicates, the seedlings were subjected to two temperature regimes, i.e., ambient temperature (non-stressed condition) and elevated temperature (heat stress conditions). The seedlings were exposed to elevated temperature stress (45 ± 2°C) for 24 h in a growth chamber after which the seedlings were maintained under ambient conditions for 24 h (Sarsu et al., 2018). The non-stressed plants continued to grow under ambient temperature (28 ± 2°C) conditions during this 48 h period. Parallelly, controls were maintained by growing seedlings in the absence of silicate sources and bacteria. There were hence, four treatments under ambient conditions and four under heat stress. The treatments under ambient temperature/heat stress conditions were (i) seedlings grown in the absence of silicates and bacteria (AC/HS), (ii) seedlings co-cultured with both bacteria (AC+ RG /HS+ RG), (iii) seedlings co-cultured with insoluble diatomaceous earth and straw (AC+ Si/HS + Si), and (iv) seedlings co-cultured with insoluble diatomaceous earth and straw and bacteria (AC+ Si + RG/HS+ Si + RG). Each sunbag with 4 phytajars constituted a replication and three replications were maintained for each treatment. Twenty-four hours after heat stress treatment, the seedlings along with the respective controls were collected for analyzing silica uptake, morphological, physiological, biochemical and molecular response of rice seedlings to bacterial inoculation in the presence of insoluble silicates.

Morphological characterization of seedlings

Morphological parameters *viz*. root and shoot biomass (g) of single rice seedlings were recorded manually in three replications (each replication consisted of three seedlings) under ambient and heat stress conditions after oven drying the seedlings at 60°C for 72 h.

Silicon content in rice seedlings

Silicon content in the root and shoot of rice seedlings was determined by the colorimetric silico-molybdate blue method after autoclave-induced digestion of the samples as described by Elliott and Synder (1991).

Physiological and biochemical characterization of seedlings

Chlorophyll content

The method of Zhang et al. (2009) was used for quantification of chlorophyll content in seedling leaves. Leaf sample (100 mg) was added to 25 mL of 80% acetone and after incubation for 48 h in dark at ambient temperature, the absorption of the supernatant was measured at 663 and 645 nm using a spectrophotometer (UV 1800, Shimadzu, Japan) and the chlorophyll content was calculated by using equations Arnon's (1949).

Electrolyte leakage (EC)

Leaf samples from treated and untreated seedlings were cut in to uniform pieces and placed in a beaker containing 10 mL of distilled water and incubated for 4 h in a shaking incubator (Gyromax 787R, Amerex Instruments; Lafeyette, CA, United States) at room temperature. After 4 h, ion leakage (E1) was measured using conductivity meter (ECTestr 11+, Eutech Instruments, Oakton, United States). The samples were then autoclaved for 15 min and after cooling and total ion leakage (E2) was measured. EC was calculated (Jambunathan, 2010) as: EC = E1/E2 * 100.

Relative water content (RWC)

Leaves of all samples were cut and weighed immediately for fresh weight (FW) and immersed in water for 4 h. After immersion, excess water was removed from the leaves by blotting and then weighed to get turgid weight (TW). Leaves were oven dried at 80°C for 72 h, dry weight (DW) of the samples were recorded and the RWC was determined (Nayyar et al., 2014) using the equation: RWC (%) = (FW-DW/TW-DW) * 100.

Antioxidant enzyme activities

Leaf sample (200 mg) was weighed and ground into fine powder with liquid nitrogen by using pre cooled mortar and pestle. The exact weight of each sample (powdered) was determined before it was homogenized thoroughly in 1.2 mL of potassium phosphate buffer (0.2 M), pH – 7.8 with EDTA (0.1 mM). After homogenization, samples were centrifuged at 15,000 rpm for 20 min at 4°C and the supernatant was collected in a 2 mL eppendorf tube. The pellet was extracted again in 0.8 mL of the same buffer, and the combined supernatants were collected and stored in ice. This crude extract was used to determine antioxidant enzyme activities (Elavarthi and Martin, 2010).

Superoxide dismutase

The modified NBT method was used to determine superoxide dismutase activity. The reaction mixture (2 mL) was prepared by using 50 mM phosphate buffer (pH 7.8) with 2 mM EDTA, 9.9 mM L-methionine, 55 μM NBT and 0.025% Triton – X 100; to this 2 times diluted 40 μL of crude extract and 20 μL of riboflavin (1 mM) were added. Two sets of samples were prepared, one set was exposed to 15 W fluorescent tube, while another set was kept in the dark, and both the sets were incubated for 10 min. Samples kept in the dark were used as blanks. The absorbance of the reaction mixture was read at 560 nm (UV 1800, Shimadzu, Japan) immediately after incubation against a standard curve obtained from pure SOD (Beyer and Fridovich, 1987) and used for estimating the SOD activity.

Catalase

Hydrogen peroxide (10 mM, 1 mL) was added to 2 mL of 200 times diluted crude extract in 50 mM potassium phosphate buffer (pH-7), and the absorbance was read immediately at 240 nm in a UV/Vis spectrophotometer (UV 1800, Shimadzu, Japan). Decomposition of hydrogen peroxide (H_2O_2) was determined by using the extinction coefficient of H_2O_2 (43.6 M^{-1} cm^{-1} at 240 nm) and the catalase activity is expressed as mM H_2O_2 per minute per mg protein (Aebi and Lester, 1984).

Ascorbate peroxidase

Crude extract (10 μL) was added to 1 mL of assay mixture (50 mM potassium phosphate buffer (pH-7), 0.5 mM ascorbate, 0.5 mM H_2O_2) and the decrease in absorbance due to oxidation of ascorbate was read at 290 nm (UV 1800, Shimadzu, Japan). The extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate the enzyme activity which is expressed as milli mole of ascorbate per minute per mg protein (Nakano and Asada, 1981).

Glutathione reductase (GR)

Assay mixture (1 mL) was prepared by adding 10 μL of crude extract, 0.75 mM of 5–5′-Dithiobis (2-nitrobenzoic acid), 0.1 mM NADPH and 1 mM oxidized glutathione (GSSG). GSSG was added last to initiate the reaction and the increase in absorbance was read at 412 nm for 3 min. The extinction coefficient of 2-nitro-5-thiobenzoate (14.15 M^{-1} cm^{-1}) was used to calculate GR activity and expressed as mM TNB/min/gram fresh weight (Smith et al., 1988).

Protein content in leaf extract

The leaf extract which was used for determining antioxidant activities was used for estimating total protein content by using Lowry's method (Lowry et al., 1951).

RNA isolation, cDNA synthesis, and gene expression analysis

Total RNA was isolated from the root and shoot tissue of seedlings grown under ambient and heat-stressed conditions using NucleoSpin RNA plant kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The quality and quantity of the pooled RNA (3 replications together) of each sample were checked using agarose gel electrophoresis (1%, w/v) and NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). Expression profiling of genes related to heat shock proteins (*viz. OsHsp 26.7*, *OsHsp 70*, *OsHsp 90 OsHsp 100*, and *60 kDa chaperone*), an auxin-responsive protein (*OsIAA6*), an ethylene response factor (*OsERF1*) and three silicon transporters (*OsLsi1*, *OsLsi2* and *OsLsi6*) were studied by using qRT-PCR analysis. The qRT-PCR primers for the selected genes were designed online by using QuantPrime high throughput qRT-PCR tool[1] by considering default parameters (Supplementary Table S1). The cDNA synthesis and qRT-PCR were performed as previously described by Phule et al. (2018, 2019). The expression levels of genes in the shoot and root

1 http://quantprime.mpimp-golm.mpg.de/

tissues under ambient and heat stressed conditions were calculated using the 2-ΔΔCT method (Livak and Schmittgen, 2001) and heatmaps were plotted using MeV (v4.9.0, Multi Experiment Viewer) software (Saeed et al., 2003).

Data analysis

Stress tolerance index (Fernandez, 1992) of the seedling biomass trait, was computed using iPASTIC online tool kit (Pour-Aboughadareh et al., 2019) to identify the best performing treatments. All recorded data were analyzed by using the statistical tool (Statistix 8.1 v2.0.1) by calculating Analysis of Variance (ANOVA) and significant difference among the treatment means was determined using least significant differences (LSD) test at 5% probability level ($p \leq 0.05$). Pearson's correlations between various measured traits were derived using Microsoft Excel and the corellogram was plotted using the corrplot function of the R statistical package v.3.6.0.

Results

Seedling biomass and tissue silica content

The bacterial (*Rhizobium* sp. IIRR N1 plus *Gluconacetobacter diazotrophicus*) and silicate treatments were generally effective in enhancing seedling biomass (shoot and root weight) and tissue silicon (Si) content in relation to untreated control at both ambient (28 ± 2°C) and high temperature (45 ± 2°C), even as heat stress modulation was observed in all treatments (Table 1).

Shoot biomass showed significant changes in response to bacterial inoculation, growth in silicate sources and high temperature. At ambient temperature relative to control (AC), bacterial inoculation improved the shoot biomass by 47 and 7% in the presence (AC + Si + RG) and absence of insoluble silicates (AC + RG). Seedlings grown solely with insoluble silicates (AC + Si) also increased root biomass by 33% more than control. Similarly, post heat stress, shoot biomass was higher by 32, 62, and 44%, respectively over control (HC) in HS + RG, HS + Si + RG, and HS + Si treatments (Table 1). However, exposure to high temperature had resulted in a reduction in shoot biomass ranging from 25 to 40% with the lowest reduction observed in seedlings grown with bacteria and silicates.

Likewise, root biomass significantly increased by 34, 31, and 51% over control (AC) in AC + RG, AC + Si, and AC + Si + RG treatments, respectively, under ambient conditions. Root biomass increment (over control) in seedlings after heat stress was 32, 44 and 62%, respectively, in the presence of bacteria (HS + RG), insoluble silicates (HS + Si) and in the treatment with both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* (HS + Si + RG). Root biomass was lower by 5–23% after the seedlings were subjected to heat stress (Table 1).

Mirroring the trends in seedling biomass, the level of increase in silica content of shoot and root over control with AC + RG, AC + Si and AC + Si + RG was 4, 10 and 19% and 6, 14, and 24%, respectively, when seedlings were grown in ambient temperature. After heat stress, seedlings subjected to HS + RG, HS + Si and HS + Si + RG treatments also had higher silica content than control (HS), with the corresponding increase in shoot and root being 3, 26, 36 and 13%, 28, 44%. Imposition of heat stress decreased silica content in the shoot and root by 10 and 19% (Table 1).

TABLE 1 Seedling biomass and tissue silica content under ambient and heat stress conditions.

Treatments	Seedling biomass (g)*		Tissue silica content (μg/mg)*	
	Shoot	Root	Shoot	Root
Ambient (28 ± 2°C)				
AC	0.15	0.13	56.0	36.8
AC + RG	0.16	0.17	58.2	39.1
AC + Si	0.20	0.17	61.3	42.0
AC + Si + RG	0.22	0.19	66.4	45.7
Mean	0.18	0.17	60.5	40.9
CD ($P < 0.05$)	0.02	0.04	2.1	2.5
CV (%)	13.2	12.1	4.2	7.4
Heat stress (45 ± 2°C)				
HS	0.09	0.10	46.9	27.1
HS + RG	0.12	0.14	48.2	30.8
HS + Si	0.13	0.11	59.1	34.8
HS + Si + RG	0.15	0.18	63.7	39.0
Mean	0.12	0.13	54.5	32.9
CD ($P < 0.05$)	0.01	0.03	2.4	2.9
CV (%)	17.3	18.7	5.3	10.7

*Dry weight.
AC, Seedlings grown in the absence of silicates and bacteria under ambient temperature.
AC + RG, Seedlings co-cultured with *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* bacteria under ambient temperature.
AC + Si, Seedlings co-cultured with solely with insoluble diatomaceous earth and straw under ambient temperature.
AC + Si + RG, Seedlings co-cultured with both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* under ambient temperature.
HS, Seedlings grown in the absence of silicates and bacteria under heat stress condition.
HS + RG, Seedlings co-cultured with *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* bacteria under heat stress.
HS + Si, Seedlings co-cultured with solely with insoluble diatomaceous earth and straw under heat stress.
HS + Si + RG, Seedlings co-cultured with both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* under heat stress.

Physiological characteristics of rice seedlings

Differences in chlorophyll content, electrolyte leakage and relative water content observed between control and treated seedlings and between the two temperature regimes are shown in Table 2. Leaf chlorophyll content increased substantially due to bacterial and silicate treatments, with AC + RG, AC + Si and AC + Si + RG treatments having 30, 13, and 33% higher chlorophyll concentrations respectively, than that of control (AC). Regarding the chlorophyll content of heat-stressed plants, the treatment with silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* (HS + Si + RG) recorded a 79% increase in chlorophyll content relative to control (HS) superior to the 37 and 36% increase observed with HS + RG and HS + Si treatments, respectively (Table 2). Following heat stress, 2–29% reduction in chlorophyll content was observed across all treatments compared to ambient temperature.

TABLE 2 Seedling physiological characteristics under ambient and heat stress conditions.

Treatments	Chlorophyll content (mg/g leaf fresh wt)	Relative water content (%)	Electrolyte leakage (%)
Ambient (28 ± 2°C)			
AC	1.71	86.4	47.5
AC + RG	1.97	95.8	33.1
AC + Si	1.80	90.8	41.3
AC + Si + RG	2.22	98.1	31.9
Mean	1.92	92.8	38.4
CD ($P<0.05$)	0.04	12.7	3.3
CV (%)	9.1	16.7	10.4
Heat stress (45 ± 2°C)			
HS	1.21	71.2	61.1
HS + RG	1.67	89.9	42.6
HS + Si	1.65	82.3	52.3
HS + Si + RG	2.17	92.9	38.7
Mean	1.68	84.1	48.6
CD ($P<0.05$)	0.01	7.5	4.4
CV (%)	7.3	11.0	11.4

AC, Seedlings grown in the absence of silicates and bacteria under ambient temperature.
AC + RG, Seedlings co-cultured with *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* bacteria under ambient temperature.
AC + Si, Seedlings co-cultured with solely with insoluble diatomaceous earth and straw under ambient temperature.
AC + Si + RG, Seedlings co-cultured with both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* under ambient temperature.
HS, Seedlings grown in the absence of silicates and bacteria under heat stress condition.
HS + RG, Seedlings co-cultured with *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* bacteria under heat stress.
HS + Si, Seedlings co-cultured with solely with insoluble diatomaceous earth and straw under heat stress.
HS + Si + RG, Seedlings co-cultured with both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* under heat stress.

Relative water content (RWC) trends showed a similar pattern as that of chlorophyll; higher RWC in leaves of seedlings where treatments have been administered, compared to control in both the temperature regimes (Table 2). High temperature has resulted in water loss from exposed seedlings, with bacterial and silicate-treated seedlings recording a minimum per cent reduction in RWC (5–9%) compared to seedlings under ambient temperature.

Seedlings inoculated with bacteria, treated with insoluble silicates, and the combination of both bacteria and silicates caused a reduction in total electrolyte leakage from seedlings by 30, 13 and 33% and 30, 14 and 37% relative to the respective ambient (AC) and heat-stressed (HS) controls (Table 2). Membrane injury in seedlings due to heat stress was witnessed, and the higher electrolyte leakage values in heat stressed seedlings corroborated the same.

Antioxidant enzyme activities in rice seedlings

The different treatments comprising silicates and bacteria markedly enhanced the rice seedling antioxidant enzyme activity compared to the control under ambient and heat stress (Table 3). The activities of superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase were significantly and maximally increased by the combined application of silicates and bacteria (Si + RG) by 25, 38, 45, and 30% under ambient conditions and by 23, 42, 28, and 36% in heat-stressed plants, relative to their corresponding controls. A lower magnitude of increase in the enzymatic antioxidant activities was also observed due to sole treatments with either silicates or bacteria. Heat stress considerably decreased the SOD, CAT, APX and GR activities in seedlings by 3.6–7.1%, 17.1–24.3%, 7.7–19.1%, and 9.4–14.6%, respectively in comparison to non-stressed seedlings subjected to similar conditions (Table 3).

Correlation analysis

Correlation analysis revealed a strong association between root and shoot seedling biomass, an indicator of heat stress tolerance, physiological traits, and antioxidant enzymatic activities under ambient and heat stress conditions (Figure 1).

Root biomass showed a significant and positive correlation with Si content in the root ($r=0.85$), shoot biomass ($r=0.82$), Si content in the shoot ($r=0.75$), chlorophyll content ($r=0.92$), relative water content ($r=0.92$), superoxide dismutase ($r=0.89$), catalase ($r=0.94$), ascorbate peroxidase ($r=0.86$), glutathione reductase ($r=0.95$) concentration and a similar positive correlation was also noted with shoot biomass and Si content in both root and shoot ($r=0.97$ and 0.84), chlorophyll content ($r=0.77$), relative water content ($r=0.79$), superoxide dismutase ($r=0.74$), catalase ($r=0.86$), ascorbate peroxidase ($r=0.79$) and glutathione reductase ($r=0.81$) concentration. In contrast, a significant negative correlation was observed between electrolyte leakage ($r=-0.94$) and root biomass (Figure 1; Supplementary Table S2).

Further, significant positive correlations were also observed among Si content in the root and shoot to chlorophyll content ($r=0.86$), relative water content ($r=0.82$ and 0.70), superoxide dismutase ($r=0.78$ and 0.72), catalase ($r=0.87$ and 0.70), ascorbate peroxidase ($r=0.79$ and 0.67) and glutathione reductase ($r=0.83$ and 0.71) activity while electrolyte leakage (−0.81 and −0.67) was negatively correlated (Figure 1; Supplementary Table S2).

Heat stress tolerance index

Heat stress tolerance index (STI) of rice seedlings under normal (Yp) and heat stress (Ys) conditions with different treatments (RG, Si and Si + RG) were calculated using seedling biomass as a screening indicator (Figure 2; Supplementary Table S3) wherein based on Fernandez's theory, treatments with STI ≥1 were considered stress tolerant. A three-dimensional plot with STI values was computed using iPASTIC, an online toolkit to categorize the treatments into four groups (Figure 2). The treatment with the combined application of both silicates and bacteria was classified as group A, indicating the ability of the treatment to induce uniform performance of rice seedlings under ambient and heat stress conditions. The treatments where the silicates and bacteria were applied individually were categorized into groups B and C, respectively, indicating that treatment with diatomaceous earth and straw could support better seedling growth only under ambient conditions, while inoculation with the two bacteria led to better performance of the seedlings under heat stress conditions (Figure 2).

TABLE 3 Seedling antioxidant activities under ambient and heat stress conditions.

Treatments	Superoxide dismutase (Units SOD/mg protein)	Catalase (mM H_2O_2/min/ mg protein)	Ascorbate peroxidase (mM ascorbate /min/ mg protein)	Glutathione reductase (mM TNB/min/mg protein)
Ambient (28 ± 2°C)				
AC	20.3	8.60	10.46	15.1
AC + RG	23.6	10.56	13.32	18.1
AC + Si	22.3	9.71	12.06	16.8
AC + Si + RG	25.3	11.87	15.13	19.7
Mean	22.9	10.18	12.74	17.4
CD ($P < 0.05$)	0.3	0.2	0.1	0.3
CV (%)	1.6	2.8	1.2	1.0
Heat stress (45/37°C)				
HS	19.1	6.77	9.55	12.9
HS + RG	22.5	8.75	12.05	16.4
HS + Si	21.5	7.35	11.13	14.4
HS + Si + RG	23.5	9.59	12.24	17.5
Mean	21.6	8.11	11.24	15.3
CD ($P < 0.05$)	0.2	0.3	0.1	0.1
CV (%)	1.7	3.4	1.3	2.8

AC, Seedlings grown in the absence of silicates and bacteria under ambient temperature.
AC + RG, Seedlings co-cultured with *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* bacteria under ambient temperature.
AC + Si, Seedlings co-cultured with solely with insoluble diatomaceous earth and straw under ambient temperature.
AC + Si + RG, Seedlings co-cultured with both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* under ambient temperature.
HS, Seedlings grown in the absence of silicates and bacteria under heat stress condition.
HS + RG, Seedlings co-cultured with *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* bacteria under heat stress.
HS + Si, Seedlings co-cultured with solely with insoluble diatomaceous earth and straw under heat stress.
HS + Si + RG, Seedlings co-cultured with both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* under heat stress.

Gene expression profiling of HSPs, hormone-related, and silicon transporter genes

The expression of selected 10 genes related to HSPs (*viz. OsHsp26.7, OsHsp70, OsHsp90, OsHsp100* and *60 kDa chaperonin*), growth hormones (*OsIAA6, OsER1*) and silicon transporters (*viz. OsLsi1, OsLsi2* and *OsLsi6*) was studied through qRT-PCR of the shoot and root tissues at seedling stage under ambient and heat stress conditions (Figure 3).

Growth of seedlings in the presence of silicates (AC + Si) increased the relative expression of *OsHsp26.7, OsHsp70, OsHsp100* and *60 kDa chaperonin* by a fold change of 12.9, 11.6, 14.9 and 6.6, respectively, in root tissue under ambient conditions (Figure 3A; Supplementary Figure S1). The treatment AC + Si also similarly induced a 5.3 and 3.7-fold change in the expression of the *OsIAA6* and *OsERS1*gene expression, respectively. Inoculation with the bacteria (AC + RG) showed higher expression of silicon transporters genes. *OsLsi1* (1.8-fold change), *OsLsi2* (1.7-fold change) and *OsLsi6* (1.1-fold change) (Figure 3A; Supplementary Figure S1).

In root tissue under heat stress conditions, high relative expression of *OsHsp90* (2.9 and 1.2-fold change), *OsHsp100* (1.3 and 1.6-fold change) and *60 kDa chaperonin* (3.4 and 1.6-fold change) was observed in the treatments HS + RG and HS + Si + RG where the plants were inoculated with bacteria alone or a combination of both silicates and bacteria (Figure 3B; Supplementary Figure S2). Similarly, the transcript levels of both hormone related genes, *OsIAA6* and *OsERS1*, exhibited the highest increase in the treatments HS + RG (5.4 and 4.5-fold change) and HS + Si + RG (4 and 1.8-fold change). The transcript abundance of the two silicon transporter genes, *OsLsi1* and *OsLsi2*, however, were higher in HS + Si treatment (9.8 and 13.9-fold change) followed by HS + RG treatment (3.1 and 3.9-fold change) and HS + Si + RG (1.7 and 1.5-fold change). In contrast, *OsLsi6* transcripts remained at a low level in all the treatments (Figure 3B; Supplementary Figure S2).

In the case of shoot tissue, expression of *OsHsp26.7, OsHsp100, OsHsp70*, and *60 kDa chaperonin* were higher (4.5, 5.9, 5.1, and 1.5-fold change, respectively) with response to AC + RG treatment under ambient conditions (Figure 3C; Supplementary Figure S3) while a 3.7-fold increase in the expression of *OsHsp90* gene was observed when treated with both bacteria and silicates (AC + Si + RG). The hormone-related gene, *OsIAA6*, expression was elevated 9.1 and 2.3-fold with AC + Si and AC + RG treatments, respectively while there was no change in relative expression in the treatment AC + Si + RG. A 2.2 and 2-fold increase was observed in the expression levels of the *OsERS1* gene as influenced by AC + Si + RG and AC + Si treatments, respectively. Among the silicon transporters, transcript levels corresponding to *OsLsi1* and *OsLsi2* did not show much change between the treatments. (Figure 3C; Supplementary Figure S3).

Furthermore, *OsHsp90* was highly expressed under heat stress conditions in shoot tissue treated with HS + Si (1.8-fold change) followed by HS + RG (1.2-fold change; Figure 3D; Supplementary Figure S4). Elevated expression of 60 kDa chaperonin by 1.2-fold (HS + RG) and by 1.1-fold in the HS + Si treatment was observed. In the combined treatment with silicates and bacteria

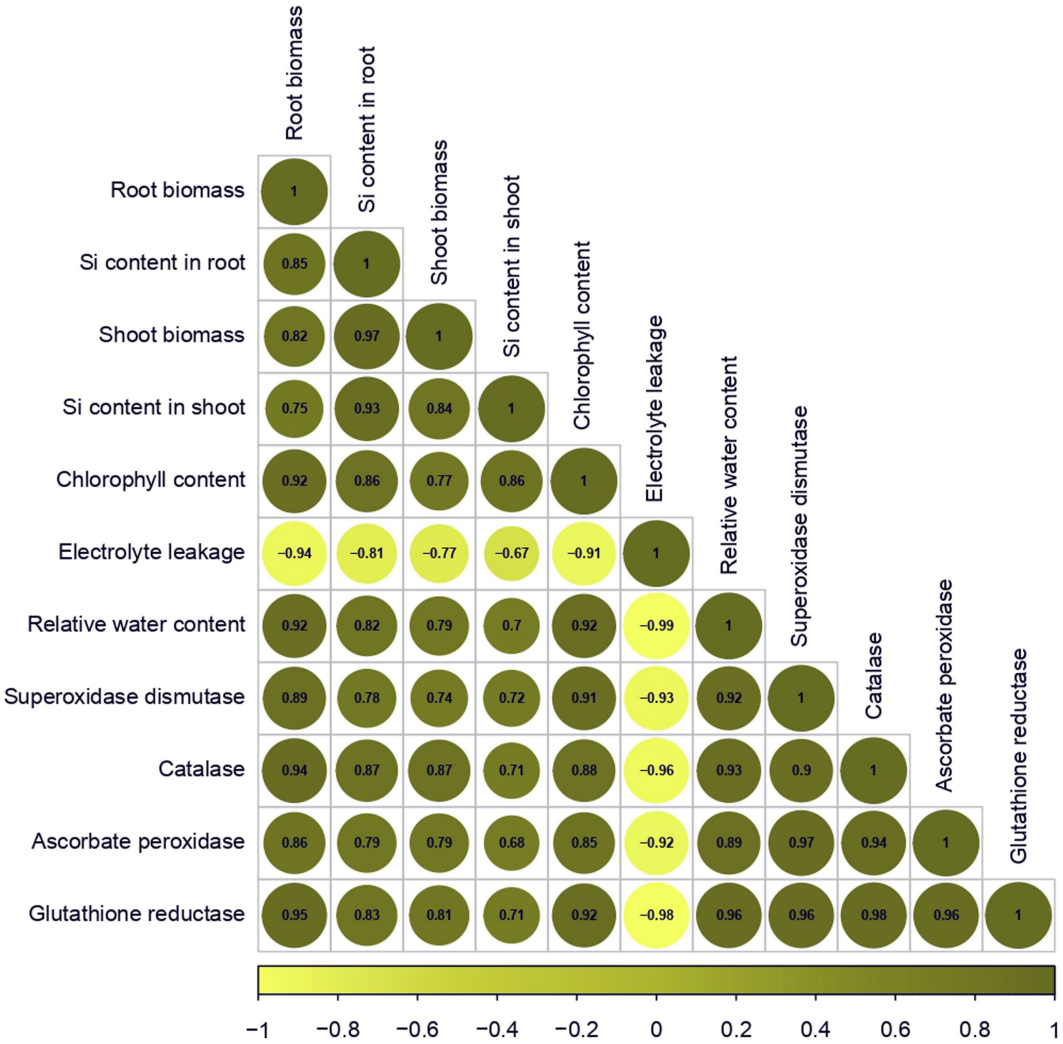

FIGURE 1
Correlation matrix for morphological, physiological and biochemical variables. Positive and negative correlations are shown as green and yellow circles, respectively. The color legend at the bottom represents the intensities of correlation coefficients.

(HS + Si + RG) there was a lower expression of both these genes compared to the control (HS). On the other hand, *OsHsp26.7* and *OsHsp100* expression reduced with the treatments HS + RG followed HS + Si, HS + Si + RG in comparison with heat-stressed control HS (control). However, among the silicon transporters genes, *OsLsi6* showed higher expression (1.6-fold change) in response to HS + Si + RG and showed lower expression with HS + Si and HS + RG. The expression levels of *OsIAA6* and *OsERS1* were lower in all treatments relative to HC (control; Figure 3D; Supplementary Figure S4).

Overall, the expression profiling of *HSPs* (*OsHsp90, OsHsp100* and *60 kDa chaperonin*), hormone-related (*OsIAA6*) and silicon transporters (*OsLsi1* and *OsLsi2*) exhibited higher expression in both shoot and root tissue HS + RG, HS + Si and HS + Si + RG. Thus, comparatively based on HSPs, hormone-related and silicon transporters genes expression profiling shows that the treatment HS + Si + RG (both silicon and *Rhizobium* sp. IIRR N1, *G. diazotrophicus*) augmenting silicon uptake affects the alleviating or coping maximum heat stress conditions compared to bacteria alone (*Rhizobium* sp. IIRR N1 and *G. diazotrophicus*) and silicon alone in rice.

Discussion

Heat stress severely affects crop production, resulting in global food insecurity (Zhou et al., 2017; Raza, 2020) as it reduces crop yields (Szymanska et al., 2017; Thakur et al., 2021). Among the various abiotic stresses, heat stress causes the most devastating effect on the physiological processes of plant growth, development, and metabolism in several ways (Szymanska et al., 2017; Thakur et al., 2021). Silicon fertilization is emerging as an important agronomic practice to combat the negative effects of biotic and abiotic stresses on plants. However, due to the high cost of synthetically manufactured silica fertilizers (Raza et al., 2023) attention has now shifted to the use of cost-effective and sustainable biological sources of Si like diatomaceous earth (Duboc et al., 2019) and crop residues (Hughes et al., 2020). The insoluble Si present in these sources can be released as soluble phyto-available Si by co-inoculation with silicate solubilizing and decomposer microorganisms. Similarly, plant growth-promoting bacteria colonizing the rhizospheric and endospheric niches and possessing phytohormone modulating capabilities like the production of auxins like IAA and enzymes like 1-aminocyclopropane-1-carboxylate-deaminase (ACC

deaminase) can improve the plants' ability to cope with environmental stresses (Fahad et al., 2015; Souza et al., 2015; Cura et al., 2017; Etesami and Jeong, 2018; Tapera et al., 2018) by reducing the production of stress-induced ethylene.

Our investigation demonstrates that the exogenous application of Si as a mixture of diatomaceous earth and rice straw in combination with silicate solubilizing bacteria, *Rhizobium* sp. IIRR N1 and plant growth-promoting endophytic bacteria *G. diazotrophicus* reduced the adverse effect of heat stress in rice plants by improving the morphological traits that include root and shoot biomass. The results presented in this study concur with earlier reports on exogenous Si application alleviating the adverse effects on plant growth under different abiotic stress conditions including salt stress in rice (Mahdieh et al., 2015), metal toxicity in rice (Chen et al., 2019), and salt stress in okra (Abbas et al., 2017). Our findings were similar to the previous reports on the exogenous application of silicon improving shoot length and biomass production in rice (Agarie et al., 1998), cucumber (Liu et al., 2009), sword fern (Sivanesan et al., 2014), and tomato (Khan et al., 2020) while enhancing the heat stress tolerance. Furthermore, the combined use of Si and PGPRs has been established as a sustainable strategy for alleviating crop abiotic stresses (Rizwan et al., 2015; Kaushal and Wani, 2016; Mahmood et al., 2016). Heat, heavy metal toxicity, nutrient deficiency, salinity, and water deficit are some of the stresses overcome by plants (Verma et al., 2019) due to the interaction of both PGPRs and Si which could be the synergistic result of both available Si and PGPR mediated regulation of plant hormones biosynthesis, i.e., auxins, abscisic acid (ABA), cytokinins, gibberellins, and ethylene (Kim et al., 2014; Yin et al., 2016; Tsukanova et al., 2017). Most of the studies related to the combined application of silicates and plant growth-promoting bacteria involved the use of soluble silicates (Al-Garni et al., 2019; Kubi et al., 2021; Mahmood et al., 2022) unlike the present study wherein inoculation with silicate solubilizing bacteria (*Rhizobium* sp. IIRR N1) could have made available soluble Si from insoluble biogenic silicates as

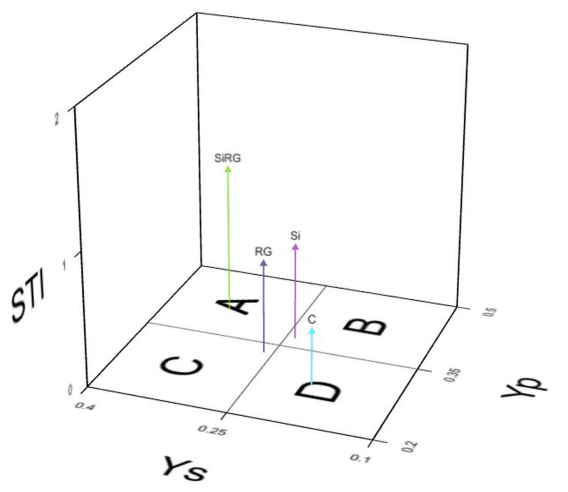

FIGURE 2
3D plot for the stress tolerance index based on rice seedling biomass under ambient temperature (Yp) and heat stress (Ys).

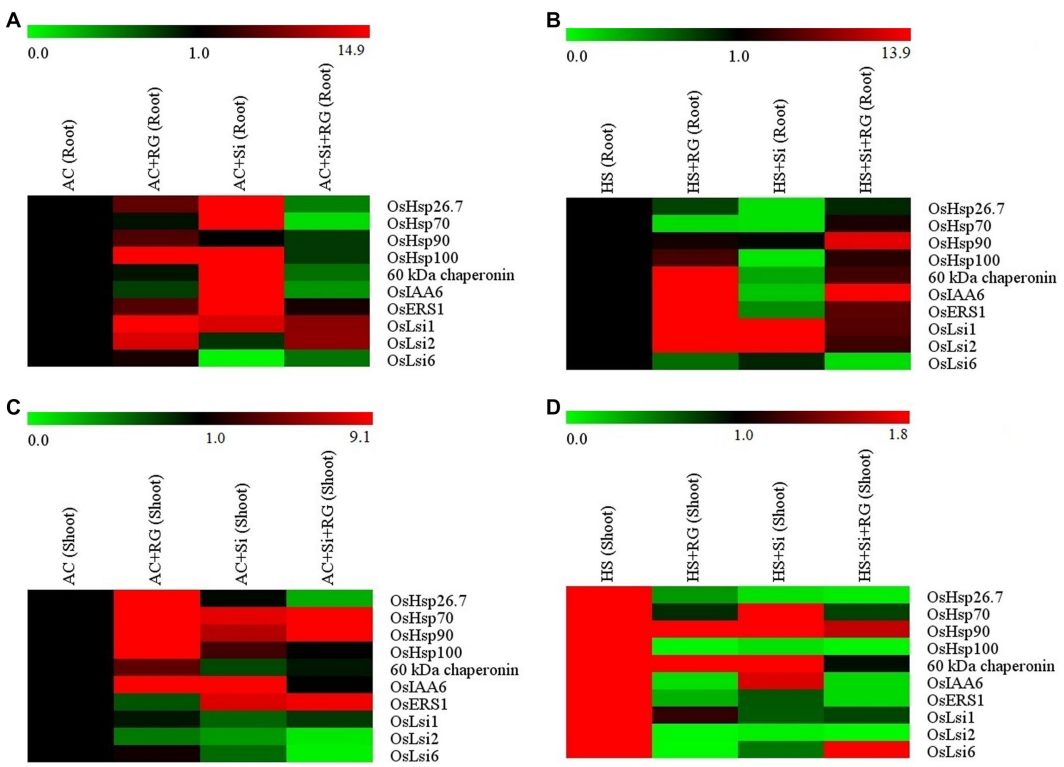

FIGURE 3
Heatmap representation of HSPs, hormone-related and silicon transporter genes expression profile in root tissue **(A,B)** and shoot tissue **(C,D)** of rice seedlings under ambient and heat stress conditions. The color scale at the top shows relative expression level values. Red indicates a higher expression level, and green indicates a lower expression level.

was reported earlier by Chandrakala et al. (2019a). The accelerated release of soluble Si from diatomaceous earth (diatom frustules of amorphous Si), which is highly recalcitrant to solubilization, through bio-dissolution by microbes has been reported earlier (Bidle et al., 2002; Roubeix et al., 2008; Holstein and Hensen, 2010). Microorganisms also play a role in decomposing phytolith silica contained in crop residues such as straw (Alfredsson et al., 2016; de Tombeur et al., 2021).

The effect of inoculation with *Rhizobium* sp. IIRR N1 and *G. diazotrophicus* application were similar to earlier reports on PGPRs mitigating heat stress. Inoculation with *Bacillus cereus*, *Pseudomonas* spp., *Serratia liquefaciens*, *P. fluorescens*, *Pseudomonas putida*, and *Bacillus tequilensis* SSB07 has alleviated heat stress in several crops like tomato, pigeon pea wheat and soybean (Kang et al., 2019). The capability of 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity in bacteria is presently one of the main selection criteria while identifying bacteria for plant stress alleviation (Gupta and Pandey, 2019; Etesami et al., 2020; Orozco-Mosqueda et al., 2023). The enzyme ACC deaminase degrades ACC, the precursor for ethylene in plants into ammonia and alpha-ketobutyrate (nitrogen and carbon nutrient source for the bacteria) resulting in reduced stress ethylene production by plants thereby enabling plants to escape the adverse effect of the gaseous hormone. ACC deaminase producing *Methylobacterium oryzae* (Roy et al., 2022) and *Glutamibacter* sp. (Ji et al., 2020) and *Burkholderia* sp. (Sarkar et al., 2018) inoculation has been reported to induce salt stress tolerance, while *Bradyrhizobium* SUTN9-2 (Sarapat et al., 2020) enhanced rice growth under drought stress condition in rice. Submergence tolerance also have been induced in rice treated with ACC deaminase producing *Microbacterium* sp. AR-ACC2, *Paenibacillus* sp. ANR-ACC3, and *Methylophaga* sp. AR-ACC3 which resulted in significant growth improvement growth parameters when compared with non-treated seeds (Bal and Adhya, 2021). There is a paucity of studies on the interactions between rice and bacteria and their role in inducing heat stress tolerance. Both bacteria used in this study possess unique traits that could have played a role in improving rice seedlings' tolerance to heat stress. *Rhizobium* sp. IIRR N1, in addition to silicate solubilization potential, can produce IAA and ACC deaminase, which could have modulated stress ethylene levels in seedlings while inducing heat stress tolerance (Chandrakala et al., 2019b). *G. diazotrophicus* is well known as a drought stress alleviator (Filgueiras et al., 2020; Silva et al., 2020), though its effect on plants during heat stress has not been studied.

The highest Si content in root and shoot in this study were observed to be in treatment with silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* (H + Si + RG) followed by those treated with silicon alone and treatment of *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* under heat stress conditions. Diatomaceous earth (Marxen et al., 2016) and rice straw incorporation (Pati et al., 2016; Sandhya and Prakash, 2019) in soil have been reported to influence the uptake of Si by rice plants. However, both diatomaceous earth and rice straw show variability in their ability to supply Si to plants (Duboc et al., 2019; Zellener et al., 2021) due to slow and inconsistent release of soluble Si that does not match to the plant demands. The inoculation of these Si source with silicate solubilizing bacteria is expected to improve the efficiency of these silicate sources. Thus, our findings on morphological traits demonstrate that exogenous application of both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* (H + Si + RG) can alleviate the effect of heat stress in rice seedlings plants enhancing plant growth attributes, such as root length, shoot length, silicon content in root and shoot and seedling biomass production.

The combined application of silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* (H + Si + RG) significantly increased chlorophyll content, relative water content in leaf followed by *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* (HS + RG) and silicon alone (HS + Si) respectively under heat stress conditions. Our findings on the chlorophyll content and relative water content concur with earlier studies in rice crops (Ma et al., 1989; Tamai and Ma, 2008; Detmann et al., 2012), which demonstrated that Si application increased plant growth, chlorophyll content, photosynthetic activity and subsequently enhanced productivity. In our results, electrolyte leakage was lowest in the combined application of silicon and *Rhizobium* sp. IIRR-1 plus *G. diazotrophicus* followed by *Rhizobium* sp. IIRR-1 plus *G. diazotrophicus* and silicon alone, respectively, under heat stress conditions. It has been reported that heat stress causes electrolyte leakage in higher plants grown without Si or under minimal Si applied conditions (Agarie et al., 1998). In rice, increasing the level of Si reduces the electrolyte leakage from leaf tissues, indicating that the concentration of Si in leaf tissue is correlated with cell wall polysaccharides level and also indicates Si role in maintaining thermal stability of lipids in cell membranes (Agarie et al., 1998).

Heat stress induces the production of highly toxic reactive oxygen species (ROS) leading to oxidative stress (Sarkar et al., 2018), and damages the various cellular, sub-cellular membranes, and macro-molecules causing disturbances in cellular homeostasis (Foyer and Noctor, 2011). Also, ROS molecules disturb the photosynthetic system (PSI and PSII) and cell membrane stability by triggering electrolyte leakage and autocatalytic lipid peroxidation (Tiwari and Yadav, 2019). Plants cope with oxidative damage by enhancing the endogenous content of catalase, peroxidase, superoxide dismutase, glutathione reductase, guaiacol peroxidase, ascorbate peroxidase, and dehydroascorbate reductase which are enzymatic antioxidants that protects against the oxidative stress produced during to abiotic stresses (Etesami and Jeong, 2018; Verma et al., 2019). It has been reported that PGPR-induced antioxidant enzymes production has the potential to mitigate the abiotic stresses in plants by reducing ROS (Etesami and Jeong, 2018). Also, exogenous application of Si may alleviate oxidative damage during abiotic stresses in plants by modifying the antioxidant machinery consisting of both enzymatic and non-enzymatic components (Zhu and Gong, 2014; Kim et al., 2017).

Our findings revealed that both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* application under heat-stressed rice seedlings increased the enzymatic antioxidants activities in line with earlier reports on tomato soybean and wheat crop (Ali et al., 2009, 2011; Meena et al., 2015; Choudhary et al., 2016; Bisht et al., 2020). In wheat, *Pseudomonas putida AKMP7* has enhanced the shoot and root length, dry biomass, tillers number, grain formation, and decreased membrane injury and antioxidant enzyme activities such as SOD, APX, and CAT under heat stress conditions (Ali et al., 2011). In wheat, auxin-producing *Azospirillum brasilense* was shown to alleviate heat stress by maintaining water status (Choudhary et al., 2016). In rice, combined application of bacterial inoculants and nano-silicon improved antioxidant activities under salt stress conditions (Alharbi et al., 2022). Kim et al. (2017) also reported that combined exogenous application of PGPRs and silicon may alleviate oxidative damage in crops by inducing ROS, CAT, POD, SOD, GR and APOD enzymatic activities under various abiotic stress conditions.

At the molecular level, the HSP genes *viz. OsHsp90, OsHsp100, 60 kDa chaperonin,* two phyto-hormone genes *viz. OsIAA6* and *OsERS1* and two silicon transporter genes, *OsLsi1* and *OsLsi2* showed higher

expression in the treatment of HS + RG and HS + Si + RG over control (HS) in root tissues under heat stress conditions. Whereas, in shoot tissues, two HSP genes *viz. OsHsp90, 60 kDa chaperonin,* and *OsIAA6* showed higher expression in HS + Si and HS + RG. The *OsLsi6* silicon transporter gene showed higher expression in response to HS + Si + RG treatment in shoot tissue under heat-stress conditions. It has been reported that plants overcome heat stress by mechanistic response via activation of conserved pathways, including overexpression of ABA-responsive genes, calcium-sensing proteins, HSPs-induced protein folding, and ROS-scavenging genes (Janni et al., 2020). Plants develop a diverse array of heat stress tolerance mechanisms including biosynthesis of heat shock proteins (HSPs), biosynthesis of specific phytohormones, and scavenge the ROS mechanism (Khan et al., 2020; Raza et al., 2021; Haider et al., 2021a,b). Our findings while profiling the gene expression of HSPs, hormone-related, and silicon transporters genes are concurrent with previous results on the combined application of PGPRs and Si that have documented enhanced expression of these genes while exhibiting tolerance to heat stress (Tiwari et al., 2016; Janni et al., 2020; Khan et al., 2020). It has been reported that Si-based activation of HSFs and HSPs interact with various signaling cascades triggered by Ca^{2+}, phospholipids, phytohormones, and H_2O_2, to reduce the effects of heat stress in crop plants (Liu et al., 2015; Sharma et al., 2019). The role of Si in inducing heat stress tolerance by stimulating heat shock proteins, antioxidant system, and phytohormone production in tomatoes was demonstrated by Khan et al. (2020).

In rice, silicon transporters *viz. OsLsi1* and *OsLsi2* are present in the plasma membrane of rice plant cells and are located on the distal side of the cell and proximal side of the cell and play a role in Si influx and efflux, respectively, (Ma et al., 2007). In rice, Si is taken up by rice roots in the available form of silicic acid (SiOH4) through Si influx (*Lsi1*) and efflux transporters (*Lsi2*) genes and then translocated into aerial organs, i.e., shoots through xylem transpiration stream unloaded by *Lsi6* (Yamaji et al., 2008, 2015). Tiwari et al. (2016) demonstrated that they induce the expression of stress-responsive genes of rice seedlings in response to *B. amyloliquefaciens* NBRI-SN12 and enhanced the accumulation of osmoprotectants mitigate the heat-stress conditions. Similarly, *Pseudomonas* sp. AKM-P6 treated sorghum seedlings showed higher expression and accumulations of HSPs and generating EPSs, which leads to tolerance to the heat stress conditions (Sandhya et al., 2009).

Thus, our findings revealed exogenous application of both silicon and *Rhizobium* sp. IIRR N1 plus *G. diazotrophicus* (H + Si + RG) showed higher expression of silicon transporters genes *OsLsi1, OsLsi2,* and *OsLsi6* in root and shoot tissue under heat stress conditions, suggesting enhanced Si uptake and played a role in mitigating heat stress. This role might be due to the uptake of Si from root to shoot and Si in the shoot provides strength to leaf and shoot tissue by minimizing the adverse effects leading to alleviating the heat stress in rice seedlings. This role might be attributed to the accumulation of Si in the shoots, providing additional strength to the leaf and stem structure to minimize the adverse effects of heat and drought stress, hence leading to a higher tolerance.

Conclusion

Globally, reduction in yield of crops due to heat stress is a major concern. Silicon dependent stress emancipation of plants is gaining popularity as a reliable, eco-friendly strategy to meet global food demands in a sustainable manner under stress agriculture. The results obtained in this study demonstrates that mixed bacterial inoculum of *Rhizobium* sp. IIRR N1 and *Gluconacetobacter diazotrophicus* along with insoluble silicates like diatomaceous earth and rice straw when used in combination as a organo-mineral biofertilizer, can result in conferring heat stress tolerance to a heat susceptible rice genotype.

Data availability statement

The original contributions presented in the study are publicly available. The datasets generated for this study can be found at the link: https://www.ncbi.nlm.nih.gov/nuccore/KY348774.1 in the NCBI GenBank, Accession number KY34877.

Author contributions

LC and CC: conceptualization and methodology. LC, CC, and AP: data curation, formal analysis, software, and validation. LC, AP, CC, and BS: investigation and visualization. LC, CC AP, RG, and VK: resources. LC, RS, CC, and BS: supervision. RS: writing—original draft. LC, CC, AP BS, VK, RG, and RS: writing review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1168415/full#supplementary-material

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EDITED BY
Arvind Kumar Rai,
Indian Council of Agricultural Research (ICAR), India

REVIEWED BY
Dharmendra Kumar,
Central Potato Research Institute (ICAR), India
Rahul Kumar Tiwari,
Central Potato Research Institute (ICAR), India

*CORRESPONDENCE
Zhenyu Liu
✉ cosmosliu@163.com

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The halophilic bacteria *Gracilibacillus dipsosauri* GDHT17 alleviates salt stress on perennial ryegrass seedlings

Xiangying Li, Jinyuan Zheng, Wei Wei, Zifan Gong and Zhenyu Liu*

College of Plant Protection, Shandong Agricultural University, Tai'an, Shandong, China

Introduction: Adverse abiotic environmental conditions including excess salt in the soil, constantly challenge plants and disrupt the function of plants, even inflict damage on plants. Salt stress is one of the major limiting factors for agricultural productivity and severe restrictions on plant growth. One of the critical ways to improve plant salt tolerance is halotolerant bacteria application. However, few such halotolerant bacteria were known and should be explored furtherly.

Methods: Halophilic bacterium strain was isolated from saline soil with serial dilution and identified with classical bacteriological tests and 16S rRNA analysis. Perennial ryegrass (*Lolium perenne* L) was used in this study to evaluate the potential effect of the bacteria.

Results and discussion: A halophilic bacterium strain GDHT17, was isolated from saline soil, which grows in the salinities media with 1.0%, 5.0%, and 10.0% (w/v) NaCl, and identified as *Gracilibacillus dipsosauri*. Inoculating GDHT17 can significantly promote ryegrass's seedling height and stem diameter and increase the root length, diameter, and surface area at different salt concentrations, indicating the significant salt stress alleviating effect of GDHT17 on the growth of ryegrass. The alleviating effect on roots growth showed more effective, especially on the root length, which increased significantly by 26.39%, 42.59%, and 98.73% at salt stress of 100 mM, 200 mM, and 300 mM NaCl when the seedlings were inoculated with GDHT17. Inoculating GDHT17 also increases perennial ryegrass biomass, water content, chlorophyll and carotenoid content under salt stress. The contents of proline and malonaldehyde in the seedlings inoculated with GDHT17 increased by 83.50% and 6.87%, when treated with 300 mM NaCl; however, the contents of MDA and Pro did not show an apparent effect under salt stress of 100 mM or 200 mM NaCl. GDHT17-inoculating maintained the Na^+/K^+ ratio in the salt-stressed ryegrass. The Na^+/K^+ ratio decreased by 26.52%, 6.89%, and 29.92% in the GDHT17-inoculated seedling roots treated with 100 mM, 200 mM, and 300 mM NaCl, respectively. The GDHT17-inoculating increased the POD and SOD activity of ryegrass seedlings by 25.83% and 250.79%, respectively, at a salt stress of 300 mM NaCl, indicating the properties of GDHT17, improving the activity of antioxidant enzymes of ryegrass at the salt-stress condition. Our results suggest that *G. dipsosauri* GDHT17 may alleviate salt stress on ryegrass in multiple ways; hence it can be processed into microbial inoculants to increase salt tolerance of ryegrass, as well as other plants in saline soil.

KEYWORDS

salt stress, *Gracilibacillus dipsosauri* GDHT17, saline habitats, ryegrass, halotolerant bacteria

1. Introduction

Salt stress is a severe abiotic factor that reduces yields and physiological processes in many plants worldwide, including crops, vegetables, and trees (Ghosh and Ali Md, 2016; Tomaz et al., 2020; Zhang et al., 2022). It has been estimated that about 7.5% of land in the world is severely affected by soil salt stress (Li et al., 2019; Hassani et al., 2020). Moreover, climate change, poor agricultural practices, and industrial pollution cause a 10% annual rise in global soil salinization (Ziska et al., 2012; Zhang et al., 2016). Soil salt stress has become crucial to agricultural productivity and sustainable development. In plants, the adverse effects of salt stress usually cause physiological drought by high solute concentrations leading to water deficit limiting plant water use in the soil, as well as ion toxicity caused by ion and osmolarity imbalances leading to oxidative stress (De Souza Miranda et al., 2017; Ullah et al., 2022). The impacts of salt stress on plant physiological and biochemical processes include nutrient uptake, osmotic stress balance, oxidative stress, photosynthetic rate, and overall growth, and ultimately reduced crop yield (Ji et al., 2018; Chang et al., 2019; Petretto et al., 2019).

To counteract the adverse effects of salt stress, plants adopt a number of fundamental mechanisms, including exclusion of toxic ions (Na^+ and Cl^-), synthesis of endogenous metabolites associated with stress adaptation, e.g., proline, betaine, etc., synthesis and activation of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), etc., and non-enzymatic antioxidants such as carotenoids, phenolic compounds etc. (Rahman et al., 2019; Zaid and Wani, 2019; Patel et al., 2020; Mostofa et al., 2021; Zhang et al., 2022). The salt tolerance mechanism of plants can be used as a standard for evaluating plant salt damage. In addition, it is meaningful and urgent to improve the salt tolerance of plants and alleviate the damage to plants caused by salt stress. Therefore, it is imperative to use appropriate technologies to alleviate salt stress damage to crops.

Advanced and more effective technologies for improving crop yields in saline soils should be explored. Genetic factors are essential in determining salt tolerance of plants, and some genes were employed for genetic modification to improve salt tolerance in plants (He et al., 2019; Zhang et al., 2022). However, access to salt-tolerant varieties is rare and requires considerable time and financial resources. Fortunately, microbial inoculation has been proposed to improve salt tolerance in crops and has received extensive attention (Li et al., 2016; Kearl et al., 2019). Some microorganisms, including *Rhizobium*, *Bacillus*, *Pseudomonas*, *Pantotrichum*, *Burkholderia*, *Microbacterium*, *Methylobacterium*, *Variovorax*, *Enterobacter*, etc., have been demonstrated with the properties of improving plant tolerance under different abiotic stresses (Yildirim et al., 2008; Dey et al., 2021; Shahid et al., 2022). Therefore, exploiting and employing such microorganisms for plant salt tolerance is a promising alternative. The mechanisms enhancing salt tolerance in plants of these microorganisms are mostly similar in different taxa (Saum and Müller, 2007; Albacete et al., 2008; Egamberdieva et al., 2016). These bacteria mediate salt tolerance in plants by regulating the expression of reactive oxygen species (ROS) scavenging enzymes (Khodair et al., 2008; Poli et al., 2010), altering the selectivity of Na^+, K^+, and maintaining high K^+/Na^+ ratios in plants (Ramezani et al., 2011; Rojas-Tapias et al., 2012; Pinedo et al., 2015). For example, *Kosakonia radicincitans* strain KR-17 applied to salt-stressed plants significantly reduced plant membrane damage, stress metabolites, and antioxidant defense enzymes (Shahid et al., 2022). Orhan et al. showed that inoculation of halotolerant and halophilic bacteria attenuated NaCl-induced toxicity and increased wheat yield in saline soils (Orhan, 2016). However, the bacteria of improving salt tolerance in plants led by microorganisms have yet to be elaborate.

Natural saline habitats are a resource bank of excellent salt-tolerant bacteria. Many salt-tolerant microorganisms have been found in natural saline environments (Hovik et al., 2018), and these microorganisms are most suitable for growth in salt-stressed environments. For example, Priestia aryabhattai JL-5 derived from saline soil has been used to promote the saline-alkaline tolerance of *Leymus chinensis* (Wang et al., 2023). Perennial ryegrass (*Lolium perenne* L.) is widely planted as a cold-season turf grass and a crucial forage grass, with a high yield and strong regeneration (Wu et al., 2005; Kane, 2011). Perennial ryegrass has strong tillering and regeneration abilities (Wang and Bughrara, 2009; Kane, 2011); however, the salt tolerance in commercial cultivars is only moderate, and the growth of ryegrass is usually affected by salt stress.

In the study, we isolated salt-tolerant bacteria from saline habitats in the Yellow River Delta, where severe land salinization is marked. We employed perennial ryegrass to evaluate the effect of the bacteria on the plant under salt stress, expecting to explore the potential properties of the bacteria to alleviate the salt stress on plants.

2. Materials and methods

2.1. Bacteria isolation and identification

The selinene soil samples with a pH of 8.5 were collected in July from the Yellow River Delta (37° 22 'N-38° 04 'N, 118° 14 'E-119° 05 'E), located in Shandong Province, China. Samples were packed in ice, brought to the lab, and stored at 4°C for processing. As previously described (Suman et al., 2018), LB solid medium (10 g Tryptone, 5 g Yeast, 15–18 g agar, 1 L distilled water) with NaCl concentrations of 0.5, 1, 5, 10, and 15% were prepared for bacteria isolation. Soil samples were serially diluted with ten-fold dilutions up to 10^{-5} in sterile water. 100 μL of soil diluent were plated on the solid LB with different NaCl concentrations and incubated at 28°C for 48 h to 72 h. The colonies, which grow on solid LB with 10 and 15% NaCl with distinct bacterial colony morphology, were selected, purified, and cultured in LB with 5% NaCl (w/v) at 28°C and then preserved at 4°C for further testing.

Cultural, morphological, and physiological characteristics of the bacteria were carried out using conventional methods (Carrasco et al., 2006; Jeon et al., 2008). The colony morphology was observed after culture on solid LB media with 5% NaCl (w/v) at 28°C for 48 h. The characters of gram staining and spore staining were observed with OLYMPUS-BX51 optical microscope.

The biochemical characteristics of bacteria were determined after culture in LB media with 5% NaCl (w/v) at 28°C for 48 h. Catalase activity, oxidase activity, Voges-Proskauer test, gelatin hydrolysis, and starch degradation tests were determined as described previously (PhilippGerhardt, 1981; Ventosa et al., 1982; Chen et al., 2007).

For 16S rRNA identification, the bacteria was cultured in LB media with 5% NaCl (w/v) at 28°C for 48 h. Genomic DNA was extracted using an OMEGA bacterial DNA kit according to the manufacturer's instructions. The primers, forward (5′-AGAGTTT GATCCTGGCTCAG-3′) and Reverse (5′-AAGGAGGTGATC CAGCCGCA-3′) were used for the 16S rRNA gene amplification. The

sequence of 16S rRNA was analyzed by comparing it with the 16S rRNA genes available at the GenBank database of the National Center for Biological Information (NCBI) using the BLAST algorithm. Phylogenetic analysis of 16S rRNA was performed using the MEGA version 11 (Tamura et al., 2021).

2.2. Determination salt tolerance properties of bacteria

The media with 1, 5, 10, and 15% NaCl concentration were used for salt tolerance properties determination by determining the culture properties. The selected strain was cultured in LB with 5% NaCl (w/v) at 28°C for 24 h. The bacteria culture was then streaked on the solid LB media with NaCl concentrations of 1, 5, 10, and 15%. Furthermore, the bacteria were inoculated in LB with 1, 5, 10, and 15% NaCl concentration and shaking cultured at 180 r · min^{-1}, 28°C. The OD value at 600 nm of the bacterial solution was measured every 12 h.

2.3. Plant growth conditions and plant inoculation

The seeds of perennial ryegrass (*L.perenne* L. cv. *Taya*) were surface sterilized and rinsed with sterile deionized water. The Hogland nutrient solution with half concentration (5 mM KNO_3, 1 mM $NH_4H_2PO_4$, 0.5 mM Ca $(NO_3)_2$, 0.5 mM $MgSO_4$, 92 μM H_3BO_3, 60 μM Fe-citrate, 1.6 μM $ZnSO_4{\cdot}7H_2O$, 0.6 μM $CuSO_4{\cdot}5H_2O$, 18 μM $MnCl_2{\cdot}4H_2O$, and 0.7 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) (Chen et al., 2022) was prepared. The culture solution was obtained by adding NaCl into half concentration Hogland nutrient solution; then, the surface sterilized seeds were sown in the sterilized perlites floated on the culture solution with a salt concentration of 0 mM, 100 mM, 200 mM, and 300 mM. 100 μL bacteria cultured for 48 h were inoculated in the culture solution. Each treatment was set with 10 repetitions. The treatment were set in illuminating incubator and cultured at 25°C, 12 h/12 h for light/dar, supplying the culture solution periodically. 30 days after, 20 seedlings were collected for growth biomass determination and physiological and biochemical property determination.

2.4. Plant characters measurement

2.4.1. Growth biomass measurement

The plant height was measured using the scale ruler with the minimum millimeter, and the stem diameter was measured with a vernier caliper. The total root length, diameter, and surface area were measured by the root scanner (WinRHIZO Plant Root Scanning System, Software name WinRHIZO).

For the relative water content test, the fresh weight (FW) of leaves and stems was weighed immediately after the sample collection, then the dry weight (DW) was measured after the samples dried at 80°C for 24 h. The water content (WC) was calculated as follows:

$$\mathbf{WC}(\%) = (\mathbf{FW} - \mathbf{DW}) / \mathbf{FW} \times 100$$

2.4.2. Photosynthetic pigments determination

The chlorophyll and carotenoid were measured with fresh leaves, following the protocol described by the classical methodology laboratory (Zhao et al., 2020). Briefly, weigh 0.1 g of fresh leaves; the fresh leaf samples were completely bleached, extracted with 80% acetone, and then centrifuged at 13,000 rpm for 10 min to obtain the chlorophyll supernatants. The chlorophyll a, chlorophyll b, and carotenoid were determined at 663 nm, 645 nm, and 470 nm absorbance, respectively. Ryegrass seedling leaf chlorophylls and carotenoid contents were calculated as follows:

$$\textbf{Chlorophyll a content}\,(\mathbf{mg/g}) = (\mathbf{12.7} \times \mathbf{A_{663}} - \mathbf{2.59} \times \mathbf{A_{645}}) \times \mathbf{10} \div \mathbf{1000} \div \mathbf{0.1};$$

$$\textbf{Chlorophyll b content}\,(\mathbf{mg/g}) = (\mathbf{22.9} \times \mathbf{A_{645}} - \mathbf{4.69} \times \mathbf{A_{663}}) \times \mathbf{10} \div \mathbf{1000} \div \mathbf{0.1};$$

$$\textbf{Chlorophyll total content}\,(\mathbf{mg/g}) = (\mathbf{20.21} \times \mathbf{A_{645}} + \mathbf{8.02} \times \mathbf{A_{663}}) \times \mathbf{10} \div \mathbf{1000} \div \mathbf{0.1};$$

$$\textbf{Carotenoid content}\,(\mathbf{mg/g}) = (\mathbf{1000} \times \mathbf{A_{470}} - \mathbf{3.31} \times \mathbf{C_a} - \mathbf{104} \times \mathbf{C_b}) \div \mathbf{229} \times \mathbf{10} \div \mathbf{1000} \div \mathbf{0.1}.$$

A_{663} represented the absorbance value at 663 nm; A_{645} represented the absorbance value at 645 nm; A_{470} represented the absorbance value at 470 nm; C_a represented the chlorophyll a content; C_b represented the chlorophyll b content.

2.4.3. Malondialdehyde and proline assay

0.1 g ryegrass seedling leaves were harvested, immediately ground into powders with liquid nitrogen, and homogenized in 4 mL ice-cold phosphate buffer (50 mM, pH 7.8). The homogenates were then centrifuged at 12,000 rpm at 4°C for 20 min. The supernatant was collected and used to determine the malondialdehyde (MDA) content following the method described by Hu et al. using the thiobarbituric acid reaction method (Hu et al., 2011). Absorbance was determined at 532 (M_{532}) and 600 (M_{600}) nm to estimate MDA content. The malondialdehyde content was calculated as follows:

$$\mathbf{MDA}\,(\mathbf{nmol/g}) = \mathbf{32.258} \times (\mathbf{M_{532}} - \mathbf{M_{600}}) \div \mathbf{0.1}$$

The Proline (Pro) content of the leaves was determined following a standard protocol with slight modifications (Bates et al., 1973). Ryegrass seedling leaves were homogenized in 5% (w/v) sulfosalicylic acid and centrifuged at 8,500 g for 10 min. The resulting supernatant was added with 2% ninhydrin (w/v) and incubated at 100°C for 30 min. An equal volume of toluene was added to the mixture to obtain the upper aqueous phase. The absorbance was measured at

520 nm. A standard curve of pure L-proline was used for the proline content calibration.

2.4.4. Ionic accumulation analysis

1 g of seedling leaves or roots from each treatment was selected for analysis of ionic elements. These samples of leaves or roots were rinsed in double distilled water. Roots and shoots were separated, and samples were ground in liquid nitrogen and then digested in 100 mL of a mixture of 10:1:2 perchloric acid, sulphuric acid, and distilled water at heating by an alcohol lamp. The Na^+ and K^+ content was measured using an atomic absorption spectrophotometer (AAS 2380, Perkin Elmer, United States). The Na^+/K^+ ratio was calculated afterward. Each treatment was performed in triplicate.

2.4.5. Measurement of the enzyme activity of SOD, CAT, and POD

Antioxidant enzymes were extracted, and superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities of the leaves were measured according to the instructions of the kit (Solarbio, cat nos. BC0175, BC0205, BC0095). Briefly, 1 g of fresh ryegrass seedling leaves were harvested and ground in liquid nitrogen, and the enzymes were extracted using an extraction buffer. Subsequently, the extract containing the enzymes was centrifuged at 12,000 g for 10 min, and the supernatant was aspirated for the determination of enzyme activity. Each activity unit is defined according to the instructions of the antioxidant enzyme assay kit.

2.5. Statistical analysis

GraphPad Prism 8 software was used for data collation and graphing. One-way ANOVA ($p < 0.05$) was performed using IBM SPSS statistics 20 software, and Duncan's method was used for multiple comparisons. Marking of significant differences.

3. Results

3.1. Isolation and identification of halotolerant bacteria

In the bacteria isolated from the selinene soil samples, a few bacteria colonies grew on the solid LB with 10% or 15% NaCl, and many colonies grew on the solid LB with 1% NaCl or 5% NaCl; however, the number of colonies grew on the solid LB with 0.5% NaCl was less than that on the solid LB with 1% NaCl and more than that on the solid LB with 10% NaCl. We obtained 160 isolates in total from the saline soil samples, and in these isolates, 5 isolates grew on the solid LB with 15% NaCl, and 12 isolates grew on the solid LB with 10% NaCl. A strain that grew on the solid LB with 10% NaCl, was chosen for further study and named GDHT17.

The colonies of GDHT17 were circular, moist, smooth, raised, undulated, rough, opaque, and grayish-white with no diffusible pigment (Figure 1A). The bacteria consisted of motile, aerobic, Gram-positive, long rods (Figure 1B), showed terminal endospore formation (Figure 1C), and were positive for catalase activity, oxidase activity, Voges-Proskauer test, gelatin hydrolysis, and starch degradation.

The 16S rRNA gene sequence of GDHT17 was obtained and deposited in GenBank with the accession number MG980060. The 16S rRNA gene sequence identities between strain GDHT17 and the type strains of other recognized members of the genus *Gracilibacillus* were below 97%. The sequence of GDHT17 shared 99.9% and 99.1 identities with those of *G. dipsosauri* (X82436) and *G. dipsosauri* DD1T (AB101591), respectively. Some typical 16S rRNA gene sequences belonging to *Gracilibacillus* and the 16S rRNA gene sequence of GDHT17 were employed for phylogenetic analysis (Figure 2), which showed higher similarities between GDHT17 and *G. dipsosauri*. According to the classical bacteriological tests and the 16S rRNA gene sequence analysis, we affirmed that the strain GDHT17 belongs to *G. dipsosauri*.

3.2. Salt tolerance properties of strain GDHT17

G. dipsosauri GDHT17 grows on the LB plates and in LB broth with salinities of 1.0, 5.0, and 10.0% (w/v) NaCl; the optimum is 5.0% (Figure 3). However, it does not grow in the media with 15.0% NaCl (Figure 3). Therefore, *G. dipsosauri* GDHT17 is considered a moderately halophilic bacterium.

3.3. Inoculation of GDHT17 positively affected the growth and biomass of NaCl-treated ryegrass

Salt stress significantly inhibits the seedling height and stem diameter of ryegrass when treated with 100 mM, 200 mM, and 300 mM NaCl in the Half concentration of Hogland nutrient solution (Figures 4A–C). The seedling height and stem diameter significantly decreased with the salt concentration increase. The same happened when GDHT17 was inoculated in the Hogland nutrient solution. However, inoculating GDHT17 significantly promote the ryegrass's seedling height and stem diameter at different salt concentrations, except for 200 mM NaCl (Figures 4A,B). Compared with the non-bacteria inoculated seedlings, GDHT17 significantly increased the seedling height by 4.59 and 12.16% at 100 mM and 300 mM NaCl stress, respectively (Figures 4A,B), significantly increased the stem diameter by 20.98, 21.75 and 15.98% at 100 mM, 200 mM, and 300 mM NaCl stress, respectively (Figures 4A,C). Therefore, GDHT17 alleviates the effects of salt stress on the growth of seedling height and stem diameter of ryegrass.

The root length and root surface area of the seedlings were inhibited when treated with different concentrations of NaCl; the root length and root surface area significantly decreased with the salt concentration increase. However, when treated with different concentrations of NaCl, the seedling root diameter did not decrease with increasing salt concentration (Figures 4D,E); only at the concentration of 300 nM NaCl, the root diameter was significantly inhibited (Figure 4F). The root length, diameter, and surface area of ryegrass seedlings increased when the GDHT17 were inoculated.

The root length increased significantly by 26.39, 42.59, and 98.73% at salt stress of 100 mM, 200 mM, and 300 mM NaCl, respectively, compared with the non-GDHT17 inoculation treatment (Figure 4D). The root diameter significantly increased by 13.60% in non-salt

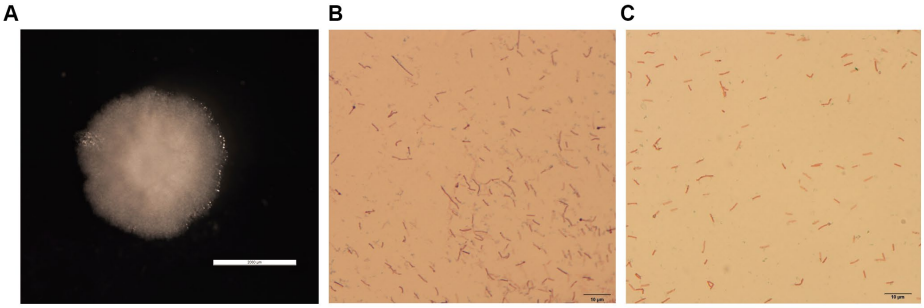

FIGURE 1
Isolation and identification of halotolerant bacteria. **(A)** Colony morphology of strain GDHT17 on LB solid medium, the scale of panel A is 2000 μm. **(B)** Gram stain, the scale of panel B is 10 μm. **(C)** Spore staining character, the scale of panel C is 10 μm.

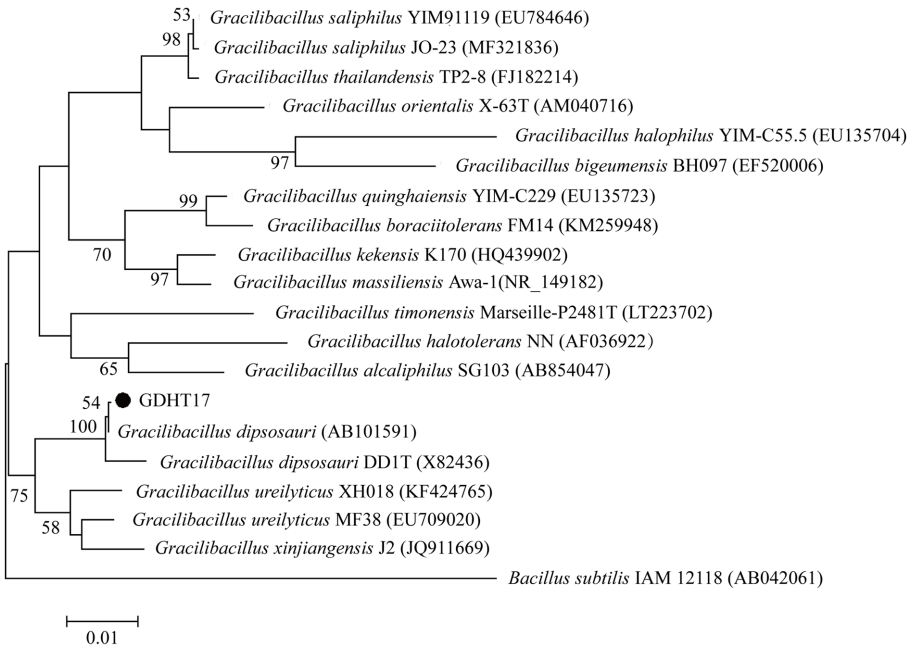

FIGURE 2
Phylogenetic trees based on 16S rRNA gene sequences using the maximum-likelihood methods, bar 0.01 substitutions per nucleotide position.

conditions, 23.76, and 6.16% at salt stress of 100 mM and 200 mM NaCl, respectively, compared with the non-GDHT17 inoculation treatment. However, GDHT17 had no significant increased effect on the root diameter under 300 mM NaCl stress (Figure 4E). The root surface area significantly increased by 11.29% in non-salt conditions, and the root surface area significantly increased by 56.30, 55.96, and 97.73% at salt stress of 100 Mm, 200 mM, and 300 mM NaCl, respectively, compared with the non-GDHT17 inoculation treatment (Figure 4F). In conclusion, GDHT17 can alleviate the salt stress on the root growth of ryegrass. Indeed, in the case of 300 mM NaCl treatment, the root length and surface were significantly decreased, demonstrating severe damage to the ryegrass even if the inoculation of GDHT17.

The seedling biomass and water content decreased when treated with different concentrations of NaCl (Figures 4G–I). Inoculating GDHT17 can significantly increase ryegrass's seedling biomass at different salt concentrations, except for 300 mM NaCl (Figures 4G–I). The fresh weight, dry weight, and water content significantly increased by 27.80, 4.70, and 2.05%, respectively, in non-salt conditions, compared with the non-GDHT17 inoculation treatment. In summary, GDHT17 can increase the biomass and water content of ryegrass, alleviating the inhibitory effect of salt stress on the growth of ryegrass.

3.4. Inoculation of GDHT17 positively affected the chlorophyll and carotenoid content of NaCl-treated ryegrass

Salt stress significantly increases the chlorophyll and carotenoid contents of ryegrass seedlings treated with 200 mM and 300 mM NaCl. Inoculating of GDHT17 significantly increased the contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids at the non-salt stress by 62.97, 66.96, 64.22, and 59.54%, respectively, compared to the non-GDHT17 treatment. Inoculating of GDHT17

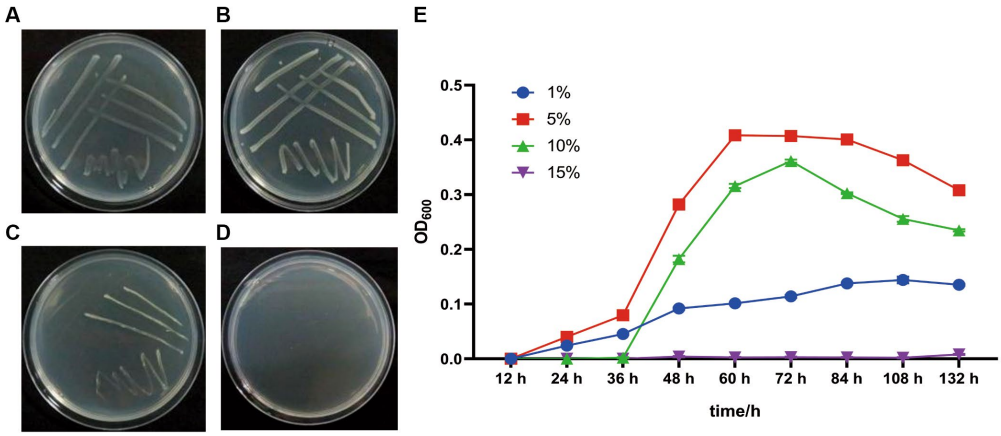

FIGURE 3
Salt tolerance properties of *G. dipsosauri* GDHT17. The patterns of GDHT17 on salt-containing solid LB plates cultivated for 48 h, **(A–D)**, the solid LB plates with 1.0, 5.0, 10.0, and 15.0% NaCl (w/v); **(E)** the growth curve of GDHT17 cultivated in LB containing with different salt concentrations. The line graph indicates the mean of the three replicates ($n = 3$). Error bars indicate the standard deviation (SD) of the three replicates.

significantly increased the carotenoid contents at the salt stress of 100 mM, 200 mM, and 300 mM NaCl and increased the chlorophyll contents at the salt stress of 100 mM and 300 mM NaCl (Figure 5).

3.5. Inoculation of GDHT17 affected the contents of malondialdehyde and proline

Salt stress significantly increased the contents of MDA and Pro in the ryegrass seedlings, especially in the seedlings treated with 200 mM and 300 mM NaCl, and also increased the contents of Pro in the seedlings treated with 100 mM (Figure 6). Inoculation with GDHT17 significantly increased the contents of MDA and Pro by 83.50 and 6.87% at the salt stress of 300 mM NaCl, compared to the non-GDHT17 treatment. However, the effect on the contents of MDA and Pro was not evident at the salt stress of 100 mM and 200 mM NaCl, even inoculating with GDHT17. Therefore, we can conclude that GDHT17 alleviates the NaCl-induced toxicity and improved proline and MDA content in ryegrass via the upregulation of antioxidant defense enzymatic activities (Figure 6).

3.6. Inoculation of GDHT17 affected ion absorption

Compared to the non-salt stress, salt stress caused a significant accumulation of Na^+ and K^+ contents and a significant increase of Na^+/K^+ ratio in ryegrass seedling leaves (Figures 7A–C); and caused a significant accumulation of Na^+ content, a significant reduction of K^+ content, and a significant increase of Na^+/K^+ ratio in seedling roots (Figures 7D–F). Compared to the non-GDHT17 inoculation, the Na^+/K^+ ratio significantly decreased by 12.45, 18.26, and 9.74% in the GDHT17-inoculated seedling leaves treated with 100 mM, 200 mM, and 300 mM NaCl, respectively. The Na^+/K^+ ratio significantly decreased by 26.52, 6.89, and 29.92% in the GDHT17-inoculated seedling roots treated with 100 mM, 200 mM, and 300 mM NaCl, respectively, compared to the non-GDHT17 treatment. Overall, GDHT17 decreases the Na^+/K^+ ratio under salt stress, whether in seedling leaves or roots, indicating the alleviating effect of GDHT17 on salt stress to ryegrass.

3.7. GDHT17 Modulated the antioxidant defense enzymes of NaCl-treated ryegrass

Salt stress significantly increased the POD, CAT, and SOD activity of ryegrass seedlings under salt stress, especially those treated with 200 mM and 300 mM NaCl. The activities of the three antioxidant defense enzymes further increased in the GDHT17-inoculated seedlings. Compared with the non-GDHT17 treatment, the POD and SOD activity of the GDHT17-inoculated seedlings significantly increased by 25.83 and 250.79%, respectively, at a salt stress of 300 mM NaCl. In addition, the activity of POD, CAT, and SOD did not show a significant increasing effect at the NaCl salt stress of 100 mM and 200 mM concentrations. The activity of CAT did not significantly increase in the GDHT17-inoculated seedlings. It suggested that GDHT17 reduces salt damage to the ryegrass seedlings by eliminating ROS in ryegrass seedlings, especially under higher salt stress (Figure 8).

4. Discussion

As available natural bioresources, bacteria habited in the saline environment have been verified to have great potential application in stimulating the growth of salt-sensitive crops and promoting salt stress tolerance (Egamberdieva and Kucharova, 2009; Etesami and Beattie, 2017). In the study, a moderately halophilic bacterium *G. dipsosauri* GDHT17 was isolated from the soils collected from the Yellow River Delta in Shandong Province, China. The Yellow River Delta is a fluvial delta plain in the Yellow River estuary. It is one of the specific regions in China, where severe land salinization ecological conditions and environment are marked. Yellow River Delta is between land and sea and lacks fresh water, which results in an excessively high salt content

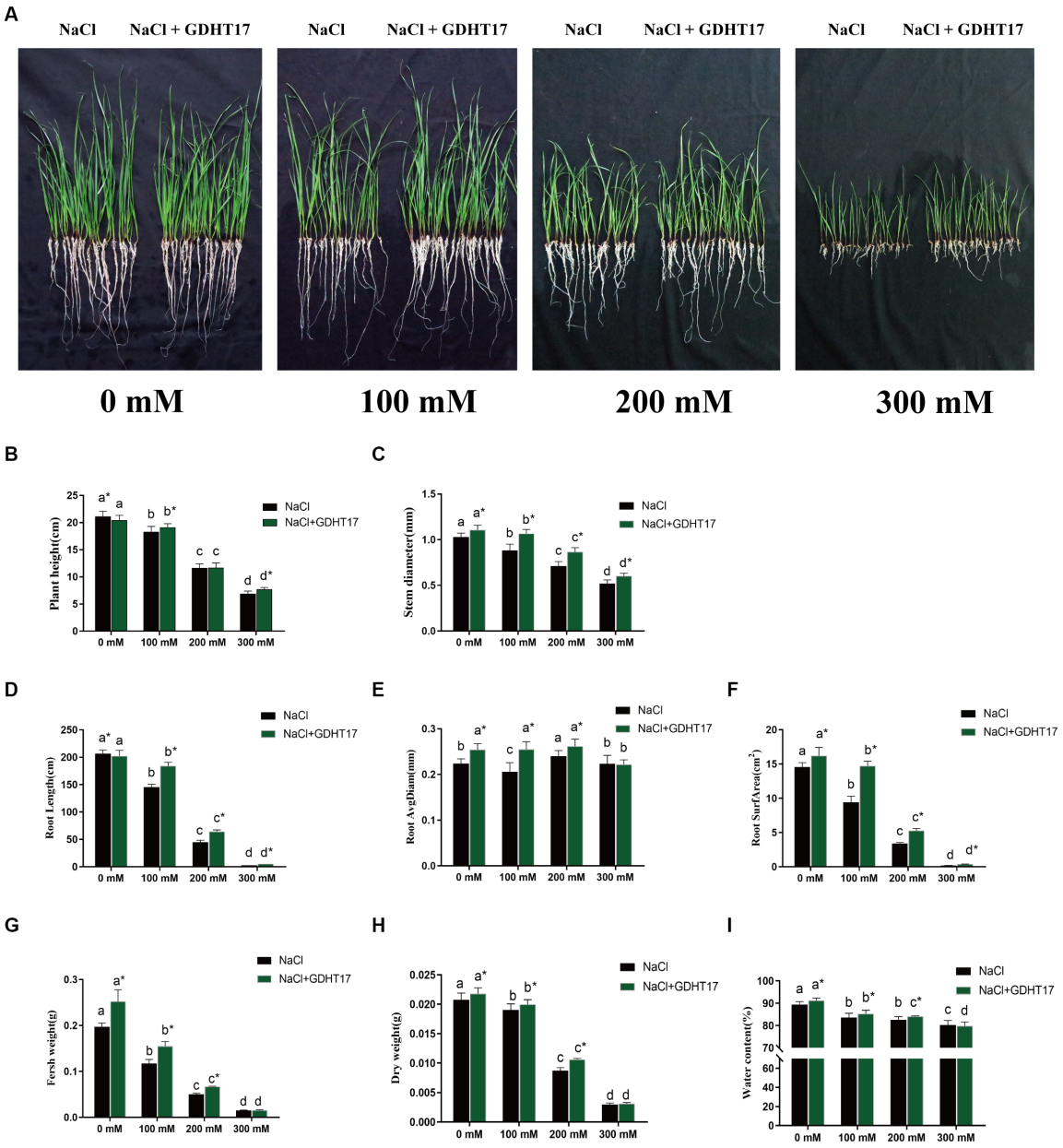

FIGURE 4
Inoculation of *G. dipsosauri* GDHT17 Affected Stressor. **(A)** Growth of ryegrass seedlings in non and inoculated with GDHT17 Hoagland nutrient solutions when treated with 0 mM, 100 mM, 200 mM, and 300 mM NaCl; Effect of inoculation with GDHT17 on the growth and biomass of ryegrass seedlings. **(B)** Seedling height, **(C)** stem diameter, **(D)** total root length, **(E)** root diameter, **(F)** root surface area, **(G)** fresh weight, **(H)** dry weight, **(I)** water content. Bar diagrams represent the mean of 20 replicates ($n = 20$). Error bars indicate the standard deviation (SD) of the 20 replicates. Different lowercase letters mean NaCl or NaCl+GDHT17; there were significant differences when different concentrations of NaCl treatments ($p < 0.05$). Bars diagrams marked with "*" indicate significant differences between NaCl and NaCl+GDHT17 treatment in the same NaCl concentration ($p < 0.05$).

that contributes to various degrees of salinization (Bian et al., 2021). The benefit of *G. dipsosauri* GDHT17 with alleviating salt-stress damage properties on ryegrass verified the challenges to obtaining such bacteria from those salinization habits. It also demonstrated the opportunities of applying these bacteria in salinization soils for soil amelioration.

Bacteria of *Gracilibacilluss* genus are moderately or heavily salinophilic (Vreeland et al., 2000; Fendrihan et al., 2006; Egamberdieva et al., 2019). *Gracilibacillus* formerly belonged to the genus *Bacillus* and was labeled as a salt stress-adapted, endospore-forming bacterium because of its specific physiological properties (Lawson et al., 1996; Vreeland et al., 2000). The genus now includes eight species with validly published names: *G. halotolerans*, *G. dipsosauri*, *G. orientalis*, *G. lacisalsi*, *G. saliphilus*, *G. boraciitolerans*, *G. halophilush*, and *G. quinghaiensi* (Carrasco et al., 2006; Ahmed et al., 2007; Chen et al., 2008; Jeon et al., 2008; Tang et al., 2009). Depending on the optimal salt requirement for growth, bacteria are classified as-slight (1.99–4.97% NaCl), moderate (4.97–19.89%), and extreme halophiles (19.89–29.84% NaCl) (Abaramak et al., 2020). These bacteria of the *Gracilibacilluss* genus are optimal salt growth at

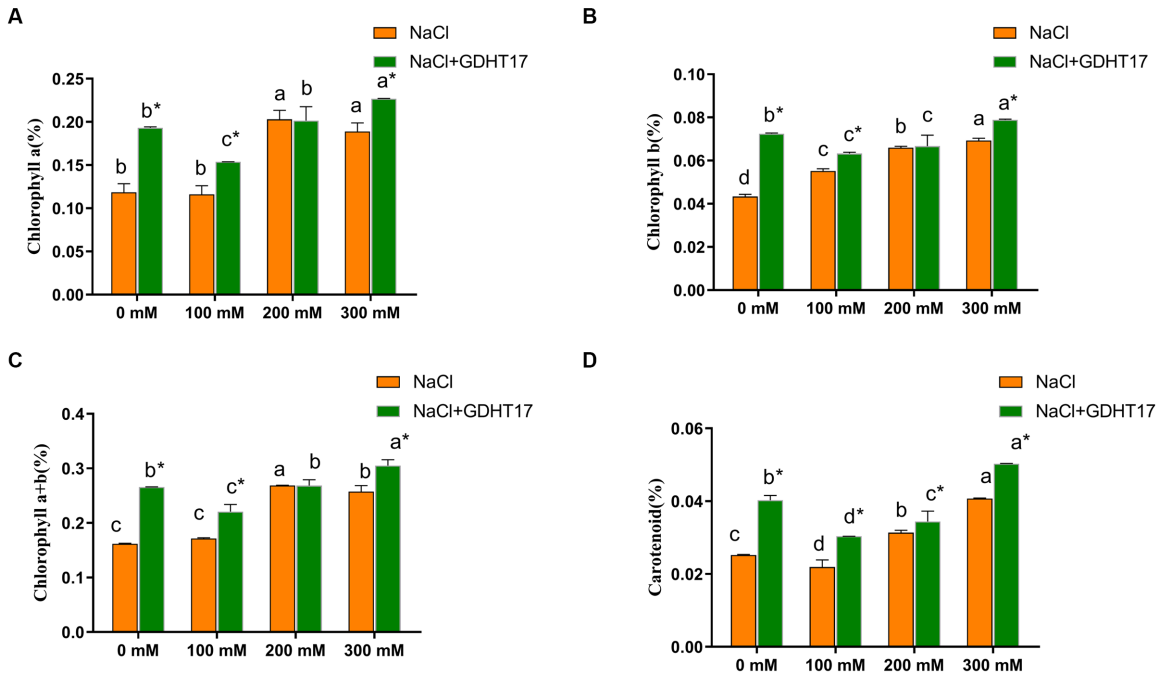

FIGURE 5
Inoculation of *G. dipsosauriuri* GDHT17 the chlorophyll content of NaCl-treated ryegrass seedlings. **(A)** Chlorophyll a content, **(B)** chlorophyll b content, **(C)** chlorophyll total content, **(D)** carotenoid content. Bar diagrams represent the mean of three replicates ($n = 3$). Error bars indicate the standard deviation (SD) of the three replicates. Different lowercase letters mean NaCl or NaCl+GDHT17; there were significant differences when different concentrations of NaCl treatments ($p < 0.05$). Bars diagrams marked with "*" indicate significant differences between NaCl and NaCl+GDHT17 treatment in the same NaCl concentration ($p < 0.05$).

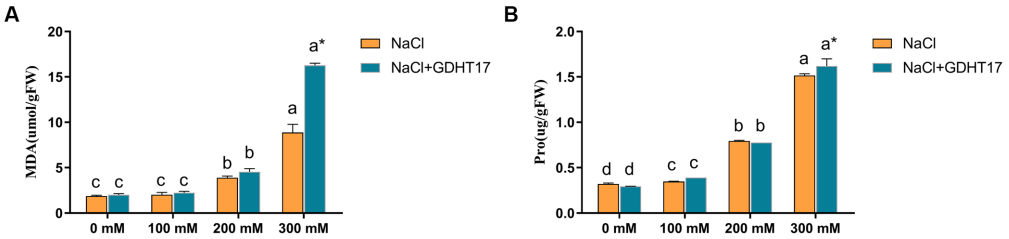

FIGURE 6
Inoculation of *G. dipsosauri* GDHT17 affected stressor metabolites of NaCl-treated ryegrass seedlings. **(A)** Malondialdehyde (MDA), **(B)** proline (pro). Bar diagrams represent the mean of three replicates ($n = 3$). Error bars indicate the standard deviation (SD) of the three replicates. Different lowercase letters mean NaCl or NaCl+GDHT17; there were significant differences when different concentrations of NaCl treatments ($p < 0.05$). Bars diagrams marked with "*" indicate significant differences between NaCl and NaCl+GDHT17 treatment in the same NaCl concentration ($p < 0.05$).

5 to 20% NaCl concentration and are moderately or severely halophiles. *G. dipsosauri* GDHT17 is suitable for growth at 5% NaCl concentration, so it is considered a moderate halophilic bacterium. In the study, the potential of *G. dipsosauri* GDHT17 for promoting plant growth and for alleviating salt stress has been confirmed. It brings up prospective agents as bio-inoculants in the future. Moreover, we also carried out a preliminary identification of 159 isolates by 16srRNA analysis isolated from the saline soil samples collected from the Yellow River Delta (data not presented in this study). Most of which belonged to *Bacillus,* followed by *Pseudomonas*. These isolates collectively constitute the rich microbial resources in saline soils. Some of them probably are worth further study for exporting microorganisms for plant salt tolerance.

In this study, we suggested that *G. dipsosauriuri* GDHT17 positively affected the growth and biomass of NaCl-treated ryegrass. Inoculating GDHT17 can significantly promote ryegrass's seedling height and stem diameter at different salt concentrations. The root length, diameter, and surface area of ryegrass seedlings also increased when the seedlings were inoculated with GDHT17. Research has suggested that salt stress mainly inhibits photosynthesis by reducing chlorophyll content and affecting photosystem II efficiency (Kalaji et al., 2011; Ma et al., 2012; Han et al., 2014). In this study, we found that the salt stress increased the chlorophyll content in ryegrass, while GDHT17 furtherly increased the chlorophyll content. The results suggest that the more effective increasing effect of GDHT17 on

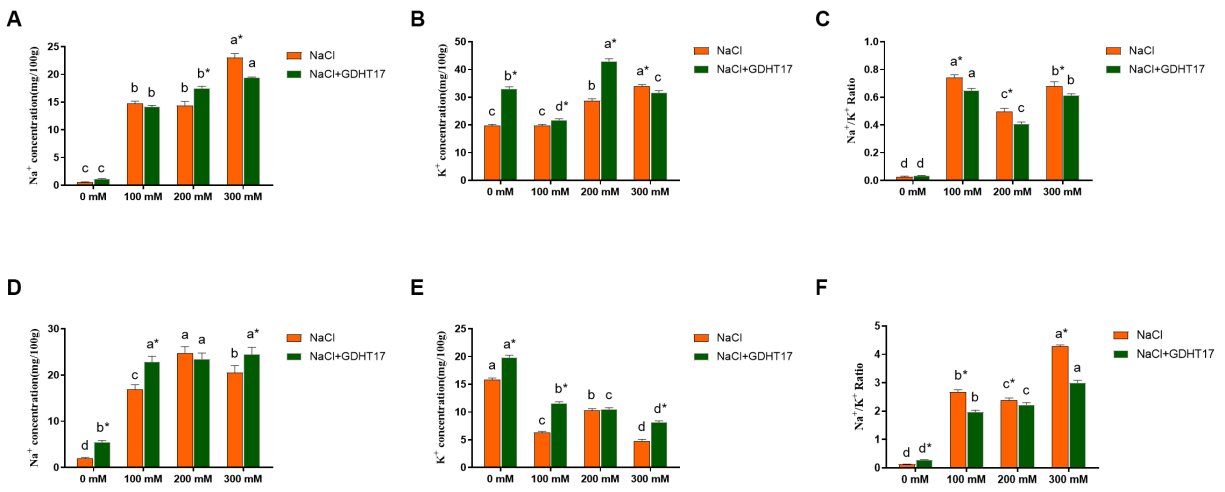

FIGURE 7
Inoculation of *G. dipsosauri* GDHT17 Affected Ion absorption in ryegrass seedlings. **(A)** Na^+ content in leaves, **(B)** K^+ content in leaves, **(C)** Na^+/K^+ ratio in leaves, **(D)** Na^+ content in roots, **(E)** K^+ content in roots, **(F)** Na^+/K^+ ratio in roots. Bar diagrams represent the mean of three replicates ($n = 3$). Error bars indicate the standard deviation (SD) of the three replicates. Different lowercase letters mean NaCl or NaCl+GDHT17; there were significant differences when treated with different concentrations of NaCl ($p < 0.05$). Bars diagrams marked with "*" indicate significant differences between NaCl and NaCl+GDHT17 treatment in the same NaCl concentration ($p < 0.05$).

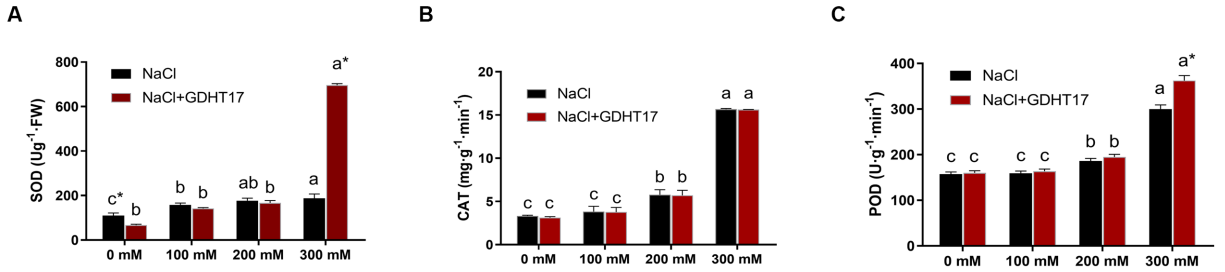

FIGURE 8
Inoculation of *G. dipsosauri* GDHT17 affected antioxidant defense enzymes of NaCl-treated ryegrass seedlings. **(A)** superoxide dismutase (SOD), **(B)** catalase (CAT), and **(C)** peroxidase (POD). Bar diagrams represent the mean of three replicates ($n = 3$). Error bars indicate the standard deviation (SD) of the three replicates. Different lowercase letters mean NaCl or NaCl+GDHT17; there were significant differences when treated with different concentrations of NaCl ($p < 0.05$). Bars diagrams marked with "*" indicate significant differences between NaCl and NaCl+GDHT17 treatment in the same NaCl concentration ($p < 0.05$).

the chlorophyll content under salt stress, indicating the impact on the ryegrass copes with salt stress.

Proline is a stress molecule that protects organelles indicating protein damage and cell membranes from the adverse effects of increased concentrations of salts (Ashraf and Foolad, 2007). The increased production of Pro facilitates the maintenance of the balance of osmotic potential inside and outside the cell, thus increasing water intake and reducing plant damage due to salt stress (Islam et al., 2016; Shahid et al., 2022). Salinity-induced oxidative stress is often measured using lipid peroxidation, and malondialdehyde is a prevalent end product of lipid peroxidation (Villalobos-López et al., 2022). The results of the study also showed a significant increase in the contents of MDA and Pro in the ryegrass seedlings treated with salt stress of 200 mM and 300 mM NaCl, demonstrating salt damage caused by salt stress and a self-reducing damage mechanism of ryegrass seedlings. Inoculation with GDHT17 significantly increased the contents of MDA and Pro by 83.50 and 6.87% at the salt stress of 300 mM NaCl, indicating *G. dipsosauri* GDHT17 possesses an apparent function of alleviating the NaCl-induced toxicity on ryegrass by improving Pro and MDA content in ryegrass.

Plants exposed to salt stress tend to take up less Na^+ and more K^+, re-establishing ionic homeostasis (Zhu, 2001; Hu et al., 2012). Maintaining an appropriate Na^+/K^+ balance is considered an essential mechanism for plants to cope with salt stress (Mahajan and Tuteja, 2005; Evelin et al., 2009). In this study, salt stress caused a significant increase of Na^+/K^+ ratio in ryegrass seedling leaves and roots. Conversely, the Na^+/K^+ ratio significantly decreased in the ryegrass seedling leaves and roots inoculated with GDHT17. Some studies also demonstrated that the beneficial bacteria could reduce the Na^+ uptake of plants, which helps to maintain a low Na^+ /K^+ ratio in the plants (Ashraf et al., 2004; Zhang et al., 2008; Dodd and Perez-Alfocea, 2012; Ali et al., 2019). Therefore, *G. dipsosauri* GDHT17 contributes to the alleviating effect of salt stress on ryegrass by maintaining an appropriate Na^+/K^+ balance.

Salt stress is usually associated with oxidative damage of reactive oxygen production. The accumulation of ROS in plant tissues leads to

cell membrane damage as well as the oxidation of biomolecules (Li et al., 2019; Hasanuzzaman et al., 2020). Salt-induced ROS production in plants is counteracted by many enzyme scavengers, such as POD, CAT, SOD, or other antioxidants (Waller et al., 2005; Gururani et al., 2013; Shahid et al., 2022). As the concentration of NaCl in plants increases, the activity of antioxidant enzymes increases (Numan et al., 2018; Mubeen et al., 2022). Inoculating GDHT17 resulted in a more evident effect of promoting the activities of the antioxidant defense enzymes in ryegrass seedlings, and the SOD activity especially showed a dramatic increase of 250.79% under the salt stress of 300 mM NaCl. Our research suggests that *G. dipsosauri* GDHT17 plays a critical protective role in modulating the antioxidant defense enzymes of NaCl-treated ryeegrass, which counteracts oxidative damage and contributes to the salt tolerance of ryegrass.

5. Conclusion

In the study, we obtained a moderately halophilic bacteria *G. dipsosauri* GDHT17, from saline habitats in the Yellow River Delta, where severe land salinization is marked. We explored and verified the properties of the GDHT17 to alleviate the salt stress on perennial ryegrass. The results demonstrated that under salt stress conditions, GDHT17 benefits ryegrass growth, increases ryegrass biomass and water content, and increases chlorophyll and carotenoid content. *G. dipsosauri* GDHT17 possesses an apparent function of alleviating the NaCl-induced damage on ryegrass by improving Pro and MDA content in ryegrass, by maintaining an appropriate Na^+/K^+ balance and by modulating the antioxidant defense enzymes of NaCl-treated ryegrass to counteract oxidative damage, so that contributes to the salt tolerance of ryegrass. Therefore, we highlight the great potential of *G. dipsosauri* GDHT17 in protecting plants from salt stress.

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

XL, JZ, and ZL contributed to conception and design of the study. XL and ZG organized the database. WW performed the statistical analysis. XL wrote the first draft of the manuscript. ZL wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1213884/full#supplementary-material

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EDITED BY
Priyanka Chandra,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Susanta Banik,
Nagaland University, India
Rahul Kumar Tiwari,
Central Potato Research Institute (ICAR), India
Durga Prasad Awasthi,
College of Agriculture, Tripura, India

*CORRESPONDENCE
Bireswar Sinha
✉ Bireswarsinha@gmail.com

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Deciphering growth abilities of *fusarium oxysporum* f. sp. *pisi* under variable temperature, pH and nitrogen

Kota Chakrapani[1], W. Tampakleima Chanu[1], Bireswar Sinha[1]*, Bijeeta Thangjam[1], Wajid Hasan[2], Konjengbam Sarda Devi[1], Tusi Chakma[1], Sumitra Phurailatpam[1], Lokesh Kumar Mishra[3], Gopi Mohan Singh[4], Pramesh Khoyumthem[5] and Rahul Saini[6]

[1]Department of Plant Pathology, College of Agriculture, Central Agricultural University, Imphal, India, [2]KVK, Jahanabad, Bihar Agricultural University, Jahanabad, India, [3]Department of BPME, College of Agriculture, Central Agricultural University, Imphal, India, [4]Department of Agricultural Statistics, College of Agriculture, Central Agricultural University, Imphal, India, [5]Department of Genetics and Plant Breeding, AICRP (Groundnut), College of Agriculture, Central Agricultural University, Imphal, India, [6]Department of Entomology, College of Agriculture, Chaudhary Charan Singh Haryana Agricultural University, Hisar, India

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *pisi* (*Fop*) is an important disease and major obstacle to pea production, causing huge losses to growers. The focus of this study was on isolation followed by morphological, molecular characterization and analyzing the growth of the casual agent under variable temperature, pH and Nitrogen levels. The morphological features of radial growth, sporulation, pigmentation and mycelial characterization were examined and the variability of all isolates was presented. Molecular characterization of the fungus by ITS rDNA sequencing revealed that all 13 isolates belong to *Fusarium oxysporum* species. Six isolates were tested for temperature, pH and nitrogen dosage optimization studies. Seven different temperatures, viz., 21, 23, 25, 27, 29, 31, 33°C and pH values, having 3, 4, 5, 6, 7, 8, and 9 pH, as well as nitrogen dosage levels of 0 g, 3 g, 5 g, 7 g, 9 g, 11 g, and 13 g were tested against all six isolates, respectively. The results showed that all isolates exhibited the highest growth at a temperature of 25°C and the optimal temperature range for growth of *Fusarium oxysporum* was 23–27°C. All isolates showed the highest growth at pH5. Change in the nitrogen doses of the base ended in formation of thick, dense, fluffy mycelium of the casual agent. Six isolates were used for combination studies with seven different levels of temperatures, pH levels and nitrogen dosages. The density plots revealed the variations in the growth of the isolates with changes in temperature, pH and nitrogen levels, which can lead to mutations or genetic changes in the pathogens that could potentially introduce new threats to pea cultivation.

KEYWORDS

wilt, *Fusarium oxysporum* (Fop) isolates, climate change, growth, temperature, pH, nitrogen dose

Introduction

Garden pea (*Pisum sativum* L.) is a popular winter legume commonly used as food for humans and animal feed. Pea seeds are a rich source of carbohydrates (56–74%), proteins (21–33%), and minerals like iron (97%), selenium (42%), molybdenum (12%), and zinc (41%) (Parihar et al., 2020, 2021, 2022). All of these nutrient components in peas are a key ingredient for national food security that can be fed to people. In addition, pea seeds are able to minimize the risk of various chronic diseases such as diabetes, maintain blood cholesterol levels, and promote cardiovascular health as they have significant amounts of antioxidants, Vitamin (A, B, E, and K), omega fatty acids and anti-inflammatory agents (Ekvall et al., 2006; Marinangeli and Jones, 2011; Singh et al., 2013). Further, the role of Garden pea as an important crop rotation promoting sustainable cropping system due to their inherent ability to fix nitrogen leading to balanced soil health and productivity is widely acknowledged (Sharma et al., 2020). According to the Food and Agriculture Organization (FAO, Food and Agriculture Organization, 2020), the global statistics for green pea and dry pea production in 2020 are 49.7 megatons and 47.2 megatons, respectively. The Indian subcontinent contributes to the bulk of pea production globally and ranks second to China in green pea production at 5.73 mega tonnes. In terms of dried pea production, India ranks fifth after Russia with 0.8 mega tonnes. It is estimated that the world population could reach 10 billion by 2050 and the production of nutritious food should be ensured to continue to feed the growing population despite the reduction in arable land. In addition, increasing climate changes and already vulnerable industrial systems are causing severe stress on farmland, public health, and the ecosystem (Anonymous, 2019).

The major barricade in hampering Garden pea production is biotic stress with the soil borne diseases being the most notable factor posing threat to pea farming across the world. Fusarium wilt (FW) caused by *Fusarium oxysporum* f. sp. *pisi* (*Fop*) is one of the major biotic stress that leads to continuous and severe yield losses. Buxton and Storey (1954) reported the emergence of pea wilt in Britain as a serious problem and found an association with Fusarium species. *Fop* is a soil-borne fungus that includes both non-pathogenic strains and economically important plant pathogens. It has been known in the United States since 1928. In India, the disease was first reported by Sukapure et al. (1957) from Bombay. Chupps and Sherf (1960) first recognized Fusarium wilt of peas in Minnesota in 1918. Pea wilt disease was first elucidated and distinguished from root rot by Jones and Linford (1925). They designated it as undescribed wilt disease. The disease caught on and was found in 50 fields in Wisconsin, causing huge losses to pea growers in the area compared to root rot disease. The causative agent of the disease was named as Fothoceras App and Wr var. *pisi* in 1928 (Linford, 1928). Over time, the name race 1 of *Fusarium oxysporum* Schl f.sp. Due to the characterization, *pisi* (van Hall) was named as the causal factor. Snyder and Hansen (1940) reported that the genus *Fusarium* belongs to the class Fungi *imperfecti*, which includes many species and many forms within species. Based on host interactions, Kraft (1994) classified numerous isolates of *Fusarium oxysporum* f.sp. *pisi* and found that it is controlled by the genetic makeup of both the host and the pathogen. The fungus protrudes the root tissue and invades the xylem vessels of the vascular bundles, clogging the bundles and restricting the movement of water minerals to the top of the plant. This leads to yellowing and drying symptoms of the infected plant. If the invasion occurs in earlier stages of the plant, the fungus will kill the plant. When invasion occurs at later stages, kernel shrinkage and loss of high yields can be visualized (Linford, 1928). The fungus enters plant tissues either through wounds or by direct penetration of the epidermis (Nyvall and Haglund, 1972). Armstrong and Armstrong (1974) revealed their findings that when the fungus invades wounds, the frequency of symptom onset and the rate of wilting increase. *Fusarium solani* f.sp. *pisi* and *Fusarium oxysporum* f.sp. *pisi* infects the root, invades the xylem vessels and becomes associated with the plants and seeds (Maheshwari et al., 1980). Lin (1991) reported that wilt caused by *Fusarium oxysporum* f.sp. *pisi* that appear in the flowering phase can be accompanied by yellowing of the leaves and discoloration.

The yield-limiting factor in form of "*Fop*" can result in 100% loss of crop (Aslam et al., 2019; El-Sharkawy et al., 2021; Deng et al., 2022). When the pea crop is included in crop rotation, it paves the way for *Fop* to build up the inoculum in the soil. The extraordinary nature of chlamydospores ensures that the pathogen lives and is viable in the soil for more than 10 years (Kraft et al., 1994; Aslam et al., 2019; El-Sharkawy et al., 2021; Deng et al., 2022). Previous records show that four races of *Fop* have been identified and characterized as races 1, 2, 5, and 6, respectively (Haglund and Kraft, 1970, 1979; Jenkins et al., 2021). Strains demonstrate resistance to *Fop* by having a single dominant gene (one for each race) for races 1, 5, and 6, whereas race 2 resistance has been identified as quantitative, exaggerating the severity (McPhee et al., 2012; Bani et al., 2018). To deal with the consequences, the focus should be on researching intelligent technologies in agriculture and adapting the country to the climate crisis and food security. In this context, we want to better understand the pathogen and its response to harsh climatic conditions. Our aim is to study the optimization conditions and combination effects of climatic factors on the growth of *Fop* isolates.

Being a part of Disease cycle, Abiotic factors, i.e., environmental factors, play a key role in disease incidence and severity. Evidence on interaction between temperature, pH and nitrogen levels on radial growth of the *Fusarium* spp. has been reported (Jones, 1924; Chen et al., 2013). Temperature has the ability of altering the expression of host defence mechanisms that favors resistance or susceptible. Banana, chickpea, carnation and lettuce had significant changes in their host defence mechanism from moderately resistant to susceptible when inoculated with their respective *Fusarium* spp. when the temperature raised above ~22–28°C. (Harling et al., 1988; Brake et al., 1995; Landa et al., 2006; Stuthman et al., 2007; Scott et al., 2010). Similarly pH of the soil has the ability to alter *Fusarium* spp. with respect to growth, spore adherence to plant roots ad appearance of wilt symptoms on host exposed to different temperatures (Chen et al., 2013; Gatch and du Toit, 2017).

Materials and methods

Pathogen isolation and characterization

The pea plants showing typical wilting symptoms were collected from the main pea growing areas of Manipur. The infected plant roots were examined for vessel discoloration. The roots, showing the typical vessel discoloration, were rinsed with tap water and torn into 4–5 mm sections. The sections were then subjected to surface sterilization with 2% sodium hypochlorite solution for 1 min. The

sections were then transferred to sterile distilled water to delineate traces of sodium hypochlorite. Washing with sterile distilled water was repeated three times to ensure tissue sections were free of sterilizing chemicals. The sterilized tissues were blotted with blotting paper to remove the excess water. Using sterile forceps, sections were plated onto water agar plates and incubated at 25 ± 1°C for 48 to 72 h. Colonies resembling *Fusarium oxysporum* were subcultured using the hyphal tip culture method to obtain the pure form of the isolate. The pure cultures were stored at −20°C for future use.

Morphological characterization of the isolates was done by inverting 5 mm disc of 7 days old culture on PDA plates. Sporulation studies carried out by taking 5 mm disc of the culture plate and placing the disc in 10 mL sterile distilled water. The spores were dislodged into water by continuous shaking. A drop of this spore suspension was placed on to a haemocytometer/ Neubauer Chamber and the spore count was taken from 5 squares at random. The spore count per ml was calculated by using a formula given by Pathak (1984).

$$\text{Number of spores per mL} = \frac{N * 1000}{\text{X}}$$

where:

N = Total No. of spores counted/No. of squares,

X = Volume of mounting solution between the cover glass and above the squares counted.

The spore morphology and size was determined by placing a drop of spore suspension of each isolate onto a clean glass slide and covered with a cover slip. The slides were observed under 40X magnification of the microscope (Olympus BX41). Size of the sopre was determined by using ocular and stage micrometers with the help of Biowizard 4.2 image analyzer.

All isolates were pure and were subcultured onto PDA plates for 7 days. The 7-day-old cultures were then subcultured in PDB containing no agar to obtain a mycelial mat. The mycelial mat was collected after 7 days of incubation. Then the mycelial mats of each isolate were harvested for extraction of total genomic DNA using HiPure ATM Fungal DNA Purification Kit (Hi Media, India). The genomic DNA was extracted according to the manufacturer's protocol. PCR amplifications were performed using primers ITS-1 (5 TCC GTA GGT GAA CCT GCC G 3) and ITS-4 (5 TCC TCC GCT TAT TGA TAT GC 3). The PCR products obtained by amplification with universal primers targeting the rRNA gene were sent to Xcelris Labs Ltd., Ahmadabad, Gujarat, India for sequencing using the same upstream and downstream primers. The sequenced data were analyzed in NCBI BLAST to identify the species of all isolates.

Optimization studies to evaluate the growth of different *Fop* isolates at different temperatures and different pH levels

The culture media PDA was prepared according to standard protocols. Seven different temperatures viz., 21, 23, 25, 27, 29, 31 and 33°C were used for optimization studies in which the growth of six *Fop* isolates was compared and analyzed. The culture media were then subjected to sterilization according to sterilization standards. The media were dispensed into petri dishes in an aseptic environment. The petri dishes were then inoculated with 5 mm pieces of 7 day old cultures of each isolate. The inoculated petri dishes were subjected to incubation in BOD at their respective temperatures. Growth of the isolates was noted after 7 days of inoculation. Three replicates were performed for each isolate at each temperature. Similarly, the PDA, culture medium was prepared following the standard protocols. Seven different pH values viz., 3, 4, 5, 6, 7, 8, and 9 were used for the optimization studies, in which the growth of six *Fop* isolates was compared and analyzed. The pH of the culture medium was changed by 0.1 N hydrochloric acid and 0.1 N sodium hydroxide with the digital pH meter (EuTech Instruments, pH700, Thermo Fisher, Scientific, United States). The culture medium was then subjected to sterilization according to sterilization standards. The medium was delivered to Petriplates under aseptic environment. The petri dishes were then inoculated with 5 mm pieces of 7 day old cultures of each isolate. The inoculated Petri dishes were subjected to incubation in BOD at a temperature of 25 + 1°C. The growth of the isolates was noted after 7 days of inoculation. Three replicates for each isolate at each pH were maintained.

Combination effect of temperature, pH and nitrogen dose levels on radial growth on six isolates of *Fop*

To study the combined effect of different pH and different temperature levels on the growth of *Fop* isolates, a pH range of 3–9, i.e., 3, 4, 5, 6, 7, 8, and 9, and a temperature range of 21–33°C, i.e., 21, 23, 25, 27, 29, 31, 33°C and nitrogen levels of 0–13 g, i.e., 0, 3.5, 7, 9, 11, and 13 g each were used in different combinations. The pH and temperature range was calibrated using the optimal pH and temperature, determined from previous studies. To assess radial growth study of each isolate, culture medium PDA was prepared according to standard protocols. The pH of the culture medium was changed by 0.1 N hydrochloric acid and 0.1 N sodium hydroxide with the digital pH meter used (EuTech Instruments, pH700, Thermo Fisher, Scientific, United States). The culture media of individual pH was prepared separately. The culture media were then subjected to sterilization according to sterilization standards. The media were dispensed into petri dishes in an aseptic environment. The petri dishes were then inoculated with 5 mm pieces of 7 day old cultures of each isolate. The inoculated petri dishes were subjected to incubation in BOD at specific temperatures, i.e., 21, 23, 25, 27, 29, 31, and 33°C. Growth of the isolates was noted after 7 days of inoculation. Three replicates were performed for each isolate at each pH and temperature. Similarly, the culture medium PDA was prepared, to which a nitrogen source (peptone) was added as per the requisite viz., peptone powder (RM001-Hi media) was weighed accordingly using a weighing balance (Precession weighing balance KERN 572) i.e., 3. 5, 7, 9, 11, and 13 g and was suspended in a litre of PDA. The prepared media was subjected for sterilization. The isolates were inoculated according to the protocol mentioned in the above step. Three replications were performed for each isolate independently.

Data analysis

Completely randomized design and a three-factorial randomized block design were used to analyze the data generated. For the sake of simplicity, the means of the raw data have been presented in the form of numbers. However, R Studio was used to analyze the data and

create density plots. The significance of the differences between the means was determined by mean comparison tests.

Results

Pathogen isolation and characterization

A total of 13 isolates were isolated from samples showing typical wilt symptoms collected from different locations of Manipur. Isolation was performed at the Department of Plant Pathology, College of Agriculture, Central Agricultural University, Imphal. The isolates were designated *Fop*-1, *Fop*-2, *Fop*-3, *Fop*-4, *Fop*-5, *Fop*-6, *Fop*-7, *Fop*-8, *Fop*-9, *Fop*-10, *Fop*-11, *Fop* – 12 or *Fop*-13. The details of the isolates collected are presented in Table 1.

Morphological and molecular characterization of the isolates

Radial growth, sporulation characterization of *Fop* isolates

Estimation of radial growth of the isolates was performed on PDA plates, maximum radial growth was record isolate *Fop*-1 being 16.50 mm (24 h inoculation), 30.00 mm (48 h inoculation), 44.17 mm (72 h inoculation) was found (inoculation), 60.50 mm (96 h inoculation) and 78.00 mm (120 h inoculation). After 144 h of inoculation, it covered the entire plate (90.00 mm), considering it a fast growing isolate compared to the other isolates. Isolate *Fop*-6 recorded the smallest growth with 33.33 mm (72 h inoculation), 45.33 mm (96 h inoculation), 55.67 mm (120 h inoculation), 66.33 mm (144 h inoculation) and 78.33 mm (144 h vaccination). Significant variation between isolates was observed at certain time intervals. The growth rate of all isolates is shown in Figure 1. The *Fop* isolates showed huge variation in mycelial color. The color pattern ranged from off-white, matte white, and white gradually changing with different pigments, such as white to light pink, white to light purple, light pink to dark pink, purple with white paint, and light brown. The topography of the isolates ranged from thick to thin with less or slightly cottony mycelial growth as shown in Table 2. The pigmentation on the back ranged from light purple to dark purple, from light pink to deep pink and red. The marginal mycelial growth pattern ranged from regular to irregular. Formation of zones and the texture of the isolates are also shown in Table 2.

Fop isolates can typically be characterized by their spores (Plates 1a,1b). *Fop* isolates tend to form micro, macro and chlamydospores. The characterization of *Fop* isolates for conidia is presented in Table 3. It lists the spore count, septum count, size and shape. All isolates showed obvious differences in spore number, septum color, size and shape.

Molecular characterization of *Fop* isolates

Molecular identification of *Fop* isolates was performed by extraction of total genomic DNA, PCR amplification using the universal primers ITS1 and ITS4 primers. Approximately 564 nucleotides were amplified by PCR and confirmed by 1.2% agarose gel electrophoresis (Plate 2). The ITS region sequence of 13 isolates of *Fusarium* species has been submitted to the NCBI and the isolates have been assigned accession numbers, namely (MH578602), (MH578602), (MH578603), (MH578604), (MH578605), (MH578606), (MH578607), (MH578608), (MH578609), (MH578610), (MH578611), (MH578612), (MH578613) and (MH578614). The ITS sequence of the isolates was searched for their homologous sequences in publicly available databases and analyzed with the nucleotide BLAST software. Molecular Evolutionary Genetics Analysis (MEGA 7) software was used for phylogenetic analysis. The obtained nucleotide sequences were assembled and aligned using the ClustalW integrated in MEGA 7, and the phylogenetic tree was constructed using the neighbor-joining method. The evolutionary history was inferred using the neighbor-joining method performed in MEGA7 (Figure 2). The bootstrap consensus tree derived from 1,000 replicates was used to represent the evolutionary history of the *Fop*

TABLE 1 Location specificities of samples collected for isolation of the casual pathogen.

Sl. No.	Isolates	Place/Site	Latitude (N)	Longitude (E)	Altitude (ft)
1	*Fop*-1	Kongba	24° 46.071'	93° 57.578'	2,533
2	*Fop*-2	Wangjing Lamding	24° 36.159'	94° 02.206'	2,525
3	*Fop*-3	Kangla Sangomshang	24° 50.859'	93° 59.531'	2,566
4	*Fop*-4	Hiyangthang	24° 40.314'	93° 55.021'	2,536
5	*Fop*-5	Itam Nungoi	24° 52.202'	94° 01.674'	2,579
6	*Fop*-6	Toubul	24° 37.638'	93° 46.068'	2,584
7	*Fop*-7	Iramsiphei Mayai Leikai	24° 39.779'	93° 55.605'	2,534
8	*Fop*-8	Awangkhul	24° 48.806'	93° 51.863'	2,549
9	*Fop*-9	Nambol Phoijing	24° 43.783'	93° 50.764'	2,508
10	*Fop*-10	Lungphou Dam	24° 35.528'	94° 01.233'	2,516
11	*Fop*-11	Lamding Khumnthem	24° 35.528'	94° 01.485'	2,529
12	*Fop*-12	Utlou Lairem Leikai	24° 43.820'	93° 51.613'	2,517
13	*Fop*-13	Maibam Chingmang	24° 42.599'	93° 49.378'	2,525

isolates from Manipur. Analysis included 19 nucleotide sequences using 13 isolates of *F. oxysporum* from Manipur and 6 isolates from a gene bank from Brazil, Algeria, Pakistan, the United States, Portugal and China and other parts of the world. The tree has two separate groups, one consisting of isolate Mnp31UL and the rest of the isolates were found in another group, showing that isolate Mnp31UL was the most diverse among all isolates compared. In the present study, isolates Mnp2KS and Mnp29YD, isolates Mnp37TO and Mnp30LD, and isolates Mnp32MC and Mnp24WL were each found to belong to one group, showing a highly related ancestry (Plates 3, 4).

Optimization studies to evaluate the growth of different *Fop* isolates at different temperatures and different pH levels

The test to achieve optimized pH conditions for the growth of *Fop* isolates gave significant results. *Fop* isolates were grown on PDA medium whose pH was changed within the pH range tested against the growth of six *Fop* isolates; pH5 gave the best growth results when the radial growth of all isolates after 7 days of inoculation was a maximum of 9 cm and covered the entire plate. The smallest radial growth of all

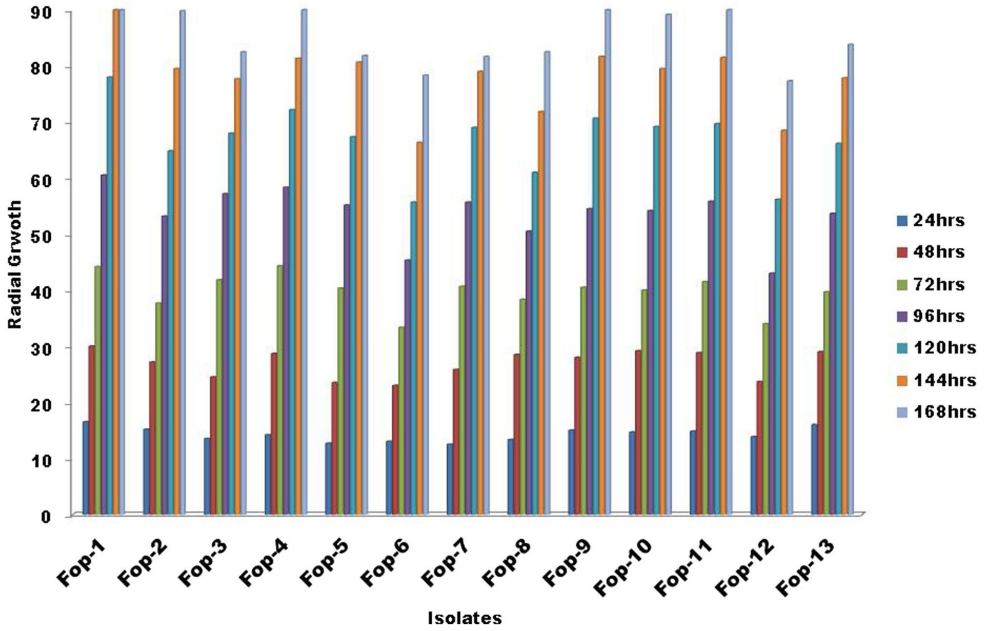

FIGURE 1
Radial growth of *Fop* isolates at specific intervals of time.

TABLE 2 Cultural characters of *Fop* isolates.

Isolates	Mycelial color	Topography	Pigmentation (Rear side)	Type of margin	Concentric Zones	Texture
Fop-1	Light purple with white color	Thick mycelium with cottony growth	Light purple	Regular	Present	Fluffy
Fop-2	Light pink	Thin mycelium with cottony growth	Dark pink	Regular	Absent	Slightly fluffy
Fop-3	Light purple with white color	Thin mycelium with less cottony growth	Dark purple	Irregular	Absent	Fluffy
Fop-4	White	Thick mycelium with cottony growth	Dark pink	Regular	Absent	Fluffy
Fop-5	White	Thick mycelium with cottony growth	Light purple	Regular	Absent	Fluffy
Fop-6	Purple with white color	Thick mycelium with slightly cottony growth	Dark purple	Irregular	Absent	Slightly fluffy
Fop-7	Off white	Thin mycelium with less cottony growth	Deep red	Regular	Present	Fluffy
Fop-8	Light purple with white color	Thick mycelium with less cottony growth	Dark purple	Irregular	Absent	Fluffy
Fop-9	Pink and white color	Thick mycelium with cottony growth	Purple	Regular	Present	Fluffy
Fop-10	Dull white	Thin mycelium with cottony growth	Light pink	Regular	Absent	Fluffy
Fop-11	The white color in the middle and purple at edges	Thick mycelium with less cottony growth	Dark purple	Irregular	Absent	Fluffy
Fop-12	Light pink with light red color	Thick mycelium with cottony growth	Deep red	Regular	Absent	Fluffy
Fop-13	Light pink	Thin mycelium with less cottony growth	Dark purple	Regular	Present	Slightly fluffy

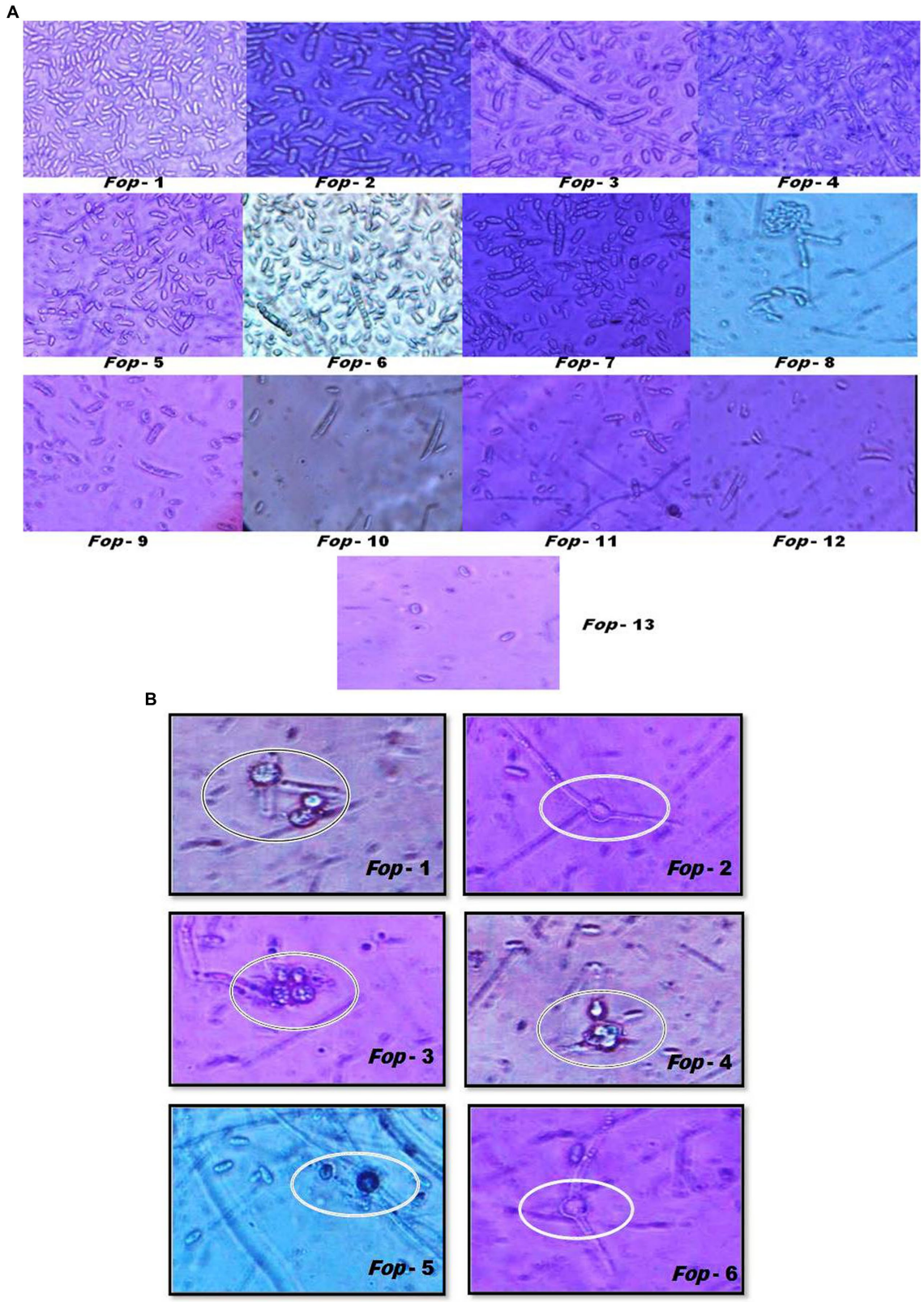

PLATE 1
(A) Sporulation (Microconidia and Macroconidia) and their characterization of different *Fop* isolates (40X magnification). **(B)** Sporulation (Chamydospores) and their characterization of different *Fop* isolates (40X magnification).

isolates was recorded at pH3. Radial growth of all isolates decreased and was limited to 2.5–3 cm in isolates, making a pH of 3 unsuitable. pH values of 4, 6, 7, 8, 9 showed growth of 7–9 cm, which varied between the isolates. The different growth of all *Fop* isolates with different pH values is shown in the interaction diagram Figure 3. The results obtained were consistent with the researchers findings

TABLE 3 Sporulation and characterization of *Fop* isolates.

Isolates	Macro conidia*				Micro conidia*				Chlamydospore*		
	Size LxB (μm)	Septation	Sporulation/mL ($\times 10^5$)	Shape	Size LxB (μm)	Septation	Sporulation/mL ($\times 10^5$)	Shape	Daimeter (μm)	Formation	Shape
Fop-1	13.53 × 3.41	3–4	39.5	Sickle-shaped with pointed ends	8.45 × 3.93	0	6.80	Oval to elliptical	5.37	Intercalary	Globose
Fop-2	14.70 × 3.51	2–3	26.40	Sickle-shaped with blunt ends	4.73 × 2.30	0	1.10	Elliptical	6.33	Terminal	Globose
Fop-3	19.22 × 4.33	3–4	38.7	Sickle-shaped with blunt ends	7.94 × 3.85	0	3.90	Round to oval, oval to elliptical	5.64	Terminal	Globose
Fop-4	19.31 × 3.25	3–4	15.40	Sickle-shaped with blunt ends	7.93 × 2.28	0–1	2.10	Elliptical	7.21	Terminal	Globose
Fop-5	14.72 × 3.98	3–4	26.6	Slightly curved with blunt ends	8.15 × 3.87	0	4.50	Round to oval, oval to elliptical	7.65	Intercalary	Globose
Fop-6	21.23×3.89	2–3	30.50	Short, straight with blunt ends	9.48 × 3.75	0	5.40	Round to oval, oval to elliptical	6.15	Terminal and intercalary	Globose
Fop-7	11.95 × 4.98	2–3	16.0	Short, straight with blunt ends	9.64 × 3.62	0	1.40	Oval to elliptical	5.31	Terminal	Globose
Fop-8	16.34 × 5.21	1–3	22.80	Short, straight with blunt ends	8.90 × 4.72	0	2.80	Round to oval, oval to elliptical	4.83	Terminal	Globose
Fop-9	12.48 × 3.75	1–2	13.90	Short, straight with blunt ends	7.53 × 3.82	0	1.20	Round to oval	5.60	Intercalary	Globose
Fop-10	18.25 × 4.52	3–4	21.50	Sickle-shaped with pointed ends	9.23 × 2.79	0–1	1.90	Elliptical	5.23	Intercalary	Globose
Fop-11	15.86 × 3.85	3	20.50	Short, straight with blunt ends	8.62 × 4.10	0	2.40	Elliptical	5.48	Terminal	Globose
Fop-12	21.24 × 3.54	3–4	23.90	Slightly curved with blunt ends	8.24 × 4.31	0	4.50	Oval	7.43	Intercalary	Globose
Fop-13	24.24 × 3.96	2–4	15.00	Sickle-shaped with blunt ends	8.78 × 4.92	0–1	1.40	Oval	4.89	Terminal and intercalary	Globose

*Mean of 10 replications.
L, length, B, breadth.

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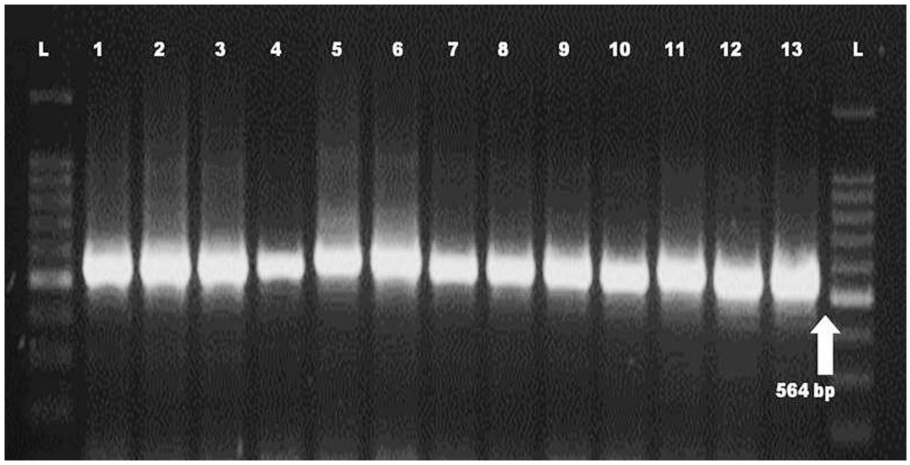

PLATE 2
PCR amplification of ITS region of *Fop* isolates (*L* = 100 bp Ladder, 1–13 = *Fop* isolates).

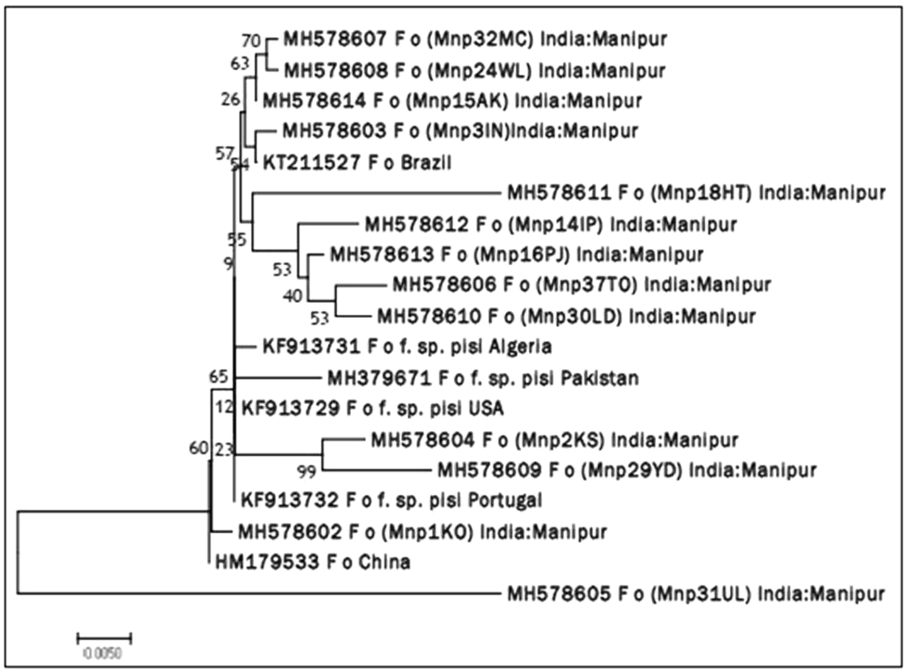

FIGURE 2
Neighbor joining (NJ) phylogenetic tree illustrating the concatenated (16S rDNA) sequence based phylogenetic relationship of *Fop* isolates.

(Attri et al., 2018; David et al., 2019; Somesh et al., 2019; Chaithra et al., 2020). The results obtained are supported by the results of Groenewald et al. (2006) who carried out *in vitro* studies on the radial growth of other *Fusarium* species and *Formae* special species. *Fusarium oxysporum* sp. *cubense* showed maximum radial growth at pH6 and 25°C, with minimal growth at pH4 using the same citrate/phosphate buffer medium.

Among all temperatures examined against the radial growth, a temperature of 25°C proved good and supported the growth of *Fop* isolates, with maximum growth being 8.8–9 cm. The lowest growth of all isolates was observed at 33°C, with the growth of all isolates dropping to 4 cm among isolates. The interaction diagram revealed that the growth of each isolate varied at different temperatures. The growth of *Fop* isolates at different temperatures is shown in the interaction diagram with different isolates and different temperatures, Figure 4. The influence of temperature on the *Fop* results has been compared with the results of David et al. (2019), Somesh et al. (2019), and Attri et al., 2018. Similar results for optimal fungal growth at a temperature of 25°C were reported for *Fusarium oxysporum* sp. *lactucae*, *Fusarium oxysporum* sp. *fabae* and *Fusarium oxysporum* sp.

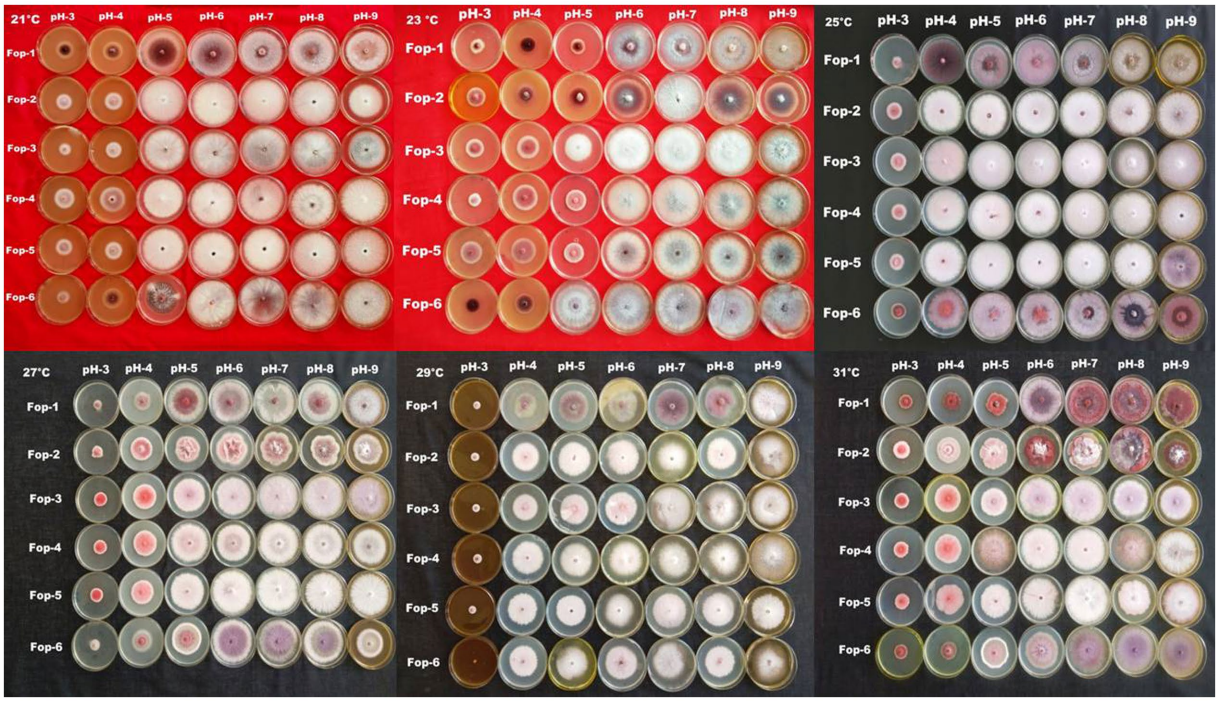

PLATE 3
Combination effect of temperature and pH on *Fop* isolates.

spinaciae (Naiki and Morita, 1983; Ivanovic et al., 1987; Scott et al., 2010).

Combination effect of temperature and pH, temperature and nitrogen dose levels on radial growth on six isolates of *Fop*

The radial growth of *Fop* isolates resulted in a variable trend in response to combined changes in temperature and pH. All isolates showed increasing radial growth patterns with increasing temperature and pH. The increasing pattern was observed in all *Fop* isolates at a temperature of 21–27°C with a change in pH from 4 to 8. The mean radial growth of all isolates in relation to different temperatures ranged from 5.76 to 6.13 cm, with isolate *Fop*-3 having the highest proportion, followed by *Fop*-4, *Fop*-5, *Fop*-6, *Fop*-1, and *Fop*-2. The radial growth of the isolates at different temperatures ranged from 4.35 to 7.66 cm, with the highest mean radial growth of 7.66 cm of all isolates observed at 25°C. Isolate *Fop*-3 showed the strongest radial growth of all isolates in relation to different pH values and the smallest radial growth in *Fop*-1. The mean radial growth at different pH values for isolates, the strongest radial growth was observed at pH7. The growth of all isolates with regard to temperature and pH should be at a maximum at the combination of 25°C and pH7, respectively. Different importance of mean radial growth among isolates was observed. A wide variation in the radial growth of all isolates with changes in temperature and pH was observed. The density plot of the isolates at different temperatures and pH showed differences between isolates, temperature and pH. Growth densities of all isolates were concentrated at 5.5 to 7 cm. The density plot (Figure 5) also shows the variation in radial growth between isolates as the growth of the isolates is not equal. The results obtained are supported by David et al. (2019). The variation exhibited by the isolates at different temperatures and pH values was not limited to radial growth, but there were variations between isolates in terms of fungal growth pattern, color changes/pigmentation of the mycelium and substrate. Its changes can be visualized in plate: 3.

The radial growth of *Fop* isolates resulted in a variable trend in response to combined changes in temperature and nitrogen doses. All isolates showed increasing radial growth patterns with increasing temperature and nitrogen dose. The increasing pattern was observed in all *Fop* isolates at a temperature of 21–27°C with a change in nitrogen dose. The maximum mean radial growth (8.9 cm) of all isolates was observed at a temperature of 25°C and 27°C with nitrogen doses of 9, 11, and 13 g, respectively. The mean radial growth of all isolates with respect to temperature was 7.62 cm for isolate *Fop*-3, followed by *Fop*-4, *Fop*-5, *Fop*-2, and *Fop*-1. Maximum growth at different temperatures for isolates was observed at 25°C. The highest mean radial growth of the isolates for changing media supplementation with different nitrogen dose levels, the highest radial growth showed isolate *Fop*-3 and the lowest isolate *Fop*-1. The radial growth of *Fop* isolates was found to be maximal at a temperature and nitrogen dose of 25°C and 11 g. Difference in mean radial growth among isolates was observed. A large variation in the radial growth of all isolates with changes in temperature and nitrogen dose was also observed. The density diagram of the isolates at different temperatures and different nitrogen doses showed differences between the isolates, the temperature and the pH. Growth densities of all isolates were concentrated at 5 to 8 cm. The density

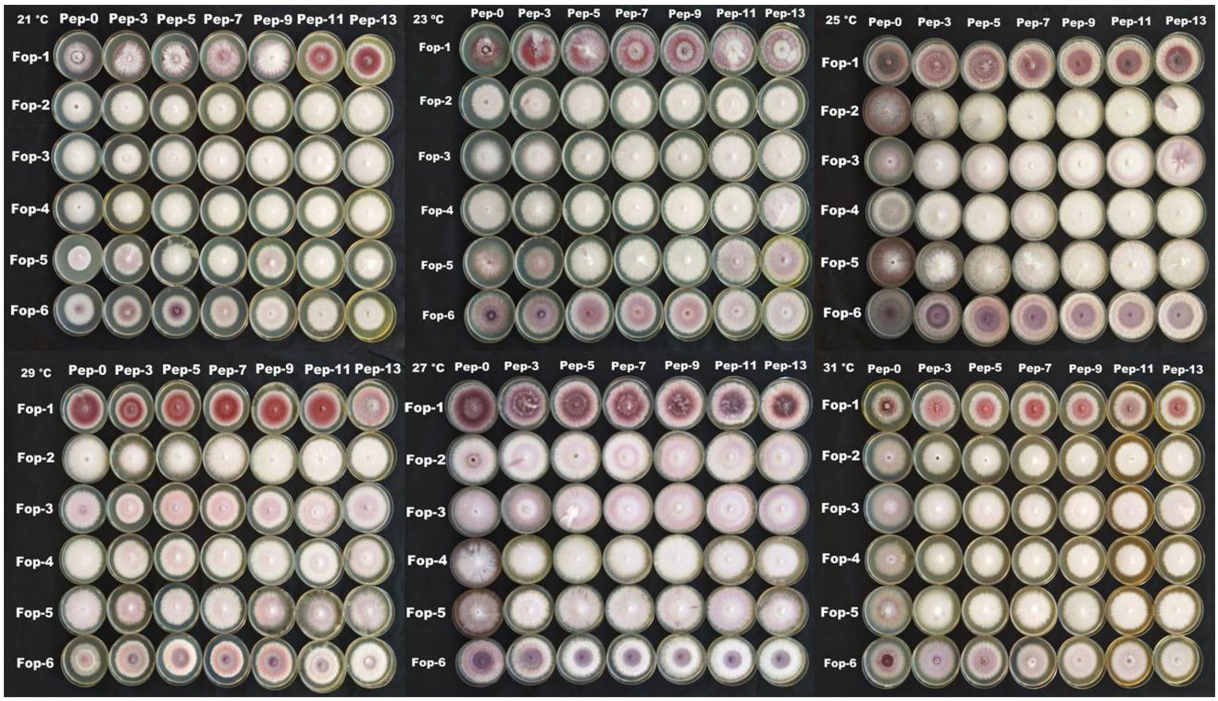

PLATE 4
Combination effect of temperature and Nitrogen levels on *Fop* isolates.

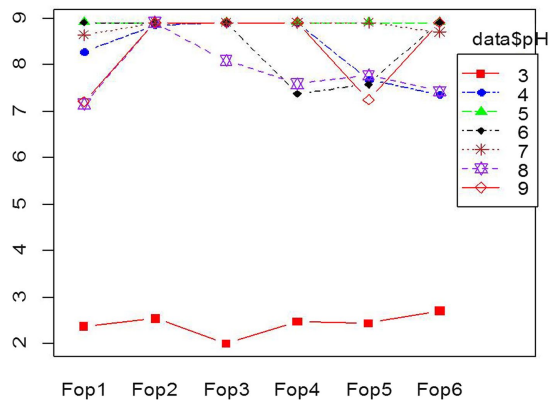

FIGURE 3
Interaction plot of radial growth of six *Fop* isolates and their growth at various levels of pH.

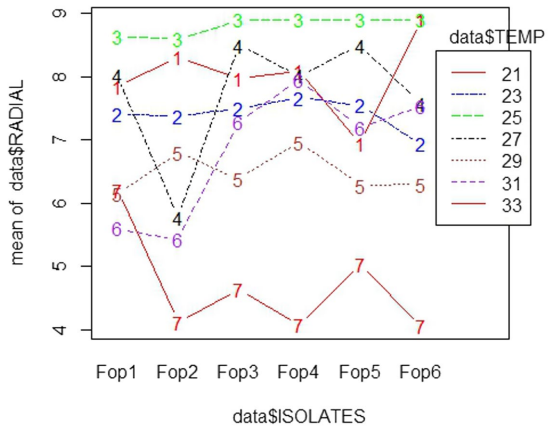

FIGURE 4
Interaction plot of radial growth of six *Fop* isolates and their growth at various levels of Temperatures.

plot (Figure 6) also shows the variation in radial growth between isolates as the growth of the isolates is not equal. The results obtained are supported by David et al. (2019). Notably, the variation was observed not only in radial growth but also in patterns of mycelial growth and pigmentation. Its changes can be visualized in plate: 4.

Discussions

The study provides evidence that all *Fop* isolates collected and isolated in different valley districts of Manipur showed mutual differences in morphology such as growth, mycelial properties, sporulation, size and shape. Colony character such as mycelial color (white, light pink, light purple, light brown), topography (thin, thick, cottony or non-cottony), texture (slightly fluffy to fluffy), border and pigmentation produced were recorded and all isolates detected showed large differences between them. The differences in cultural traits observed in *Fop* cultures have been important for the biology of fungi as they occur in nature because it is closely linked to the question of the physiological races of the pathogens. The results obtained in our investigation were consistent with the previous results of many workers (Singh et al.,

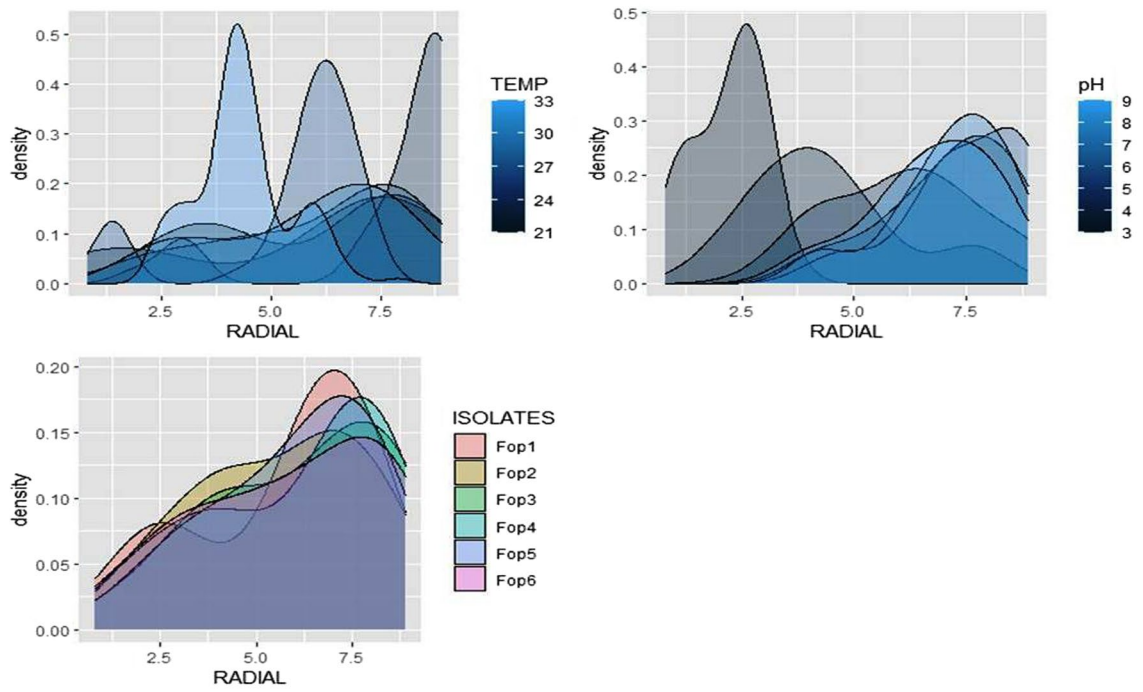

FIGURE 5
Density graph of radial growth of six *Fop* isolates with synergistic effect of Temperature and pH.

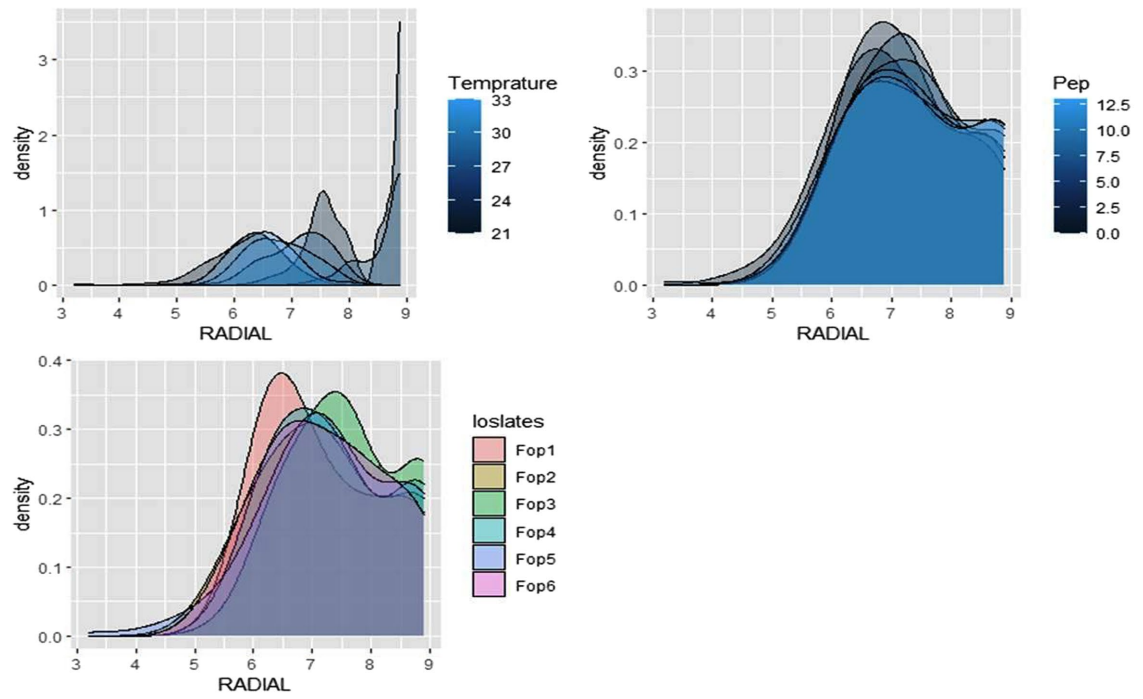

FIGURE 6
Density graph of radial growth of six *Fop* isolates with synergistic effect of Temperature and Nitrogen dose.

2010; Anser and Srivastava, 2013; Ashwathi et al., 2017; Thaware et al., 2017). Differences in sporulation, size, shape, color and number of septations of macroconidia and microconidia, and chlamydospore diameter were observed among Fusarium isolates. These observations indicate that sporulation is important for the virulence of the isolates. The isolates that produced heavy sporulation were highly virulent, while isolates that produced low to moderate sporulation had very low pathogenicity. All

morphological characters of *Fusarium oxysporum* obtained in the present investigation agree broadly with Booth (1971) and Nelson et al. (1990). A maximum degree of variability has previously been reported by former investigators in *Fusarium oxysporum* sp. *pisi* (Linford, 1928; Kraft, 1994). Awachar (2014) and Thaware et al. (2017) also described the size of microconidia, macroconidia and chlamydospores of Fusarium wilt of chickpeas. The current results confirm the reports of previous investigators who based their morphological studies on appropriate media for the growth and sporulation of Fusarium spp. (Kulkarni, 2006).

The characterization of the population structure of fungal pathogens is important for understanding the biology of the organism and for the development of disease control strategies (Malvick and Percich, 1998), as well as for molecular studies of individuals, which are one of the components of the population structure (Leung et al., 1993). However, the classification system based solely on morphology does not provide an accurate tool for identifying species. Therefore, a molecular approach is one of the most promising detection methods for species identification. In the present study, the morphological characterization of the isolates belonged to Fusarium spp. and their identity was confirmed using molecular methods. The primer combination ITS1 and ITS4 generated a fixed region length of 564 bp for 13 *Fusarium oxysporum* isolates. By comparing their sequences to the NCBI database using a blast search, a complete query with the highest match and coverage for *Fusarium oxysporum* was found. Chanu et al. (2018) worked on the molecular identification of *Fusarium oxysporum* f.sp. ciceris from chickpeas and confirmed that using the primer combination ITS1 and ITS4 resulted in a fixed region length of 564 bp. Devi et al. (2016) reported that the ITS region was successfully amplified from DNA of *Fusarium oxysporum* strains. Joshi et al. (2013) confirmed 39 isolates of *Fusarium oxysporum* using species-specific primers FOF1 and FOR1, which amplified the 340 bp DNA fragment. The Internal Transcribed Spacer (ITS) region is possibly the most frequently sequenced DNA region in fungi and is phylogenetically interpretable, i.e., the sequences are comparatively easy to align and large enough to provide potentially useful features for phylogenetic reconstruction. PCR amplification of the ITS region has become a popular choice for phylogenetic analysis of closely related species and populations. ITS1 and ITS4 of ribosomal RNA, which encodes DNA, have numerous advantages that represent an ideal region for a sequence for phylogenetic analysis. Its speed of development lends itself to study at both the specific and general levels. Therefore, the PCR-ITS method described in this study provides a simple and rapid procedure to differentiate *Fusarium* strains at the species level.

Optimization studies on six *Fop* isolates revealed that temperature and pH are key factors determining fungal growth. All *Fop* isolates were shown to show maximum growth at a temperature of 25°C and a neutral and slightly acidic medium. It has been observed that optimal temperatures and pH support the growth of the fungus for it to thrive and that the fission changes in the factors can alter the growth of the fungus. This was observed when lower and higher temperatures and higher pH were used. A reduction in the growth of the fungus with changes in its growth pattern and morphology was observed. These results also show that isolates found in the warmer regions tend to survive harsh conditions compared to isolates that thrive in temperate climates. The soils of Northeast India exhibit acidic conditions that favor the growth of the *Fop*. This in turn helps the fungus survive longer in the form of dormant structures. Our results on the influence of temperature and pH on fungal growth were consistent with the findings of several investigators studying Fusarium spp. All results indicated that the optimal temperature and pH for fungal growth were 25–30°C and 4–7°C, respectively (Groenewald et al., 2006; Wu et al., 2009; Gupta et al., 2010). However, temperature and pH may not be the only factors influencing the growth of the fungus. Other factors such as relative humidity and water potential may also have some impact on growth promotion (Cochrane, 1958; Nelson et al., 1990). The strongest radial growth tends to occur at 25°C. in *Fusarium oxysporum* sp. Betae isolates collected from temperate regions in the United States (Webb et al., 2015) and for *Fusarium oxysporum* sp. Psidii and F. solani isolates collected from subtropical regions in India (Gupta et al., 2010).

The merging effect of temperature, pH, temperature and nitrogen content shows that *Fop* isolates collected from different sites in the valley districts of Manipur tend to have different growth rates under different conditions of temperature, pH and nitrogen content base to achieve. Total *Fop* isolates were observed to exhibit white, cottony, fluffy mycelial growth as the nitrogen content of the basal medium increased, suggesting that field nitrogen concentrations support vegetative growth of the disease-causing pathogens. It was observed that the fungal isolates changed their morphology with changes in temperature, pH and nitrogen content (Panels 3 and 4). From this it can be understood that the change in climatic conditions causes mutations or genetic changes in pathogenic fungi. The climate-related changes of the fungus may pose a serious threat to the living world. As pathogens become more virulent under the influence of these changing climate scenarios, new epidemics can arise that can starve people and animals and cause devastating problems in the human world. The higher temperatures and pH levels and indiscriminate fertilization in the fields may not have an immediate impact but may pose future challenges that may be difficult to manage.

Future aspects

Since the results of inter-isolate variation with changes in temperature, pH and nitrogen doses are confirmed at the morphological level, these should also be evaluated at the genetic level to determine the degree of inter-isolate variation when there is a change in atmospheric conditions. The same should be evaluated under field conditions involving the host to determine the effect of the pathogenic isolates.

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

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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

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EDITED BY
Nirmalendu Basak,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Sandeep Kumar Sihag,
Indian Agricultural Research Institute (ICAR), India
Sunanda Biswas,
Indian Agricultural Research Institute (ICAR), India

*CORRESPONDENCE
Jixia Zhao
✉ zhaojixiacc@163.com
Maopan Fan
✉ mpfan@126.com

†These authors have contributed equally to this work and share first authorship

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Effects of partial substitution of chemical fertilizer with organic manure on the activity of enzyme and soil bacterial communities in the mountain red soil

Zerang Lu[1†], Yun Zhou[1,2†], Yongmei Li[1], Chunpei Li[1], Mei Lu[1], Xuemei Sun[1], Zhizhang Luo[1], Jixia Zhao[1*] and Maopan Fan[1*]

[1]College of Resources and Environment, Yunnan Agricultural University, Kunming, China, [2]College of Ecology and Environment, Yunnan University, Kunming, China

Introduction: The partial substitution of chemical fertilizer with organic manure takes on a critical significance to enhancing soil quality and boosting sustainable agricultural development. However, rare research has studied the effects of partial substitution of chemical fertilizer with organic manure on soil bacterial community diversity and enzyme activity in maize field in the mountain red soil region of Yunnan.

Methods: In this study, four treatments were set up in which chemical fertilizer (the application rates of N, P_2O_5 and K_2O were 240, 75 and 75 kg·ha^{-1}, respectively) was substituted by 10% (M10), 20% (M20), 30% (M30) and 40% (M40) of organic manure with equal nitrogen, as well as two control treatments of single application of chemical fertilizer (M0) and no fertilization (CK). The maize (*Zea mays* L.) crop was sown as a test crop in May 2018. The effects of partial substitution of chemical fertilizer with organic manure on soil physicochemical properties, soil bacterial community diversity and enzyme activity were studied.

Results: The activities of Cellulase (CBH), Invertase (INV) and β-glucosidase (BG) increased with the increase of organic manure substitution ratio. The activities of β-1,4-N-acetylglucosaminidase (NAG), Urease (URE), and leucine aminopeptidase (LAP) also had the same trend, but the highest activities were 159.92 mg·g^{-1}·h^{-1}, 66.82 mg·g^{-1}·h^{-1} and 143.90 mg·g^{-1}·h^{-1} at 30% substitution ratio. Compared with CK and M0 treatments, Shannon index increased notably by 82.91%–116.74% and 92.42%–128.01%, respectively, at the organic manure substitution ratio ranging from 10% to 40%. Chao1 and ACE index increased significantly at the organic manure substitution ratio ranging from 10% to 30%. *Proteobacteria* was the dominant phylum in all treatments, the relative abundance of *Proteobacteria* decreased as the organic manure substitution ratio increased. Redundancy analysis showed that microbial biomass C was the main factor affecting the bacterial community composition under partial replacement of chemical fertilizer treatment, while *Actinobacteria* was the main factor affecting the enzyme activity. In addition, the maize yield of M30 and M40 treatments was significantly higher than that of CK and M0-M20 treatments, and the yield of M30 treatment was the highest, reaching 7652.89 kg·ha^{-1}.

Conclusion: Therefore, the partial substitution of chemical fertilizer with organic manure can improve soil biological characteristics, while increasing bacterial community diversity and soil enzyme activity. Therefore, a thirty percent organic manure substitution was determined as the optimal substitution ratio for maize farmland in the mountain red soil area of Yunnan, China.

KEYWORDS

fertilizer, maize, soil properties, soil bacterial community, soil enzyme activity, mountain red soil

1. Introduction

Fertilizers are the indispensable agricultural measure to improve soil fertility and sustaining crop yield (Xiang et al., 2020), High levels of agricultural production are heavily determined by fertilizers (Muhammad et al., 2020). However, excessive application of chemical fertilizer is a common and serious problem in China, and overuse of chemical fertilizers exceeds crop demand (Zhu et al., 2019), causing soil degradation (e.g., soil acidification, soil structure deterioration (Guo et al., 2010), reducing soil organic carbon (SOC) content and changing the carbon (C) pool (Li et al., 2014), soil pollution and drastic reduction of soil biodiversity and other environmental problems (Wu et al., 2020)). The above soil environmental problems jeopardize entire agroecosystems, reducing the sustainability of crop production (Kim et al., 2022). Thus, how to ensure high and stable yield while controlling the application of chemical fertilizers is of critical significance to achieving sustainable agricultural development.

Manure has been confirmed as a viable alternative to chemical fertilizers (Du et al., 2020), which may contribute to soil physical, chemical and biological characteristics. It is capable of improving soil quality and structure, stimulating soil enzyme activity, and enhancing functional diversity and richness of soil community structure (Zhao et al., 2016; Hou et al., 2022a,b). However, the fertilizer efficiency of manure is generally low and difficult to control; farmers are inclined to use chemical fertilizers instead of manure to maintain crop yields in agricultural production. Accordingly, the combined application of manure (organic manure) and chemical fertilizer has been proven as a sustainable fertilizer management strategy (Zhao et al., 2014). The partial substitution of chemical fertilizer with organic manure is effective in increasing the efficiency of crop resource use and enhancing the quality of agricultural products. On that basis, the input of chemical fertilizers can be reduced, while soil quality and sustainable agricultural production can be maintained (Gattinger et al., 2012; Ji et al., 2021). Existing research has suggested that partial replacement of chemical fertilizers with organic fertilizers can expedite microbial growth while changing soil microbial community structure and diversity (Qiao et al., 2019; Xiang et al., 2020), thus increasing enzyme activity (Lazcano et al., 2012) and soil fertility and crop productivity in the long term. The optimal substitution ratio remains unclear, especially for the optimal substitution ratio of organic manure in maize farmland in the mountain red soil area of Yunnan Province, though the advantages of organic manure have been generally recognized. Previous research has indicated that a higher substitution ratio leads to higher rice yields in southern China (Bi et al., 2009), and Huang et al. (2010) reported that 50% organic manure instead of fertilizer treatment yields higher maize than single fertilizer, or 100% organic fertilization in the North China Plain. Thus, a wide variety of crops have different responses to the combined application ratio of organic fertilizer and chemical fertilizer, and the effect of some organic manure instead of chemical fertilizer on soil microbes and crop yield should be studied in depth.

Changes in soil microbial community abundance and composition can effectively indicate soil biochemical processes and crop productivity in agricultural systems (Liu et al., 2018), and soil microbes are critical to enhancing soil physicochemical properties, controlling soil microbial community and diversity, and maintaining soil quality and fertility (Tang et al., 2020). Appropriate community structure, rich diversity, and high microbial activity have been confirmed as crucial factors for maintaining soil ecosystem and productivity (Zhao et al., 2014). Existing studies reported that the microbial biomass and diversity of manure and compost soil more obvioulsy increase than mineral fertilizer, but a wide variety of fertilization methods and organic manure types have different responses to soil microbial biomass and diversity (Geisseler et al., 2017; Wang et al., 2018). Moreover, soil enzymes takes on a vital importance to nutrient mineralization, organic matter decomposition, and plant nutrient cycling (Li et al., 2015b). Soil enzyme activity also serves as a sensitive indicator of soil environmental changes (Tan et al., 2023). In general, it is correlated with soil physical and chemical properties, indicates the direction and intensity of soil biochemical processes, while significantly affecting soil physical and chemical properties, fertility, and biological conditions. Accordingly, it commonly serves as an essential indicator to evaluate the environmental quality of the soil (Guo et al., 2020; Cheng et al., 2022). Previous research suggested that the application of organic fertilizers generally increases soil enzyme activity in comparison with soil where fertilizers are applied, thus indicating higher nutrient cycling capacity. For instance, the addition of pig manure notably increases enzyme activity (Zhang et al., 2019). In contrast, chemical fertilizer application reduces the activity of C and N-associated hydrolases (DeForest et al., 2011). The functional capacity of soil microbial communities is manifested by the activity of enzymes that play a certain role in the nutrient mineralization process (Miao et al., 2019). For example, soil microbes produce enzymes associated with carbon and nitrogen acquisition (e.g., cellulase, urease, leucine aminopeptidase, N-acetyl-β-D-glucosaminidase, and invertase) (Tao et al., 2015; Liu et al., 2017; Zhang et al., 2017). Thus, exploring the effect of partial substitution of organic fertilizer on soil bacterial community and enzyme activity is of great significance.

However, there has been scarce research on the response of soil enzyme activity, bacterial community composition and diversity to partial replacement of chemical fertilizer by organic fertilizer in maize farmland in the mountain red soil area of Yunnan though some studies have suggested that partial substitution of chemical fertilizer with organic manure is capable of improving soil nutrients, bacterial community diversity, and soil enzyme activity. In addition, the results of the effects of fertilization measures on soil bacterial communities remain controversial in different soil types and soil environments in different regions. Accordingly, a hypothesis was proposed in this study, i.e., replacing chemical fertilizer with nitrogen (e.g., base-applied organic manure) will enhance soil properties, microbial properties, and enzyme activities. This study primarily investigated (1)

the effects of partial substitution of chemical fertilizer with organic manure on soil physicochemical properties, bacterial diversity and community composition, and enzyme activity, and (2) the correlation between soil properties, soil bacterial communities and enzyme activities.

2. Materials and methods

2.1. Experimental design and soil sampling

The positioning test was established in May 2016 at the Practice Teaching Experimental Station of Yunnan Agricultural University, Daheqiao, Xundian County, Kunming City, Yunnan Province, China (25°31′07″N, 103°16′41″E), and this test began in May 2018, and the experimental design and treatment are the same every year. This region has a temperate Subtropical monsoon climate, with an altitude of 1860 m, a mean annual precipitation of 900 ~ 1,000 mm, and a mean annual temperature of 15.80°C. The soil is classified as a mountain red soil (mountain red soil is a subclass of red soil) in the Chinese Soil Taxonomy. The cropping system was a continuous monocropped maize (*Zea mays* L.). The main soil properties (0–20 cm depth) are elucidated as follows: pH, 6.84, organic matter, 28.57 g·kg^{-1}, total nitrogen, 109.45 mg·kg^{-1}, nitrate N, 77.10 mg·kg^{-1}, available P, 14.25 mg·kg^{-1}, and available K, 129.37 mg·kg^{-1}.

The experiment was completely randomized design. This site has 18 plots (6 treatments and three replicates per treatment), with the respective plot of 28 m^2 (7 m × 4 m). The corn planting distance is 60 cm, the plant spacing is 25 cm, the ditch is sowed, the sowing depth is 4–5 cm, and 280 plants are planted in each community. The six different treatment fertilization methods in this study are elucidated as follows: no fertilization (CK), N, P and potassium (K) inorganic fertilizer (M0), the percentage of manure replacing chemical fertilizer 10%(M10), 20%(M20), 30%(M30), 40%(M40), organic manure application was 1,162 kg·ha^{-1}, 2,325 kg·ha^{-1}, 3,487 kg·ha^{-1}, 4,650 kg·ha^{-1}, respectively. The tested organic fertilizer was pig manure. The amount of nutrient inputs (N, P_2O_5, and K_2O) was the same for the five treatments. The inorganic fertilizer (M0): N, P_2O_5, and K_2O (240, 75, and 75 kg·ha^{-1}) was employed in the maize, respectively. Table 1 lists the amounts of N, P_2O, and K_2O from chemical fertilizer and manure for the respective treatment. Organic fertilizer and phosphorus and potassium fertilizer are used as a one -time strip of base fertilizer under the seeds. The total amount of nitrogen applies is 40% as the base fertilizer, 60% of the total amount of nitrogen are used for additional fertilizer (25% of the total nitrogen). Weeding, watering, and disease -insect worst prevention are performed according to conventional field management.

Soil samples were collected from the respective plot at 0–20 cm depth at the uprooting stage of the fifth crop cycle (18 September 2018). Fresh soil samples were immediately transported to the laboratory in an ice-box for in-depth processing. Gravel and residual roots were removed, and the soil samples were sieved (2 mm). Subsequently, a portion of the respective sample was stored at 4°C and then analyzed for enzyme activities and soil microbial biomass (MBC). Another part was stored at −80°C for microbiological Illumina MiSeq sequencing analysis. The rest of each sample was air-dried for chemical analysis.

2.2. Measurements of soil chemical properties and microbial biomass carbon

Soil pH was extracted at a ratio of 1:2.5 (soil: water) and determined using pH meter (HANNA, HI2221). Total nitrogen (TN) was determined using semi-trace Kjeldahl method. Soil nitrate nitrogen (NO_3^--N), ammonium nitrogen (NH_4^+-N)1 mol L^{-1} KCl leaching, 1 h filtration by shaking, and the filtrate was determined directly using a flow analyzer (Yang and Zhang, 2022). Total soil organic carbon (TOC) was determined using the TOC-VCPH organic carbon analyzer. The supernatant was pumped with a 0.45 μm membrane after the soluble organic carbon (DOC) was extracted with distilled water, and the filtrate was determined directly using the TOC-VCPH organic carbon analyzer (Yu et al., 2019). β-glucosidase (BG) activity was indicated by p-nitrophenol colorimetry, and the sample was incubated at 37°C to synthesize 1 μg of p-nitrophenol (PNP) per gram per hour of dry soil (Dick et al., 1996). Cellulase (CBH) activity was examined by 3,5-dinitrosalicylic acid colorimetry, and soil culture at 37°C was expressed by producing 1 μg of reducing sugar per gram per hour of soil sample (Yang et al., 2014). Invertase (INV) activity was examined through colorimetry using 3,5-dinitrosalicylic acid, and then the sample was cultured at 37°C to synthesize 1 mg of glucose per hour per gram of soil sample (Gong et al., 2019). Urease (URE) activity was incubated at 38°C using the indigophenol blue colorimetric method to yield 1 μg of NH_3-N per gram per hour of soil sample (Dick et al., 1996). Leucine aminopeptidase (LAP) activity was examined through chromogenic reaction by p-nitroaniline, and then the sample was cultured at 37°C to synthesize 1 μmol of p-nitroaniline per hour per gram of soil sample per day. Soil N-acetyl-β-glucosidase (NAG) was

TABLE 1 Application rate of chemical fertilizer /organic manure for different fertilization treatment (kg·ha^{-1}).

Treatment	Chemical fertilizer			Organic fertilizer		
	N application rate	P_2O_5 application rate	K_2O application rate	N content	P_2O_5 content	K_2O content
CK	0	0	0	0	0	0
M0	240	75	75	0	0	0
M10	216	62.46	64.91	24	12.54	10.09
M20	192	49.92	54.82	48	25.08	20.18
M30	168	37.38	44.73	72	37.62	30.27
M40	144	24.84	34.64	96	50.16	40.36

chromogenic by p-nitrophenol chromogenic and then incubated at 37°C to generate 1 μg of p-nitrophenol (PNP) per gram per hour of dry soil (Bell et al., 2013).

2.3. Soil DNA extraction, amplification, and sequencing

0.5 g of fresh soil was extracted using the soil DNA isolation kit FastDNAr SPIN (MP Biomedicals, Santa Ana, United States) in accordance with the instructions provided by the manufacturer. DNA mass and concentration were determined using a spectrophotometer (NanoDropTM 2000 (Thermo Fisher Scientific, Waltham, United States)). The V4-V5 hypervariable region of the 16S rRNA gene was selected and then amplified with primers 515F (5'-GTGCCAGCMGCCGCGG-3′) and 907R (5'-CCGTCAATTCMTTTRAGTTT-3′). The PCR reaction was performed in a mixture of 20 μL consisting of 2 μL of 10 × buffer, 2 μL of deoxyribonucleoside triphosphate (dNTPs) (2.5 mmol L^{-1}), 0.8 μL (5 μmol L^{-1}) per primer, 0.2 μL of conventional Taq polymerase, 0.2 μL of BSA, 10 ng template DNA, and double distilled water. The PCR conditions were 95°C for 3 min, 35 cycles, 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, and 72°C for 10 min. The PCR reaction was repeated 3 times. PCR products were extracted from 2% agarose gels, polymerized at equal concentrations, sequenced with the Illumina MiSeq platform, and high-quality sequences grouped into operational taxons (OTUs) at a 97% sequence similarity level. For the respective representative sequence, the classification information was annotated using the Unite database[1] based on the Blast algorithm using the QIIME software (Version 1.9.1).[2] It is noteworthy that total soil DNA extraction, amplification, library construction, sequencing and data analysis were performed by Beijing Novo Technology Co., Ltd., China.

2.4. Statistical analysis

The differences in soil properties, soil enzyme activity, soil bacterial α diversity and bacterial community composition between treatments was evaluated using One-way ANOVA (ANOVA), and the possible correlations between bacterial community, bacterial diversity, soil properties and soil enzyme activity was explored through Pearson correlation analysis. ANOVA and Pearson analysis was conducted using SPSS 23.0 statistical software. The complexity of sample species diversity was studied using Alpha diversity based on four indicators (i.e., Shannon, Simpson, Chao1, and ACE). All indicators in the sample were obtained using QIIME (version 1.7.0) and displayed using R software (version 2.15.3). Moreover, the differences in species complexity of the samples were evaluated through Beta diversity analysis, and the beta diversity of weighted and unweighted single plants was determined using QIIME software (Version 1.7.0). The dimensionality of the original variables was reduced through principal component analysis (PCA) using the FactoMineR package and ggplot2 package in the R software (Version 2.15.3) before clustering. Non-metric multidimensional scale (NMDS) maps were used based on the Bray–Curtis dissimilarity to visualize changes in bacterial community structure between different samples. Furthermore, the effects of soil properties and soil microbial communities on soil enzyme activity were investigated through redundancy analysis (RDA).

3. Results

3.1. Soil physiochemical properties, microbial biomass and maize yield

Base organic manure substituting chemical fertilizer with equal nitrogen had an effect on soil nutrient content and microbial biomass to varying degrees (Figure 1). In contrast to CK, the MBN and MBC contents of M10, M20, M30 and M40 increased significantly by 72.56–155.66% and 62.72–176%, respectively (Figures 1A,D), the NH_4^+-N content increased by 22.05–109.07% (Figure 1B), the NO_3^--N content grew by 28.47–92.76% (Figure 1E), and the TN content increased notably by 13.41–45.65% (Figure 1F), DOC and TOC contents rose obviously by 12.11–120.03% and 27.02–54.56%, respectively (Figures 1C,H). Compared with M0, the MBN and MBC contents of M10, M20, M30 and M40 increased notably by 7.05–58.59% and 9.26–85.32%, the content of NH_4^+-N increased by 16.95–85.84%, the NO_3^--N content decreased by 8.46–38.96%, and the DOC and TOC increased by 14.38–124.49% and 5.17–27.98%, respectively. The pH differed significantly between M20, M30 and M40 ($p < 0.05$). In summary, the contents of DOC, TOC and MBC and pH reached the higher values at the organic manure substitution ratio of 40%, the MBN and NH_4^+-N content were peaked at the organic manure substitution ratio of 30%, the NO_3^--N content decreased as the organic manure substitution ratio increased, and the TN content reached the higher values at the organic manure substitution ratio of 20%.

Base organic manure substituting chemical fertilizer with equal nitrogen had an effect on maize yield to varying degrees (Figure 2). The order of maize yield was M30 > M40 > M20 > M10 > M0 > CK. Compared with M0, M10 and M20 treatments, the maize yield of M30 treatment increased significantly by 16.69, 16.66 and 11.16%, and that of M40 treatment increased significantly by 15.65, 15.62 and 10.17%.

3.2. Soil enzyme activities

The activities of CBH, INV, BG, LAP, NAG, and URE were significantly increased by 81.06–302.93% compared with CK, and significantly increased by 23.99–183.16% compared with M0, and the activities of INV, BG, and URE were significantly different among treatments. In brief, the activities of CBH, INV, and BG increased as the proportion of organic fertilizer substitution rose, and their activities reached the maximum at the substitution ratio of 40%, while LAP, NAG, and URE exhibited the highest activities under the substitution treatment of 30%.

1 https://unite.ut.ee/
2 http://qiime.org/scripts/assign_taxonomy.html

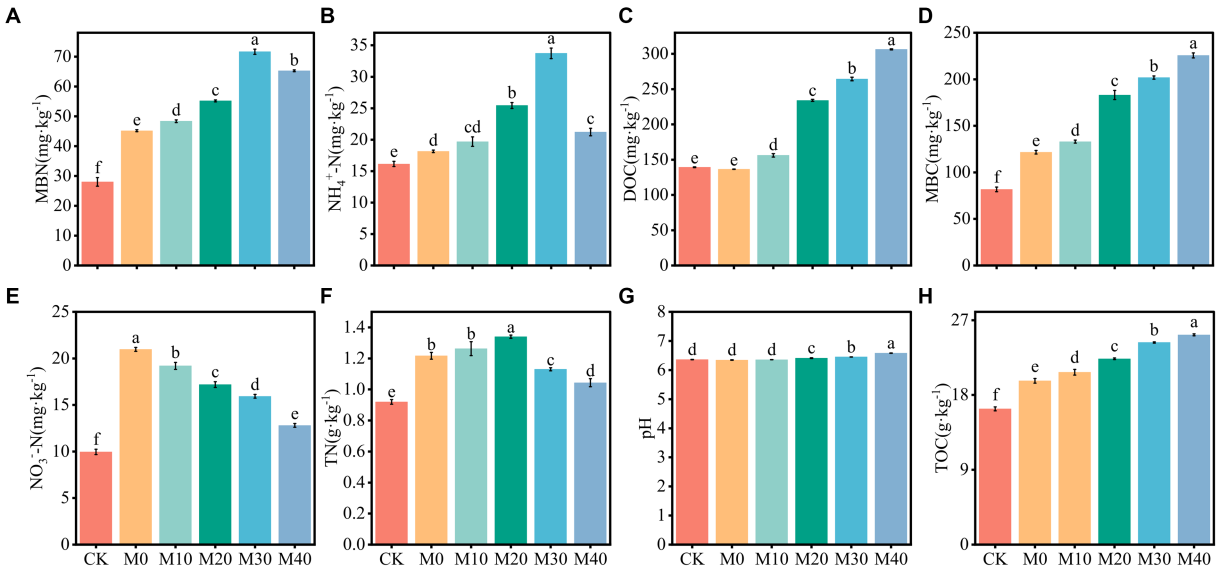

FIGURE 1
Change characteristics of soil physical and chemical properties and microbial biomass under different fertilization treatments **(A-H)**. Values followed by different letters are notably different ($p < 0.05$) according to Tukey's multiple comparison test. TN, pH, NH_4^+-N, NO_3^--N, TOC, DOC, MBC and MBN represent total nitrogen, soil pH, ammonium nitrogen, nitrate nitrogen, total organic carbon, dissolved organic carbon, microbial biomass carbon, microbial biomass nitrogen, CK, without fertilizer. M0, single inorganic fertilizer. M10, M20, M30 and M40 stand for nitrogen of organic manure replacing 10% chemical nitrogenous fertilizer, nitrogen of organic manure replacing 20% chemical nitrogenous fertilizer, nitrogen of organic manure replacing 30% chemical nitrogenous fertilizer, nitrogen of organic manure replacing 40% chemical nitrogenous fertilizer. The same as below.

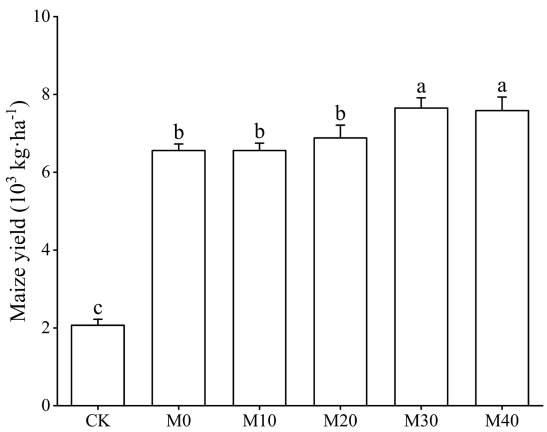

FIGURE 2
Change characteristics of maize yield under different fertilization treatments.

3.3. α-Diversities of bacterial communities

Partial substitution of chemical fertilizer with organic manure changed the diversity and richness of soil bacteria to varying degrees (Table 2). Compared with CK treatment, the Chao1 and ACE indices of M10, M20 and M30 increased by 49.61–72.8% and 48.27–71.04%, respectively, and the Shannon indices of M10, M20, M30 and M40 increased remarkably by 82.91–116.74%. Compared with M0 treatment, the Chao1 and ACE indices of M10, M20 and M30 were significantly increased by 68.05–94.04% and 66.16–91.68%, respectively, and the size orders of Chao1 and ACE indices were illustrated as M30 > M20 > M10 > M40 > CK > M0. The Shannon index of M10, M20, M30, and M40 treatment increased remarkably by 92.42–128.01% compared with M0 treatment, in which the Shannon index under M20 treatment reached the maximum. The Simpson index did not differ significantly between treatments.

3.4. Soil bacterial community structure and composition

Non-metric multidimensional scale analysis (NMDS) was performed (Figure 3) to compare the bacterial community composition under different fertilization treatments. A significant separation was identified between CK treatment and M10, M20 and M30 (stress = 0.102), whereas no significant difference was reported between M40 and CK treatment, and there was no significant difference between M0 and the respective treatment. Different proportions of substitution of chemical fertilizer with organic manure treatment affected the composition of soil bacterial community to different degrees (Figures 4, 5). The relative abundances of *Proteobacteria* under CK, M0, M10, M20, M30 and M40 treatments were determined as 90.87, 70.04, 70.15, 48.53, 67.64 and 62.46%, respectively, and the abundance of *Proteobacteria* was reduced by different substitutions of chemical fertilizer with organic manure treatments. Compared with CK treatment, the relative abundance of *Proteobacteria* under M20 treatment decreased by 46.6% (Figure 5A), whereas the relative abundance of *Actinobacteria* under M40 treatment increased by 301.53% ($p < 0.01$) (Figure 5B). *Actinobacteria* differed notably between the M10 and M40 treatments ($p < 0.05$) (Figure 5C). *Firmicutes*, *Bacteroidetes*,

TABLE 2 Variation characteristics of microbial *a*-diversity under different treatments.

Treatments	Chao1	ACE	Shannon	Simpson
CK	554.64 ± 82.73b	556.14 ± 68.84b	2.95 ± 0.13b	0.75 ± 0.06a
M0	493.80 ± 6.68b	496.27 ± 5.48b	2.81 ± 0.36b	0.75 ± 0.14a
M10	829.82 ± 30.13a	824.61 ± 31.41a	5.40 ± 0.42a	0.90 ± 0.04a
M20	922.28 ± 123.23a	908.87 ± 122.44a	6.40 ± 0.53a	0.94 ± 0.03a
M30	958.42 ± 100.83a	951.23 ± 98.40a	5.43 ± 0.57a	0.85 ± 0.04a
M40	585.98 ± 10.99b	596.67 ± 1.82b	5.42 ± 1.43a	0.88 ± 0.06a

Date: Values are mean ± standard deviation ($n = 3$), different letters in the same column represent significant differences at the $p < 0.05$ level.

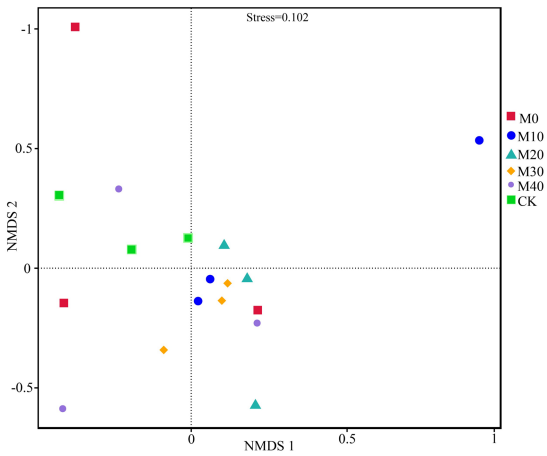

FIGURE 3
Changes in soil bacterial beta diversity (NMDS) under different fertilization treatments.

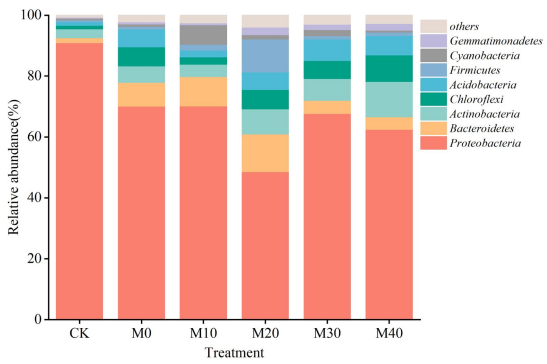

FIGURE 4
The 16S rRNA gene-based bacterial phylum community compositions in six treatments.

Chloroflexi, *Cyanobacteria*, *Acidobacteria* and *Gemmatimonadetes* did not differ remarkably between treatments.

3.5. Relation between soil physicochemical properties and biological properties

Spearman was used to analyze the correlation between soil physicochemical properties and soil microbial mass, carbon and nitrogen and enzyme activity (Figure 6). The results showed that the activities of carbon-metabolizing enzymes (INV, CBH and BG) and nitrogen-metabolizing enzymes (URE, LAP and NAG) were positively correlated with pH, TOC, DOC, NH_4^+-N, MBC and MBN ($p \leq 0.001$), and the correlation coefficients were between 0.73–0.98, and the correlation coefficient was not significant between TN and NO_3^--N.

The effects of soil physicochemical properties, soil enzyme activity and microbial mass carbon and nitrogen on microbial community structure were analyzed using Spearman and RDA (Figures 7, 8). *Proteobacteria* showed a significant negative correlation with INV, CBH, BG, URE, LAP, NAG, MBC, MBN, and NH_4^+-N ($p < 0.05$). *Bacteroidetes* had a remarkable positive correlation with TN ($p < 0.05$). Notably, the relative abundance of *Chloroflexi* phylum was positively correlated with BG, URE, LAP, NAG, MBC and NH_4^+-N ($p < 0.05$). The relative abundance of *Actinobacteria* phylum showed a notable positive correlation with INV, CBH and BG ($p < 0.01$), with URE, LAP, NAG, pH and MBN ($p < 0.05$), and with TOC, DOC, and MBC ($p < 0.001$). Both *Acidobacteria* and *Gemmatimonadetes* had a remarkable positive correlation with BG, URE, LAP, NAG, MBC, MBN and NH_4^+-N ($p < 0.05$), and the Shannon index achieved a significant positive association with INV, CBH, BG, URE, LAP, NAG, MBC, MBN, TOC, DOC, and NH_4^+-N ($p < 0.05$) (Figure 7).

The first and second axes accounted for the changes of bacterial communities by 21.78 and 18.00%, respectively (Figure 8A), as revealed by redundancy analysis. In the redundant analysis of soil physicochemical properties and microbial mass, and pH, DOC, TOC, MBN, MBC and NH_4^+-N were positively correlated with *Actinobacteria*, *Acidobacteria*, *Gemmatimonadetes* and *Chloroflexi*. TN and NO_3^--N were positively correlated with *Bacteroidetes*, *Firmicutes*, and *Cyanobacteria*, whereas pH, DOC, TOC, MBN, MBC, NH_4^+-N, NO_3^--N and TN were negatively correlated with *Proteobacteria*. In the redundant analysis of bacterial community and soil enzyme activity, the first and second axes accounted for the change of bacterial community by 57.99 and 4.67%, respectively (Figure 8B), and INV, CBH, BG, URE, LAP and NAG were positively correlated with *Actinobacteria*, *Gemmatimonadetes*, *Acidobacteria*, *Chloroflexi* and *Firmicutes*, while they were negatively correlated with *Proteobacteria*. MBC ($p = 0.022$) was the main factor for the composition of bacterial community under the treatment of organic fertilizer partial replacement fertilizer, while *Actinobacteria* ($p = 0.008$) was the major factor for enzyme activity.

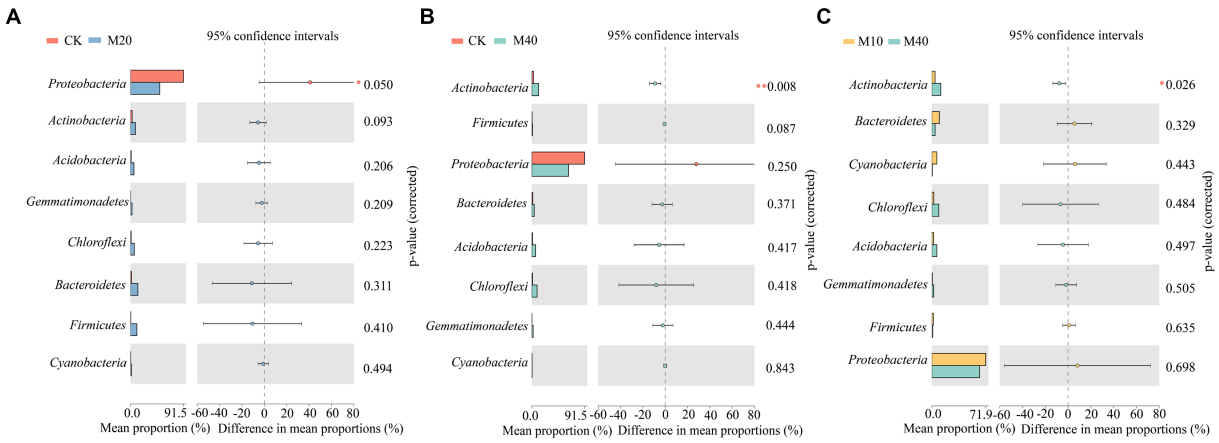

FIGURE 5
Differences in the relative abundance of bacteria in communities at the phylum levels **(A-C)**. The number of asterisks indicates significant differences between treatments according to a one-way ANOVA. Tukey–Kramer was used as a Post-hoc test and FDR (False Discovery Rate) adjustment ($p < 0.05$): *$0.01 < p \leq 0.05$; **$0.001 < p \leq 0.01$; ***$p \leq 0.001$.

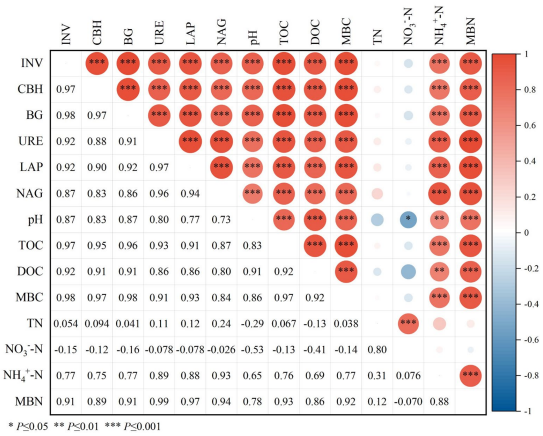

FIGURE 6
Spearman correlation between soil enzyme activities and physicochemical properties. pH, TN, NH_4^+-N, NO_3^--N, TOC, DOC, MBC, MBN, INV, CBH, BG, URE, LAP and NAG represent soil pH, total nitrogen, ammonium nitrogen, nitrate nitrogen, total organic carbon, soluble organic carbon, microbial biomass carbon, microbial biomass nitrogen, Invertase activity, Cellulase activity, BGcosidase activity, Urease activity, Leucine aminopeptidase activity, acetyl--D-glucosaminidas activity.

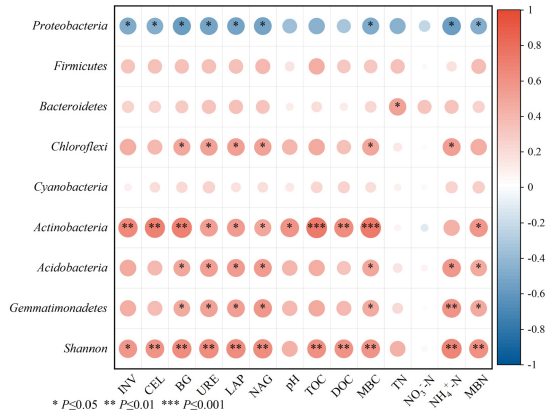

FIGURE 7
Correlation analysis between bacterial communities at the phylum levels and bacterial *a*-diversity with the soil physicochemical properties and enzyme activity.

4. Discussion

4.1. Effects of partial substitution of chemical fertilizer with manure on soil physicochemical properties and microbial biomass

Soil physicochemical properties are considered an indicator of soil quality; in general, they are defined as the ability to maintain soil environmental quality and improve crop yields (Li et al., 2020; Ren et al., 2021). However, soil microbial biomass plays a certain role in nutrient transformation and cycling in terrestrial ecosystems and serves as a reservoir of available nutrients for plants (Wang et al., 2021). In this study, chemical fertilizer was partially substituted with organic manure, thus leading to the significantly increased soil nutrient content and microbial biomass (Figure 1). To be specific, the DOC, TOC, and MBC contents reached the maximum at 40% organic manure substitution ratio, while MBN and NH_4^+-N contents reached the maximum at the organic manure substitution ratio of 30%, consistent with the results obtained by Lazcano et al. (2012). Luan et al. (2020) also suggested that microbial biomass and soil C content increased with the increase of organic manure substitution ratio, probably because organic manure provides sufficient C and nutrient resources, thus facilitating organic carbon sequestration (Muhammad et al., 2020) and microbial growth and reproduction (Mi et al., 2016). Besides, DOC is active soil organic matter that is easily decomposed by soil microbes, and it is critical to improving soil nutrients, microbial biomass and enzyme activity (Li et al., 2018). In addition, the presence of microbes in organic manure may contribute to the increase of soil microbial biomass (Dong et al., 2014). In this study, compared with no fertilization treatment, different

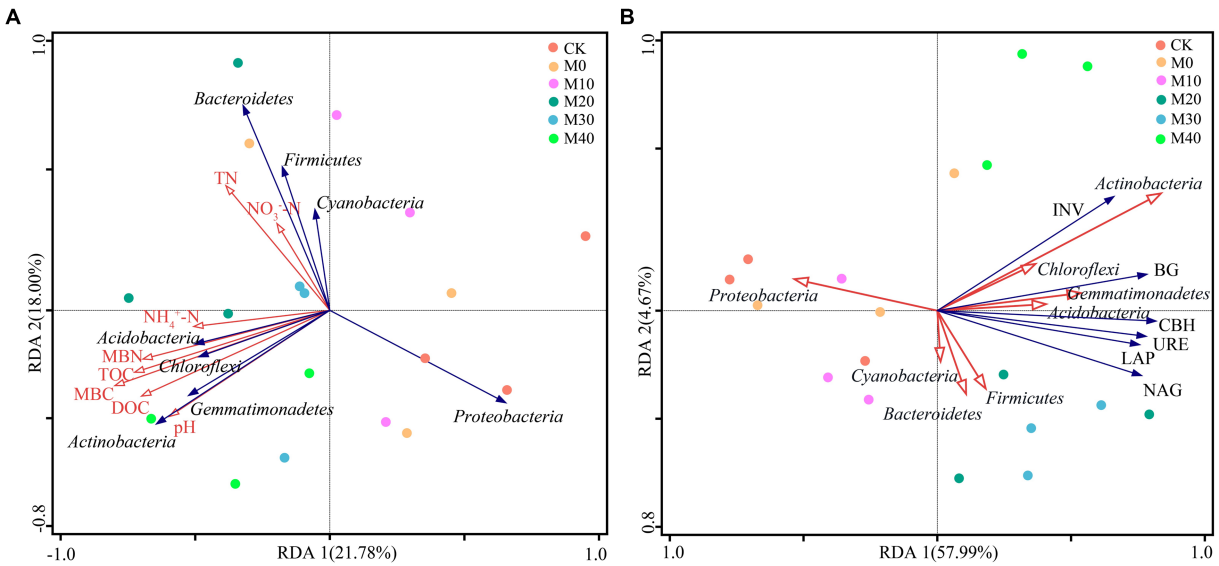

FIGURE 8
Panel **(A)** presents the redundancy analysis (RDA) between phylum level bacterial communities (blue arrow) and soil physicochemical properties (red arrow) under different fertilization treatments. Panel **(B)** illustrates the redundancy analysis (RDA) between phylum level bacterial community (red arrow) and soil enzyme activity (blue arrow) based on different fertilization treatments.

proportions of substitution of chemical fertilizer with organic manure treatment notably increased the NO_3^--N content, whereas the NO_3^--N content decreased remarkably with the increase of the proportion of organic manure substitution. The above result is consistent with the results obtained by Yang et al. (2020). In their study, the chemical nitrogen fertilizer was substituted with organic manure to study NO_3^--N in wheat field soil, the reason may be that after the application of organic manure, on the one hand, it increased the input of carbon and increased the soil C:N ratio, resulting in the fixation of N (Hou et al., 2023), on the other hand, organic manure promotes nitrogen uptake by crops (Abbasi and Tahir, 2012), reduced the apparent excess of nitrogen and the accumulation of NO_3^--N, thereby reducing the content of NO_3^--N (Wen et al., 2016). Xu (1996) also indicated that organic manure is capable of alleviating nitrate accumulation and leaching in soil profiles. Hou et al. (2022b) reported that the content of AN, NO_3^--N and MBN in the soil is significantly increased when the substitution rate of organic manure is less than 20% or between 20 and 40%. In this study, however, the partial substitution of chemical fertilizer with organic manure increased the soil nitrogen nutrient content to varying degrees, consistent with the previous research results. In this study, the proportion of organic manure substitution by 20–40% notably increased soil pH, and the pH increased with the increase of organic manure substitution ratio (Figure 1G), consistent with the results of existing research such as (Ding et al., 2016). The possible reason for the above result is that organic acids containing carboxyl and phenolic hydroxyl groups in manure can buffer soil acidity (García-Gil et al., 2004). Furthermore, pig manure is weakly alkaline, which has the potential to neutralize soil acidity, which can remarkably alleviate soil acidification caused by nitrogen, phosphorus, and potassium fertilization (Sun et al., 2015). However, no significant correlation was reported between pH and soil microbial community in this study, not consistent with the results achieved by Yang et al. (2018). The possible reasons for this result are different soil and climate factors.

4.2. Effects of partial substitution of chemical fertilizer with organic manure on soil enzyme activities

Soil enzymes play an important role in soil nutrient cycling and metabolism and have a direct impact on soil fertility (Hu et al., 2016; Xiao et al., 2018). In this study, carbon-metabolizing enzyme activity (CBH, INV, and BG) increased with the increase of organic fertilizer replacement ratio, and nitrogen-metabolizing enzyme activity (LAP, NAG, and URE) showed the same trend, whereas it was peaked at the substitution ratio of 30% (Figure 9). The major reason for the above result is that compared with single application of chemical fertilizer and CK, organic manure directly or indirectly increased soil humus (organic matter) content through crop growth; thus, the protective sites of soil enzymes, and the carbon and nitrogen sources of soil microorganisms increased, microbial reproduction was facilitated, and the increase of enzyme activity was stimulated (Gu et al., 2009; Li et al., 2022). Second, organic matter, the substrate of soil enzyme, may also directly induce the increase of soil enzyme activity. Although the substitution of chemical fertilizer with organic manure effectively alleviates the carbon limitation in soil, excessive alternative fertilizer may cause increased nitrogen competition (Olander and Vitousek, 2000). As a result, the consumption of considerable nitrogen metabolism enzymes in the soil should meet the soil nitrogen supply, such that the substitution ratio of 40% organic manure is lower than that of 30% soil nitrogen metabolism enzyme activity. Besides, Tao et al. (2015) and Zhang et al. (2020) suggested that partial substitution of chemical fertilizer with organic manure is capable of significantly increasing the activity of INV, URE, and CBH. The study of Ning et al. (2017) achieved similar results, i.e., a high proportion of organic manure supplementation resulted in higher enzyme activity. The possible reason for this result is that the addition of organic manure facilitates the growth of microbes and the

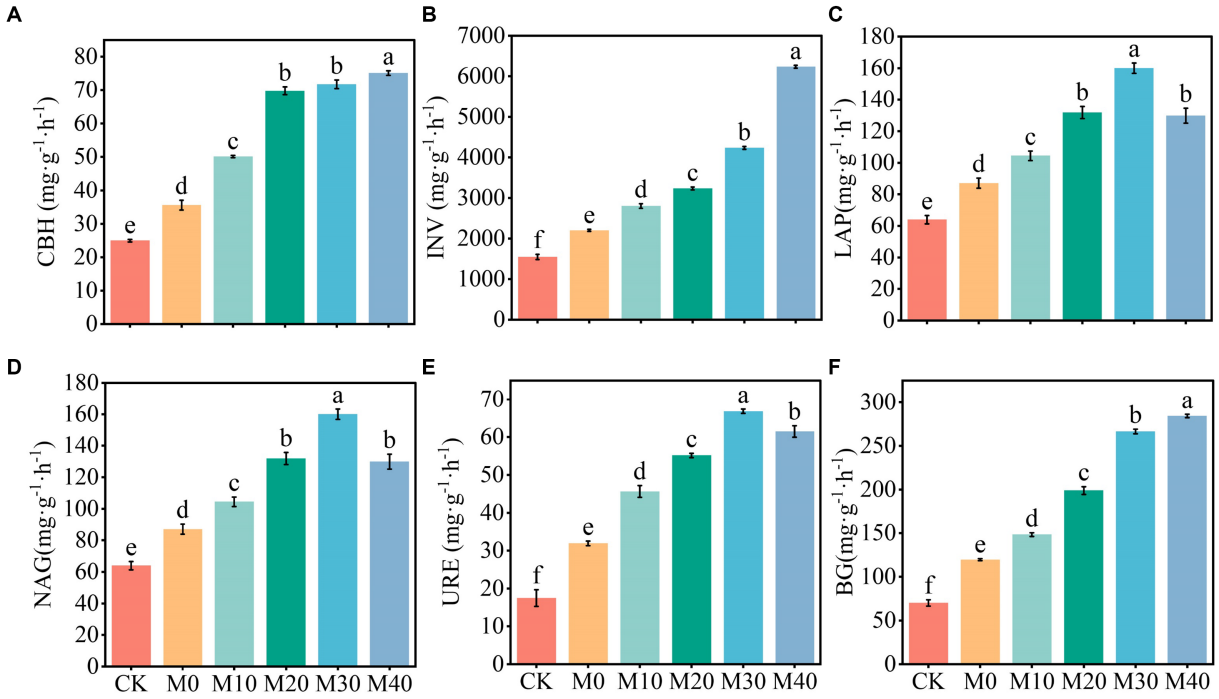

FIGURE 9
Soil enzymes activity for the respective treatment **(A-F)**. Different letters mean significant difference($p < 0.05$)between different treatments, INV, CBH, BG, URE, LAP and NAG represent Invertase activity, Cellulase activity, β-glucosidase activity, Urease activity, Leucine aminopeptidase activity, β-1,4-N-acetylglucosaminidase activity, the same as below.

increase of organic substrates is correlated with enzyme synthesis, such that the enzyme activity involved in major plant macronutrients is enhanced (Dinesh et al., 2010). In addition, the results showed that CBH, INV, LAP, NAG, URE and BG activities were positively correlated with pH, TOC, DOC, NH_4^+-N, MBC and MBN (Figure 6), which was similar to the results of positive correlation between hydrolase activity and TOC, DOC, and TN found in Hojjati and Nourbakhsh (2006). The possible reason for this result is that soil enzymes facilitate macromolecular organic hydrolysis, thus increasing carbon and nitrogen content, and sufficient C and N resources (derived from manure) can alleviate the restriction of soil microbial metabolism on C and N, thus promoting enzyme secretion (Wang et al., 2010). In this study, 10–40% of the organic manure substitution ratio notably improved the soil enzyme activity, and the soil carbon and nitrogen metabolism enzyme activity was basically consistent with the change of soil carbon and nitrogen nutrients, and the substitution of chemical fertilizer with basic organic manure can remarkably enhance the soil biological characteristics in the short term, thus facilitating the transformation and storage of soil nutrients and enhancing soil fertility.

4.3. Effects of partial substitution of chemical fertilizer with manure on soil bacterial diversity and community composition

Soil microbes take up an essential part of terrestrial ecosystems, and the diversity and community structure of microbes are not only closely correlated with the effectiveness of biotransformation, but also reflect soil fertility status and plant health, so the changes of microbial diversity and community have been widely concerned by soil scientists (Manasa et al., 2020; Yang and Zhang, 2022). Bacteria are considered a more accurate measure of soil fertility in comparison with fungi because they have much shorter turnaround times than fungi in utilizing carbon substrates (Demoling et al., 2007; Kuzyakov, 2010). Tian et al. (2015) found in short-term (3-year) field trials that the application of organic manure significantly reduced soil bacterial diversity. However, Han et al. (2021) reported that the partial substitution of chemical fertilizer with organic manure notably increases the species richness of bacteria, Chao1 and Shannon index, consistent with the results in this study, i.e., the proportion of organic manure substitution of 10–30% remarkably increased the Chao1, ACE and Shannon index, whereas the bacterial richness did not increase obviously at the proportion of 40% organic manure substitution. The reason for this result is that high mineral fertilizer inputs or excessive application of organic manure may inhibit bacterial growth, thus resulting in a decline in soil bacterial diversity (Cai et al., 2017; Ji et al., 2018), while moderate organic manure substitution ratios provide available C and N sources for soil microbes, thus contributing to the increase of bacterial diversity and richness (Xu et al., 2017). In this study, the single application of chemical fertilizer and 40% substitution ratio inhibited the growth of some bacteria, resulting in lower bacterial diversity. Furthermore, the bacterial α diversity index (Shannon) showed a notable positive correlation with enzyme activity (INV, CBH, BG, URE, LAP, NAG), MBC, MBN, TOC,

DOC, and NH_4^+-N ($p < 0.05$) under different substitutions of chemical fertilizer with organic manure treatment (Figure 7), consistent with the results of the Shannon index of soil bacterial community diversity found in Lu et al. (2022) and showing a significant positive correlation with pH, MBC, and URE. Long-term application of organic manure boosts the development of soil bacterial community diversity, mainly due to the effects on soil chemistry, biological properties and enzyme activities, especially on soil microbial biomass carbon (Li et al., 2015a; Miao et al., 2019). Moreover, as revealed by the above analyses, the improvement of soil physicochemical properties and soil enzyme activities can facilitate the growth and reproduction of soil bacteria.

In this study, the main bacterial phylums detected were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Chloroflexi*, *Acidobacteria*, which are similar to those found in other agricultural soils applied with organic manure (Wu et al., 2014; Ji et al., 2018). *Proteobacteria* is the dominant gate under each fertilization treatment, which is consistent with most studies (Ji et al., 2021). *Proteobacteria* is the most abundant phylum in soils and exists in environments with high nutrient levels and can be grown and metabolized using unstable C (Trivedi et al., 2013; He et al., 2021). This study found that the abundance of *Proteobacteria* was reduced by different substitutions of chemical fertilizer with organic manure treatments compared with no fertilization treatment, and the organic manure substitution ratio was more significant ($p < 0.05$) (Figure 4). The reason for this phenomenon may be that the application of organic manure causes certain specific bacterial groups to have a "preference" response due to the increase in available organic compounds, consuming large amounts of nutrients and inhibiting the growth of *Proteobacteria* (Lin et al., 2019). However, Yang and Zhang (2022) showed that the soil bacterial community characteristics of citrus orchards under partial replacement of bio-organic fertilizer showed that different organic fertilizer partial fertilizer treatment increased the relative abundance of *Proteobacteria*, which is contrary to the results obtained in this study. This may be partly due to different crop types, organic manure types, soils, and climate types (Xue et al., 2016). In addition, *Actinobacteria* under 40% organic manure substitution treatment was notably improved compared with no fertilization and 10% organic manure substitution treatment ($p < 0.05$) (Figure 5). *Actinobacteria* had a remarkable positive correlation with pH, DOC, TOC, MBN, MBC, NH_4^+-N, and enzyme activity (INV, CBH, BG, URE, LAP, and NAG) (Figure 7) since *Actinobacteria* refers to a highly abundant phylum of bacteria in a good nutrient environment that takes on a critical significance to soil carbon and nitrogen cycling (Zhu et al., 2019), which can contribute to the rapid growth of lignin, hemicellulose, protein, cellulose, and other C/N substances (Dai et al., 2018). Moreover, the relative abundances of *Bacteroidetes*, *Actinobacteria*, *Chloroflexi*, *Acidobacteria*, *Firmicutes*, *Cyanobacteria* and *Gemmatimonadetes* at the proportion of 10–40% organic manure substitution were higher than those without fertilization, whereas the relative abundance did not increase or decrease as the organic manure substitution ratio rose (Figure 4). The possible reason for this result is the different degree of response of each bacterial phylum to the proportion of organic manure application. As revealed by previous research, *Bacteroidetes* can degrade complex substances (e.g., starch, cellulose, lignin, and pectin) (Zhu et al., 2019), and nitrogen content may directly or indirectly cause the transfer of dominant flora members, especially *Bacteroidetes* (Fierer et al., 2012). In this study, the relative abundance of *Bacteroidetes* based on different treatments was higher than that of CK, consistent with the results of existing research (Yang and Zhang, 2022). The possible reason for this result is *Bacteroidetes* preference for eutrophication environments, where the application of organic manure increases soil nutrients while boosting *Bacteroidetes* use of carbon sources (Liang et al., 2018). *Acidobacteria and Gemmatimonadete* showed a significant positive correlation with enzyme activity (BG, URE, LAP, NAG), MBC, MBN, and NH_4^+-N, similar to the results of the study (Zhang et al., 2014), a class of oligotrophic bacteria that are less distributed in nutrient-rich agricultural soils (Lopez-Lozano et al., 2013). Studies have shown that soil properties are closely correlated with soil microbial community structure and can affect soil microbes to varying degrees (Xun et al., 2015), and soil microbial mass carbon and enzyme activity are important indicators of soil biological activity (Song et al., 2022). Ren et al. (2021) studied the effect of partial substitution of chemical fertilizer with organic manure on soil microbial community and soil enzyme activities. Their result suggested that soil bacterial community significantly affected soil enzyme activities. Through the RDA analysis of soil properties, this study also confirmed that soil enzyme activity and soil bacterial community phylum level, MBC ($p = 0.022$) is the main factor for the composition of bacterial community under the treatment of partial substitution of chemical fertilizer with organic manure, while *Actinobacteria* ($p = 0.008$) is the main factor for soil enzyme activity (Figure 8), consistent with the previous results.

5. Conclusion

In summary, this study shows that the alternative fertilizer of organic manure has significantly improved the nature of soil physical and chemical, maize yield, soil bacterial community diversity and enzyme activity. Based on these four aspects, 30% of organic manure substitution ratio is better. Among them, the maize yield of 30 and 40% of organic manure substitution treatments was significantly higher than that of CK and 0–20% of organic manure substitution treatments, and the yield of 30% of organic manure substitution treatment was the highest, reaching 7652.89 $kg \cdot ha^{-1}$. Compared with fertilization treatment, the relative abundance of *Proteobacteria* under M20 is significantly reduced by 46.60%. INV, CBH, BG, URE, LAP, and NAG activity are very positive as pH, TOC, DOC, NH_4^+-N, MBC, MBN. Shannon Index and enzyme activity (INV, CBH, BG, URE, LAP, and NAG), MBC, MBN, TOC, DOC, and NH_4^+-N are significantly positive. MBC is the main factor affecting bacterial community composition under the partial substitution of chemical fertilizer with organic manure, while *Actinobacteria* is the main factor affecting enzyme activity. To sum up, the appropriate proportion of organic manure substitution is conducive to improving soil properties, providing a good living environment for bacterial communities, thereby increasing soil enzyme activity, improving soil micro-ecological environment, and achieving sustainable agricultural development.

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

Experimental work and draft manuscript were completed by ZeL and YZ. Experimental ideas are provided by YL, CL, ML, XS, ZhL, JZ, and MF. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1234904/full#supplementary-material

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Dilfuza Egamberdieva,
Leibniz Center for Agricultural Landscape Research (ZALF), Germany

REVIEWED BY
Xiaojing Hu,
Chinese Academy of Sciences, China
Dahui Liu,
Hubei University of Chinese Medicine, China

*CORRESPONDENCE
Tao Zhou
✉ taozhou88@163.com

†These authors have contributed equally to this work

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Effect of symbiotic fungi-*Armillaria gallica* on the yield of *Gastrodia elata* Bl. and insight into the response of soil microbial community

Yanhong Wang[1†], Jiao Xu[1†], Qingsong Yuan[1], Lanping Guo[2], Chenghong Xiao[1], Changgui Yang[1], Liangyuan Li[1], Weike Jiang[1] and Tao Zhou[1*]

[1]Resource Institute for Chinese & Ethnic Materia Medica, Guizhou University of Traditional Chinese Medicine, Guiyang, China, [2]National Resource Center for Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China

Armillaria members play important roles in the nutrient supply and growth modulation of *Gastrodia elata* Bl., and they will undergo severe competition with native soil organisms before colonization and become symbiotic with *G. elata*. Unraveling the response of soil microbial organisms to symbiotic fungi will open up new avenues to illustrate the biological mechanisms driving *G. elata*'s benefit from *Armillaria*. For this purpose, *Armillaria* strains from four main *G. elata* production areas in China were collected, identified, and co-planted with *G. elata* in Guizhou Province. The result of the phylogenetic tree indicated that the four *Armillaria* strains shared the shortest clade with *Armillaria gallica.* The yields of *G. elata* were compared to uncover the potential role of these *A. gallica* strains. Soil microbial DNA was extracted and sequenced using Illumina sequencing of 16S and ITS rRNA gene amplicons to decipher the changes of soil bacterial and fungal communities arising from *A. gallica* strains. The yield of *G. elata* symbiosis with the YN strain (*A. gallica* collected from Yunnan) was four times higher than that of the GZ strain (*A. gallica* collected from Guizhou) and nearly two times higher than that of the AH and SX strains (*A. gallica* collected from Shanxi and Anhui). We found that the GZ strain induced changes in the bacterial community, while the YN strain mainly caused changes in the fungal community. Similar patterns were identified in non-metric multidimensional scaling analysis, in which the GZ strain greatly separated from others in bacterial structure, while the YN strain caused significant separation from other strains in fungal structure. This current study revealed the assembly and response of the soil microbial community to *A. gallica* strains and suggested that exotic strains of *A. gallica* might be helpful in improving the yield of *G. elata* by inducing changes in the soil fungal community.

KEYWORDS

high-throughput sequencing, symbiotic fungi, microbial community, *Armillaria*, *Gastrodia elata* Bl.

Introduction

Mycorrhizal symbiosis is found in over 80% of extant land plant species (Wang et al., 2017; Feijen et al., 2018). Orchidaceae showing mycorrhizal association with fungi are essential for seed germination and plant growth (Currah et al., 1997; Brundrett, 2002). It is believed that the ability to recruit saprotrophic lineages of wood and litter-inhibiting fungi is considered instrumental for plant adaptation to various ecological challenges, and many symbiotic fungi have coevolved with plants and showed a high degree of host specificity (Raaijmakers et al., 2009; Crowther et al., 2012). A well-known and highly specific alliance example is *Gastrodia elata* Bl. and *Armillaria* (Yuan et al., 2018; Camprubi et al., 2020; Xu et al., 2021). *G. elata*, also called Tianma in Chinese, is a perennial herb in the Orchidaceae family and one of the most valuable traditional Chinese medicines. Being one of the heterotrophic plants without chlorophyll, the growth and development of *G. elata* require symbiosis with *Armillaria* for nutrient supply since the protocorm stage (Yuan et al., 2018). The *Armillaria* members impose a significant impact on the yield and quality of *G. elata*; therefore, the isolation and identification of excellent *Armillaria* strains are of great significance (Yu et al., 2022).

Members of *Armillaria* generally form linear mycelial organs involved in wood decay and nutrient absorption. Evidence indicates that rhizomorphs of wood-decay species can absorb inorganic nutrients from the external environment (Clipson et al., 1987; Cairney et al., 1988), and the melanized layer of rhizomorphs generally contains calcium, which can provide chemical defense and natural controls (Porter et al., 2022). However, recent studies observed increased diversity of bacteria and fungi during the juvenile tuber to mature tuber periods of *G. elata*, which implied that *Armillaria mellea* can affect the structure of the microbial community associated with *G. elata* and greatly reduce the antifungal and antibacterial activities as a symbiotic association with its host is established (Yuan et al., 2018).

Soil contains large amounts of microbial biomass, and its microbial communities are highly diverse (Torsvik and Vres, 2002; Fierer, 2017). Diversity, structure, and composition of the soil microbial community are central issues in agricultural management, as this information is crucial for understanding and predicting the role played by organisms in maintaining plant health and production (Berendsen et al., 2012). Biotic factors, such as plant cultivars, strongly influence the composition of the soil rhizosphere microbiota and control the assembly of root-associated microbiotas (Tkacz et al., 2015; Zhou and Fong, 2021). Normally, plant cultivars impose an effect on the rhizosphere microbiota by investing a huge amount of root exudates and providing sources of carbonaceous compounds (Bulgarelli et al., 2013). Symbiotic fungi also have the ability to influence soil microbes; however, unlike plants, secretions of fungi through mycelia or rhizomorphs might inhibit the growth of others (Collins et al., 2013). Studies of changes in microbial community composition in response to symbiotic fungi can foster a better understanding of *Armillaria* utilization in the future.

The geographical areas of *G. elata* cultivation are mainly spread over the Ta-pieh Mountains, Qinling-Daba Mountains, and Yunnan-Kweichow Plateau in China. These sites are typically among the regions with sufficient rainfall and variable air temperatures in the summer. As global climate change continues and frequent commercial trade occurs among planting regions, it is incredibly significant to choose alternative *Armillaria* strains that can adapt to the diverse cultivation conditions in the process of *G. elata* artificial cultivation. However, a knowledge gap exists regarding to what extent *G. elata* will benefit from and how soil microbial communities vary with different *Armillaria* strains. For this purpose, four accessions of *Armillaria* strains were collected around China and tested in Guizhou Province. The genetic relationship of *Armillaria* strains was identified, and the yields of *G. elata* and soil microbial community dynamic were compared. Findings from this current study might be helpful in optimizing our strategies to utilize symbiotic fungi and *Armillaria* strains.

Materials and methods

Collection and identification of *Armillaria* strains

In this study, rhizomorphs of *Armillaria* were collected from three wild conditions in Lu'an (115°22′E, 31°06′N) Anhui Province, Xiaocaoba (103°51′E, 27°16′N) Yunnan Province, and Bijie (105°00′E, 27°14′N) Guizhou Province, and one commercial *Armillaria* strain was from Ningqiang (106°30′E, 32°37′N) Shanxi Province (Supplementary Figure S1). All these provinces are the main producing regions of *G. elata*. First, all the rhizomorphs of *Armillaria* were rinsed with running water and soaked with 75% ethanol for 45 s, followed by 1% NaOCl for 6 min. Then, the rhizomorphs were rinsed with sterilized water 3–4 times and dried with microbe-free filter paper. Finally, the cortices of rhizomorphs were removed with a sterilized knife, and the mycelia were lightly pulled out. Equal sizes and sections of mycelia for each *Armillaria* were inoculated in potato dextrose agar (PDA) solid medium and cultured for 20 days. In total, four *Armillaria* isolates, in accordance with collection sites, were obtained in the laboratory and named as follows: AH strain, SX strain, YN strain, and GZ stain.

The DNA of *Armillaria* isolates was extracted using the first generation with the Ezup Column Fungi Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China). Then, the intergenic spacer (IGS) regions of ribosomal RNA genes were amplified with primers CNL12 (5'-CTGAACGCCTCTAAGTCAG-3′) and 5SA (5'-CAGAGTCCTATGGCCGTGGAT-3′). The amplicons were subjected to Sanger sequencing, and gene sequence comparisons were done with the basic local alignment search (BLAST) homology comparison.[1] The purified *Armillaria* strains were kept on medium for one generation, then the second generation was used as the primary strain and enlarged by cultivating in sterilized packets with oat bran and cornstalk as nutrient sources. The conditions of *Armillaria* strain packets used for *G. elata* artificial cultivation were presented with graphs in Supplementary Figure S2.

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

Experiment design and sampling procedure

The site of *G. elata* artificial cultivation was located at Gonglongping National Forest Park in Qixingguan district, Bijie, Guizhou Province, China (105°00′38″E, 27°14′43″N), 1781 m above sea level. The mean annual temperature and precipitation in this area are 12.8°C and 998 mm, respectively. The soil is classified as brown loam (FAO-UNESCO, 1974).

The trial was performed at a single field site that was newly explored to ensure a uniform growth condition among repetitions. The cultivation of *G. elata* was carried out according to the WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants.[2] In the beginning, holes with a size of 80 cm × 50 cm × 30–50 cm (length × width × depth) were dug in the field. Then, 5–8 pieces of chestnut wood (Φ = 5–10 cm, length = 30 cm) that were fully infected with *Armillaria* strains were neatly laid out on the bottom of each hole. Subsequently, 0.1 kg of juvenile tubers of *G. elata* were put evenly on the chestnut wood and crushed with twigs and leaves that were infected with *Armillaria* strains. Finally, the soil was filled back into the holes and covered with straw or leaves (Yuan et al., 2020). The chestnut wood, twigs, and leaves that were infected with *Armillaria* strains were prepared ahead of cultivation, and the strains used for the chestnut wood, twig, and leaf infection were in a one-to-one relationship. There are four strains of *Armillaria* used in this trial in total, namely, AH strain, SX strain, YN strain, and GZ strain; each of them was replicated in three.

The cultivation was processed in March 2021, and the sampling was conducted in November 2021. When sampling, the twigs and leaves were removed gently without disturbing the soil covered by *G. elata*. A tensiometer was used to check the tensile strength of mycelia. The conditions of *Armillaria* mycelia at harvest time can be found in Supplementary Figure S3. At first, *Armillaria* strains tightly connected with chestnut wood and *G. elata* were picked out; subsequently, a section of *Armillaria* rhizomorph was hung on the hook of the tensiometer; then, the other side of the rhizomorph was pulled until it broke; and the real-time data on the tensiometer were recorded. Eight mycelia within one hole were randomly selected for statistical analysis. Soils around mature *G. elata* tubers were collected. Briefly, the soils loosely attached to *G. elata* were carefully collected into a 50-ml centrifuge tube with 20 mL of sterile water. After vortex mixing at 200 rpm for 20 min and centrifuging at 11,000 rpm for 10 min, the deposit was maintained at −80°C for subsequent soil microbial DNA extraction. Finally, all fresh tubes including mature and juvenile tubes of *G. elata* within one hole were collected and weighed, and the data were recorded.

Soil microbial DNA extraction, amplification, and Illumina sequencing

Soil microbial DNA was extracted using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, United States). The 16S rRNA gene was amplified with primers (F: ACTCCTACGGG AGGCAGCA, R: GGACTACHVGGGTWTCT AAT), and the ITS rRNA gene was amplified with primers (F: CTTGGTCATTTA GAGGAAGTAA, R: GCTGCGTTCTTCATCGATGC) with a 12 nt unique barcode at the 5′ end. The PCR reactions were performed in triplicate in 20-μl mixtures, containing 1 μL of 10-fold diluted DNA, 10 μL of SYBR® Premix Ex Taq (Tli RNase H Plus, 2×, Takara Bio, Japan), 1 μL of forward primer and 1 μL of reverse primer at 10 mM, and 7 μL of Milli-Q water. The PCR products were detected by electrophoresis in a 2% agarose gel, and then purified using the agarose gel DNA purification kit (Takara, Dalian, China). Furthermore, equal amounts of purified amplicons were pooled in equimolar amounts and paired-end sequenced by the Illumina NovaSeq platform at Biomarker Technologies Co., Ltd. (Beijing, China).

Processing of sequencing data and bioinformatics analysis of microbiome sequencing

Raw reads were quality-filtered using Trimmomatic (v0.33) and then clean reads were obtained with Cutadapt (1.9.1) by identifying and removing the primer sequences. Usearch[3] was used to merge the paired-end reads into a tag from high-quality clean reads. The operational taxonomic units (OTUs) were defined at the 97% similarity level by Usearch (Edgar, 2013) with the remaining unique sequences. At 70% of the confidence threshold, the taxonomic identity of phylotypes was classified using the Ribosomal Database Project RDP Classifier[4], the 16S rRNA gene sequence was analyzed with the Silva (SSU123) 16S rRNA database, and the ITS rRNA gene sequence was analyzed against the UNITE database (Version 6).

The Venn diagrams were constructed to visualize shared and unique OTUs among samples. Based on the calculated Bray–Curtis distance, the NMDS analysis was conducted to investigate the variation of bacterial and fungal community structure among treatments. To assess the significance of the difference in the bacterial and fungal community structure among different *Armillaria* strains, permutational multivariate ANOVA (PERMANOVA) was conducted using the ADONIS tests in the R vegan package (ADONIS; Oksanen et al., 2014). Based on the pairwise Spearman method and correlation coefficient with a threshold value of $r \geq 0.5$, the co-occurrence network analysis of bacteria and fungi was calculated at a $p < 0.5$ significant level, and the analysis and visualization were performed on the platform BMKCloud.[5]

The statistical significance of the yield of *G. elata*, tensile strength of *Armillaria* mycelia, OTU numbers, alpha diversity of bacterial and fungal communities, and relative abundance at the genus level among different *Armillaria* strains were tested using a one-way analysis of variance (ANOVA) and Duncan's multiple range test at a 0.05 significance level (SPSS 20.0 for Windows, IBM, Chicago, IL, United States). The IGS sequence comparisons of *Armillaria* strains were done with BLAST (see footnote 1), and a phylogenetic tree based

2 https://www.who.int/medicines/publications/traditional/gacp2004/en/

3 version 7.1, http://drive5.com/uparse.

4 RDP, V.2.2, http://rdp.cme.msu.edu/.

5 www.biocloud.net

on gene sequence comparisons was constructed with Mega (version 11, Tamura et al., 2021).

Results

Identification of four Armillaria strains

The gene sequence comparisons of intergenic spacers were done with BLAST homology comparison to classify the origins of *Armillaria* strains. The results of BLAST indicated that AH, SX, and GZ strains shared more than 98% homology with *A. gallica*, while the YN strain shared 82% with *A. gallica* in sequences producing significant alignments. The phylogenetic analysis based on sequences showed that all the *Armillaria* strains were clustered in three main clades, and the four strains and *A. gallica* had very close genetic relationships (Figure 1).

Yields of *Gastrodia elata* symbiosis with *Armillaria gallica* strains and the tensile strength of *Armillaria gallica* mycelia at the harvest time

The yield of *G. elata* symbiosis with the YN strain was the highest (3.91 kg·m^{-2}) (Figure 2A) and significantly higher than that of other strains ($p < 0.05$). In addition, the yield of *G. elata* symbiosis with SX and AH was both 2.38 kg·m^{-2} and markedly higher compared with the yield of *G. elata* symbiosis with the GZ strain, which was 0.98 kg·m^{-2} ($p < 0.05$).

To assess the ability of nutrient supply in different *A. gallica* strains, we tested the tensile strength of *A. gallica* mycelia at harvest time using a tensiometer. The tensile strength of the YN strain was significantly higher ($p < 0.05$) than that of the SX, AH, and GZ strains (Figure 2B), and strong growth of mycelia was observed in the YN strain compared with others (Figure 1A). However, there was no significant difference in the tensile strength among the SX, AH, and GZ strains.

Numbers of OTUs and Venn analysis across samples

The next-generation sequencing generated 959,747 pairs of reads for 16S rRNA genes and 959,978 pairs of reads for ITS rRNA genes across all samples. After rarefication to the minimum average, 79,764 and 79,666 clean reads were obtained for bacteria and fungi, respectively. Based on 97% similarity, 19,265 and 7,588 OTUs were identified for the bacterial and fungal communities, respectively.

Compared with the SX and YN strains, the GZ strain resulted in increases in soil bacterial OTU numbers (Figure 3A). Compared with others, the YN strain led to significantly higher OTU numbers in soil fungal community (Figure 3C). The Venn diagram analysis showed that there were large numbers of OTUs shared across samples, while less peculiar OTUs were spread in the bacterial community (Figure 3B). In the fungal community, the numbers of peculiar OTUs in soil planted with the YN strain were much higher than those of the AH, SX, and GZ strains, and more numbers of shared OTUs were found across treatments (Figure 3D).

Dynamics of soil microbial community diversity and structure in response to different *Armillaria gallica* strains

We analyzed the response of the soil bacterial and fungal communities to different *A. gallica* strains through Illumina sequencing. The diversity and richness of bacteria and fungi varied among different *A. gallica* strains and showed different patterns (Figure 4). In particular, the ACE and Simpson indexes

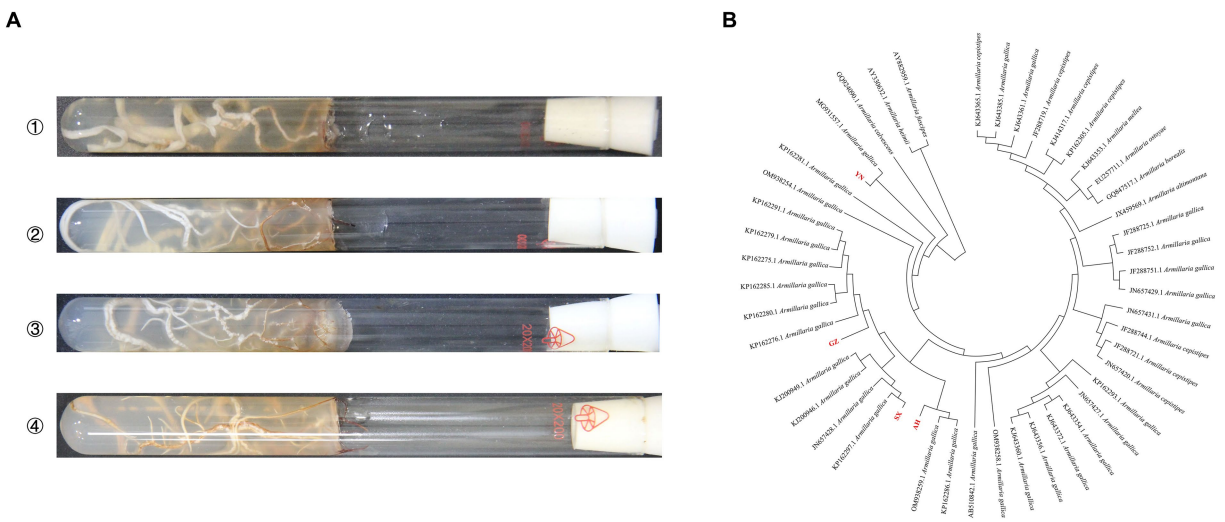

FIGURE 1
Incubation and identification of *Armillaria* isolates. **(A)** Mycelial morphology of four isolates on PDA medium at 23°C for 20 days (①: YN, ②: AH, ③: SX, ④: GZ). **(B)** Phylogenetic analysis of four *Armillaria* isolates based on the intergenic spacer.

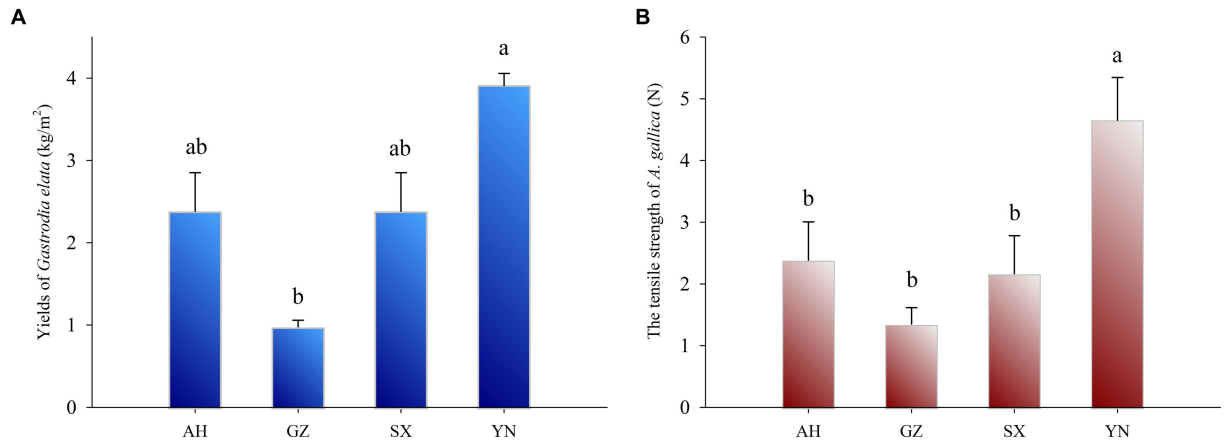

FIGURE 2
Yields of *G. eleta* symbiosis with *Armillaria* strains from different production areas for 8 months **(A)** and the tensile strength of *Armillaria* strains at harvest time **(B)**. Lowercase letters a, b, and c within one plot indicate significant differences among treatments, determined using the one-way ANOVA and Duncan's multiple range test ($p < 0.05$).

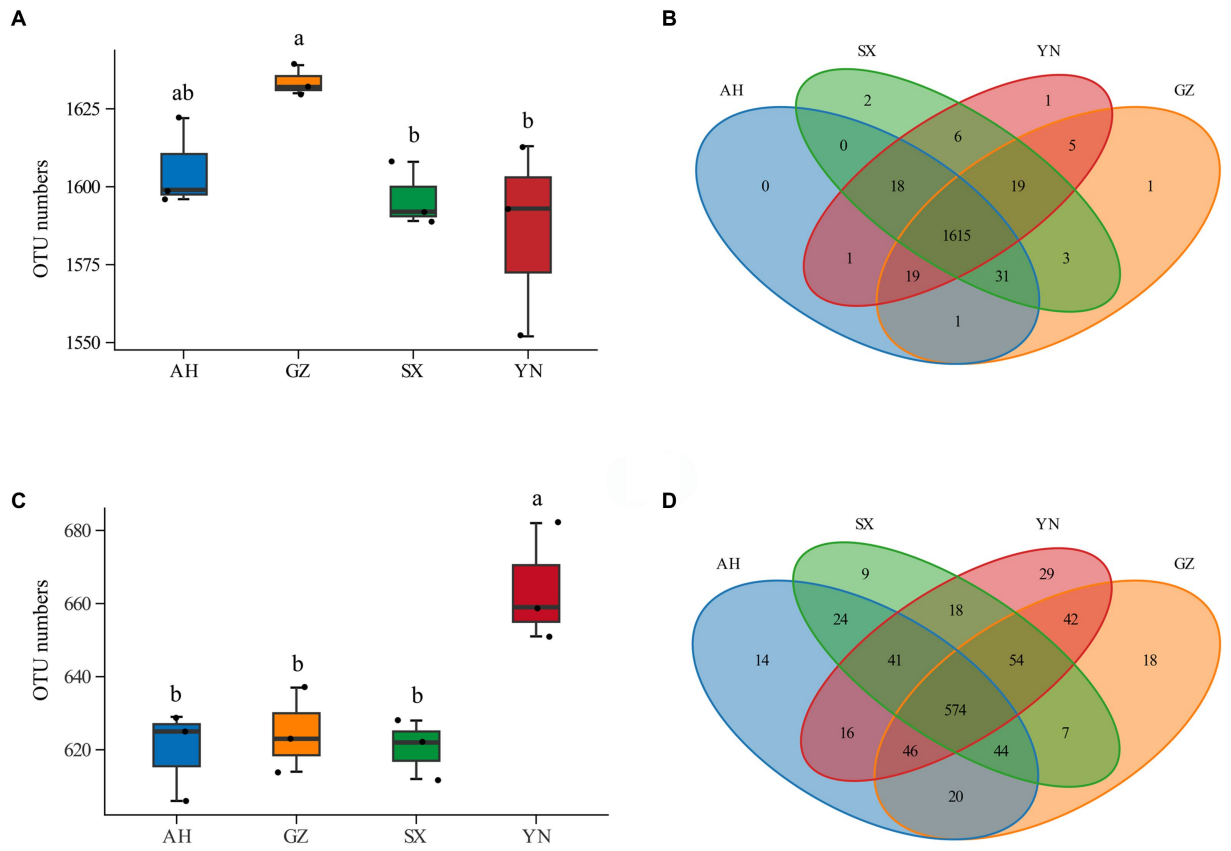

FIGURE 3
OTU numbers and Venn analysis of bacterial and fungal communities in soils around *G. eleta* symbiosis with *Armillaria* strains. **(A,B)** Represent OTU numbers and the Venn diagram of the bacterial community; **(C,D)** represent OTU numbers and the Venn diagram of the fungal community. Lowercase letters a and b indicate significant differences among different soils, determined using the one-way ANOVA and Duncan's multiple range test ($p < 0.05$).

of bacterial diversity in soil planted with the GZ strain significantly increased compared with those of other strains (Figures 4A,B), whereas the changes in fungal diversity indexes were different from those of bacteria. Specifically, the ACE and Simpson indexes tended to increase in soil planted with the YN strain, which showed significant differences with others (Figures 4C,D). Overall, the α diversity of the soil bacterial community was significantly influenced by the GZ strain, while

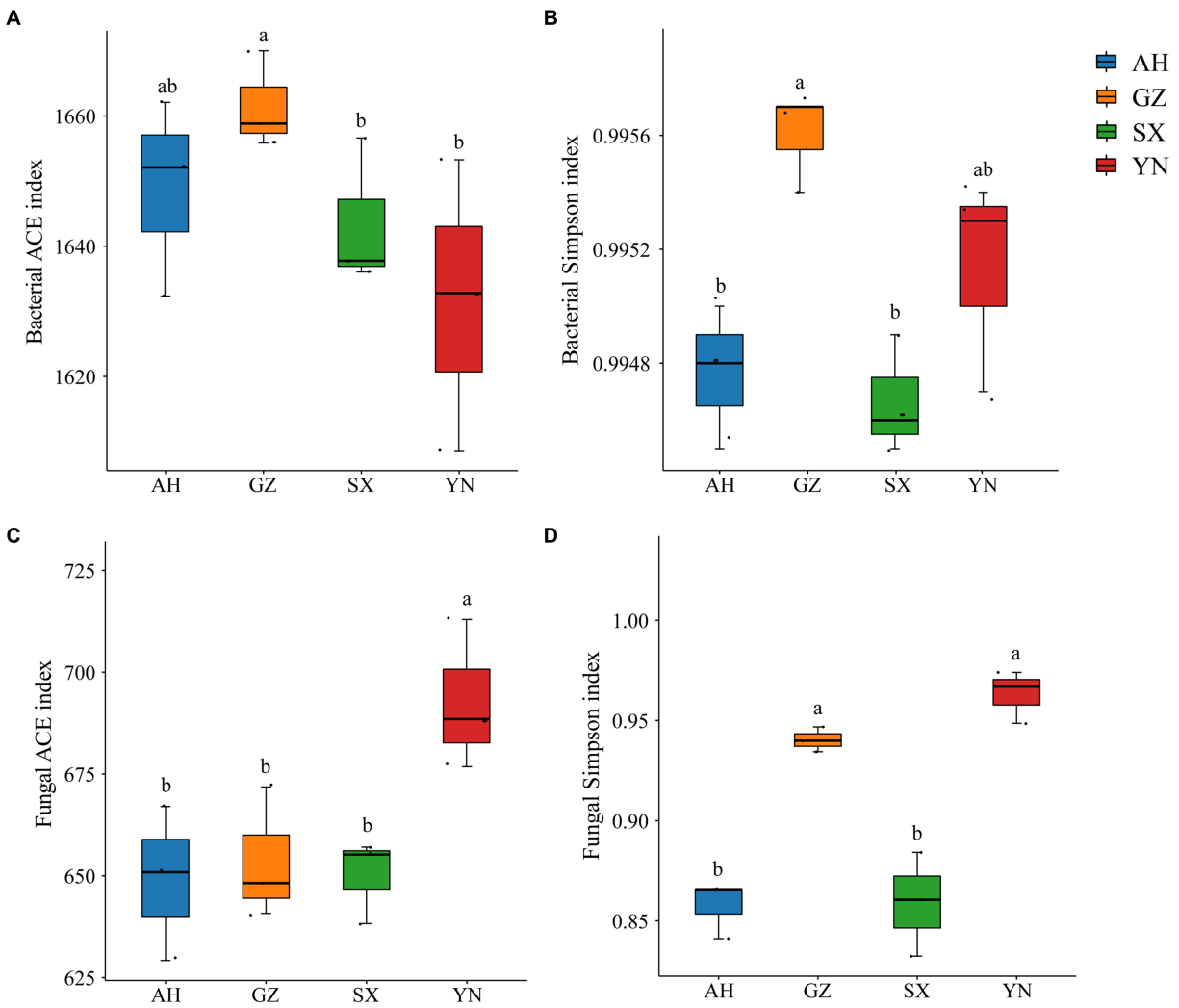

FIGURE 4
Alfa diversity indices of bacterial and fungal communities in soils around *G. eleta* symbiosis with *Armillaria* strains. **(A,B)** Represent the Ace and Simpson indexes of the bacterial community; **(C,D)** represent the Ace and Simpson indexes of the fungal community. Lowercase letters a and b indicate significant differences among different soils, determined using the one-way ANOVA and Duncan's multiple range test ($p < 0.05$).

that of the soil fungal community was markedly altered by the YN strain.

Similarly, the community structure of bacteria and fungi significantly changed (Figure 5). The result of NMDS analysis showed that the bacterial community in soil planted with the GZ strain clustered together and was greatly separated from the other strains ($R^2 = 0.309$, $p < 0.01$) (Figure 5A). Meanwhile, the fungal community structures of YN and SX strains were greatly separated from the others ($R^2 = 0.401$, $p < 0.01$), while no significant difference was observed between AH and GZ strains ($p = 0.1$) (Figure 5B).

Changes of microbial composition in different *Armillaria gallica* strains

The bacterial community was predominated by 9 phyla with a minor proportion of others (1.5–2.3%). The phyla Pseudomonadota (38.57–42.34%), Acidobacteriota (27.39–32.69%), and Chloroflexota (6.12–9.77%) were dominant members, followed by Planctomycetota (4.26–5.91%), Actinomycetota (3.83–4.38%), Verrucomicrobiota (3.21–4.16%), Bacteroidota (2.43–4.15%), Gemmatimonadota (1.73–2.81%), and WPS-2 (0.53–1.41%) (Figure 6A). None of the *A. gallica* strains induced significant changes in the relative abundances of Pseudomonadota ($p = 0.344$). However, compared with other strains, planting GZ strains significantly increased the abundances of Chloroflexota ($p < 0.05$) while significantly decreasing the abundances of Acidobacteriota ($p < 0.01$).

In the total ITS rRNA amplicons, OTUs in all samples were classified into four phyla, namely, Ascomycota (25.48–60.13%), Basidiomycota (30.73–67.80%), Mortierellomycota (2.85–10.96%), and Rozellomycota (0.95–1.44%), besides the unclassified (2.42–3.09%) and the sum of relative abundances that were lower than 1% (0.40–1.04% in total) (Figure 6B). Notably, the significant role of *A. gallica* strains in affecting the rhizomicrobiome in three dominant fungal phyla, i.e., Ascomycota, Basidiomycota, and Mortierellomycota, was detectable ($p < 0.05$) (Figure 6B).

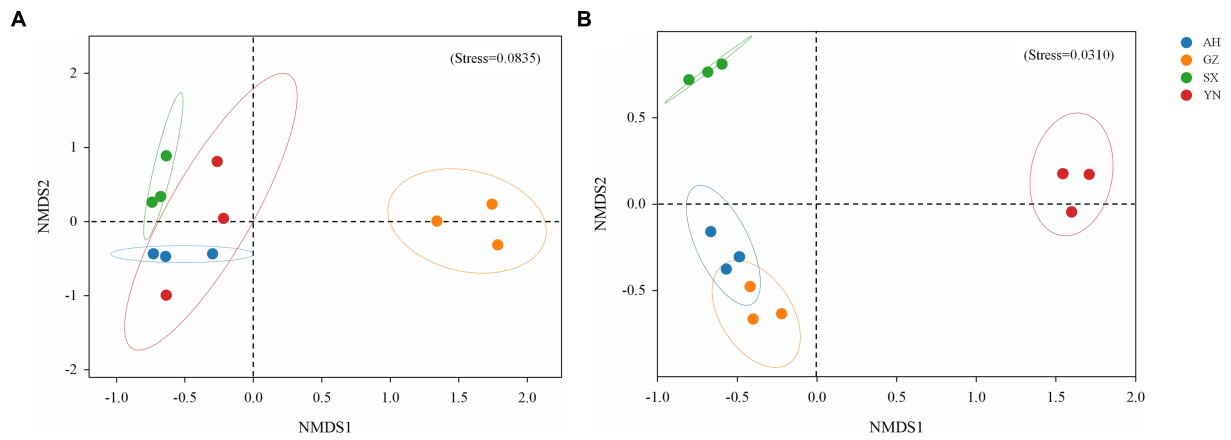

FIGURE 5
Non-metric multidimensional scaling (NMDS) analysis of bacterial **(A)** and fungal **(B)** communities in soils around *G. eleta* symbiosis with *Armillaria* strains, calculated based on Bray-Curtis distance.

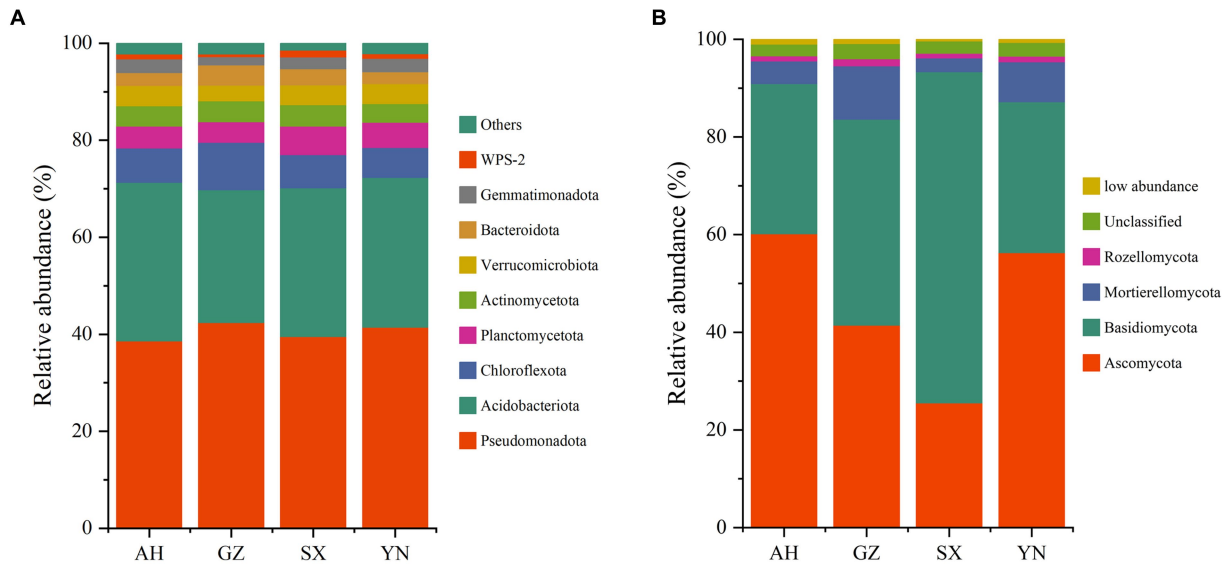

FIGURE 6
The bacterial **(A)** and fungal **(B)** community compositions in soils around *G. eleta* symbiosis with *Armillaria* strains at the level of phylum classification.

Specifically, AH and YN strains significantly increased the relative abundance of Ascomycota and Mortierellomycota ($p < 0.01$) while decreasing the relative abundance of Basidiomycota compared with other strains.

At the genus level, the number of genera greatly responded to different *A. gallica* strains. Compared with others, relative abundances of most genera of bacteria were mainly reduced in soil planted with GZ strain, except for the *bacterium_c_AD3*, *bacterium_c_Alphaproteobacteria*, and *Reyranella*, whose relative abundances were significantly increased by GZ strain ($p < 0.05$) (Figure 7A). However, most fungal groups were enriched by the YN strain. For example, the genera with increased relative abundances in response to the YN strain included *Mortierella*, *Nemania*, *Agrocybe*, *Archaeorhizomyces*, *Armillaria*, *Pseudoclathrosphaerina*, *Scopuloides*, *Leptodontidium*, *Cladophialophora*, *Psathyrella*, and *Saitozyma* ($p < 0.05$) (Figure 7B). Other genera showed negative responses to the YN strain, including *Coprinellus*, *Sebacina*, and *Russula*; however, their relative abundances were significantly increased by the SX strain ($p < 0.05$).

Co-occurrence patterns of bacterial and fungal communities responding to different *Armillaria gallica* strains

Networks were constructed, respectively, for bacteria and fungi, and significant correlations were compared. We found that bacterial co-occurrence networks were larger, more connected, and more modular than those in fungal networks, and a larger proportion of bacterial OTUs was included in co-occurrence networks than that

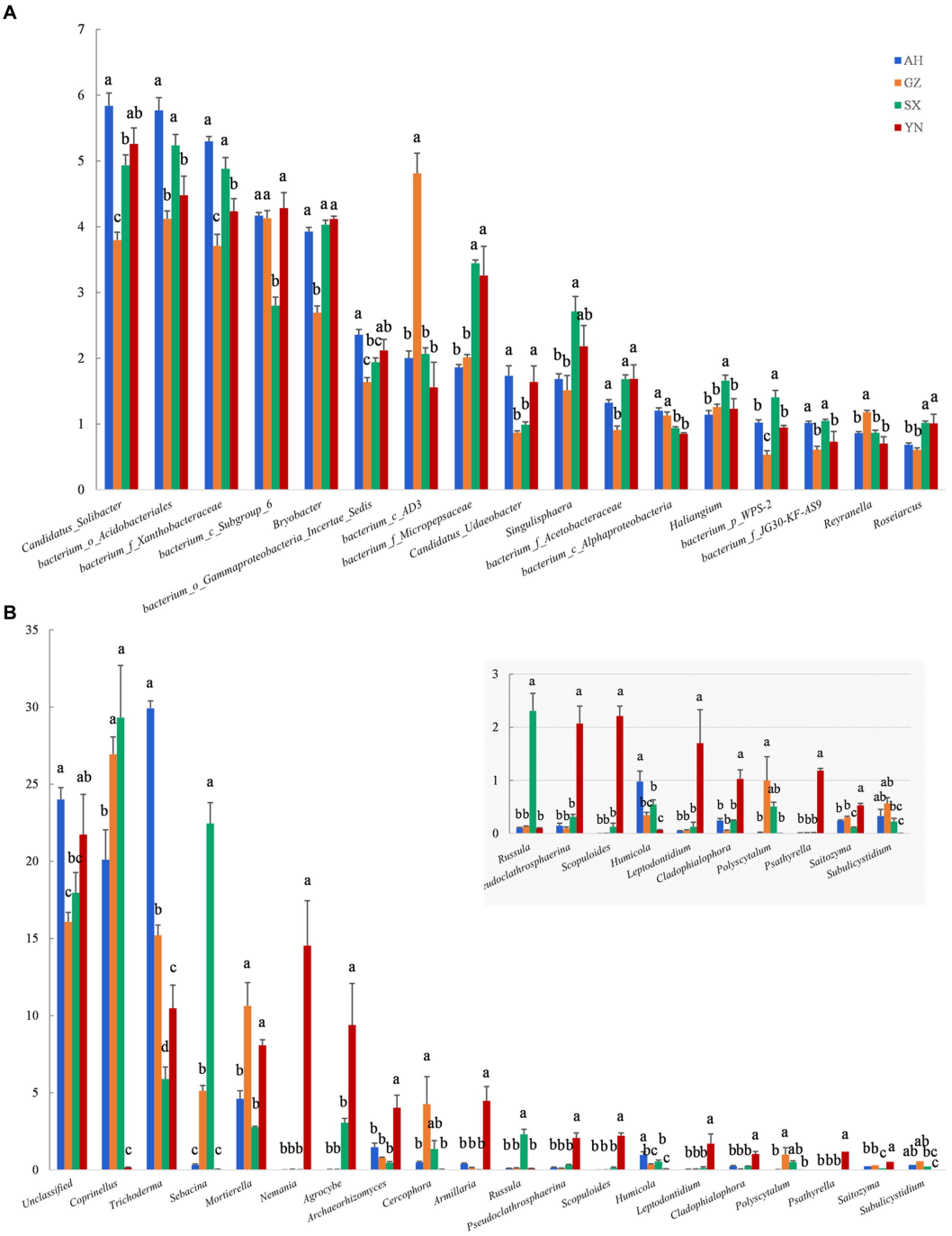

FIGURE 7
Relative abundance of soil bacterial **(A)** and fungal **(B)** communities in soils around *G. eleta* symbiosis with *Armillaria* strains at the level of genus classification. Lowercase letters a, b, and c within one genus taxa indicate significant differences among treatments, determined using the one-way ANOVA and Duncan's multiple range test ($p < 0.05$).

of fungal OTUs (Figure 8). In particular, the network for bacteria was composed of 46 nodes and 100 edges, and the modularity was 0.58; all nodes were assigned to 9 phyla. For the fungal community, the network presented 42 nodes and 100 edges; all these nodes were assigned to 4 phyla, i.e., Ascomycota, Basidiomycota, Chytridiomycota, and Mortierellomycota. Moreover, compared with Basidiomycota (14 nodes), Ascomycota (26 nodes) had a more complex correlation with different *A. gallica* strains.

Discussion

The demand for *G. elata* production is expected to increase further in the future as a result of health preservation and wild resource shortages. Such concerns have led to the development of artificial cultivation of *G. elata*, in which *Armillaria* strains are functionally important factors in affecting the growth and yield of *G. elata*. Soil microbial communities are responsible for nutrient cycling and plant

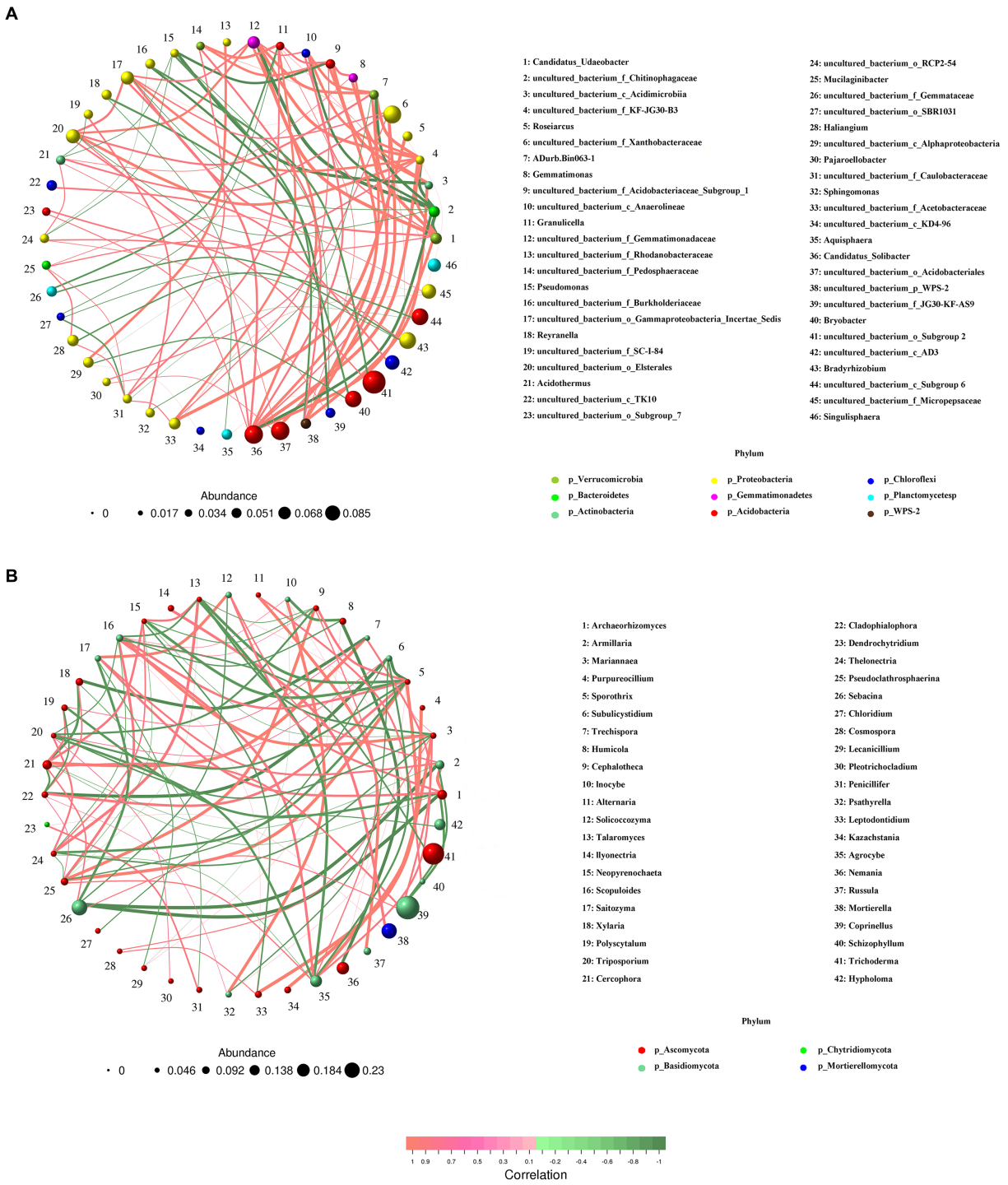

FIGURE 8
Co-occurrence analysis of soil bacterial **(A)** and fungal **(B)** communities in soils around *G. eleta* symbiosis with *Armillaria* strains.

growth; these communities could be affected by abiotic or biotic factors, particularly interactions with other microorganisms (Singh et al., 2009; Kubiak et al., 2017). For these reasons, there has long been interest in selecting the optimum *Armillaria* strains to co-plant with *G. elata* in a variety of environments and disentangling the mechanisms behind the high yield of *G. elata*. This study is one of the few attempts to provide insight into the yield of *G. elata* co-planted with different *Armillaria* strains and the microbiome influenced beyond this interaction.

The effect of *Armillaria gallica* strains on the yield of *Gastrodia elata*

The current study indicated that cultivation of *A. gallica* strains collected from other regions, especially Yunnan (YN strain), can significantly increase the yield of *G. elata* compared with that of the original Guizhou local strain (Figure 2A). Our finding is consistent with previous research in which four strains of *Armillaria* were

identified as *A. gallica* groups, whereas co-planting of these *A. gallica* strains with *G. elata* resulted in significant differences in yield and active ingredient content of *G. elata* (Liu et al., 2019). However, how did that happen if the identification of *Armillaria* strains indicated that they were in close relation to *A. gallica* (Figure 1)? Beyond the classification results of *Armillaria* strains, a possible explanation is that the robust growth condition (Figure 1A) and greater tensile strength of mycelia in YN strain (Figure 2B) contributed to the high yield of *G. elata* compared with others, which is also evidenced by previous studies that the optimum *Armillaria* strains that can greatly increase the yield of *G. elata* are typically the ones with stronger branches of mycelia (Chen et al., 2004; Wang et al., 2020). Therefore, the tensile strength, which can be a measurement of the thickness and strength of *Armillaria* mycelia, was tested at harvest time, and the results also confirmed that the stronger the *Armillaria* branches, the higher the yield of *G. elata* that is harvested (Figure 2). In the meantime, given that *Armillaria* mycelia are generally considered a special morphological adaptation of this fungus to different environmental conditions (Garett, 1960; Morrison, 2004), the migration of YN, AH, and SX strains from their local habitat partly triggered their strong growth of mycelia. On the contrary, the adaptability of GZ strain to the local condition and its weak growth of mycelia might lead to its poor performance when co-planted with *G. elata* (Sun et al., 2009). Besides that, the great variation in soil bacterial and fungal communities arising from different *A. gallica* strains may also play vital roles in controlling the yield of *G. elata*.

Response of soil bacterial and fungal communities to exotic *Armillaria gallica* strains and potential benefits on the growth of *Gastrodia elata*

Available studies have shown that the impacts of symbiotic mycorrhizal fungi on the diversity and composition of the soil rhizomicrobiome may induce changes in the ecological environment of soil microorganisms and, potentially, increase stress tolerance and yield of plants (Doubková et al., 2012; Cosme and Wurst, 2013; Selosse et al., 2022). In the current study, we observed that the GZ strain induced great changes in the bacterial community (Figures 3A,B, 4A,B, 5A), while the YN strain had a much stronger impact on the fungal community (Figures 3C,D, 4C,D, 5B). Moreover, a diverse variation and more complex fungal community composition were observed across all soil samples in responding to different *A. gallica* strains (Figures 6, 7). In a co-culture study, Collins et al. (2013) revealed the defensive and potentially offensive nature of *Armillaria* members and their ability to influence other microbes. Thus, it is acceptable that greater variation was observed in the fungal community induced by *A. gallica* strains, considering that soil fungal communities are more sensitive and respond more quickly to a variety of stimuli, including biological disturbance (Xiao et al., 2014).

As also reported, the *Armillaria* members are known as wood-rotting fungi (Brundrett, 2002), along with an ability to secrete wood-decomposition enzymes such as glycoside hydrolases. When *A. gallica* exists in soil and attempts symbiosis with *G. elata*, it will typically secrete enzymes to penetrate plant cell walls. The enzymes can mineralize or decompose most plant cell wall polymers into simple compounds (Floudas et al., 2012; Purahong et al., 2016), which increase their accessibility to other organisms. A previous study conducted with five *Armillaria* isolates reported a positive effect of an excellent *Armillaria* strain on the promotion of quality and yield of *G. elata* Bl. f. glauca in Changbai Mountain (Yu et al., 2022). The researchers found that excellent *Armillaria* strains increased the yield of *G. elata* Bl. f. glauca by improving the soil microbial environment, especially by increasing the relative abundances of some beneficial genera such as *Mortierella*, *Agrocybe*, and *Armillaria*. In accordance with this study, the high yield of *G. elata* co-planted with the YN strain was accompanied by higher relative abundances of *Mortierella*, *Nemania*, *Agrocybe*, *Archaeorhizomyces*, *Armillaria*, *Pseudoclathrosphaerina*, *Scopuloides*, *Leptodontidium*, *Cladophialophora*, *Psathyrella*, and *Saitozyma* compared with these in other *A. gallica* strains (Figure 7B). Species of the genera *Mortierella*, *Nemania*, and *Leptodontidium* were commonly reported as wood-rotting members and are sensitive to readily accessible carbohydrates (Tang et al., 2007; Lindahl et al., 2010; Werner et al., 2016). Their strong co-occurrence with the YN strain implied that they were candidate fungal taxa that might be involved in the high activity of *A. gallica*. Accordingly, *Archaeorhizomyces* species are non-pathogenic plant root and rhizosphere-associated fungi that commonly harbor deeper soil horizons with low pH and high nutrient turnover (Rosling et al., 2011; Carrino-Kyker et al., 2016). Previous findings suggest that correlations between *Archaeorhizomycetes* and other fungal taxa indicate that *Archaeorhizomycetes* might benefit from carbohydrates or nutrients (Pinto-Figueroa et al., 2019) and may also have potential associations with plant growth (Schadt et al., 2003). Similarly, species of the genus *Cladophialophora* possessed superior growth promotion activities as well as disease suppression, which significantly increased the growth parameters of strawberry plants (Harsonowati et al., 2020). As a result, the combination of yields of *G. elata* and responses of these fungal genera due to *A. gallica* strains in our study may reflect the potential performance of biological and ecological functions in soil fungal communities.

It is well known that the interactions of interspecific fungi are complex and dynamic processes (Awan and Asiegbu, 2021). Co-occurrence networks indicated that bacteria such as *Candidatus_Solicabcter*, *bacterium_f_Gemmatimonadaceae* were highly central and connected (Figure 8A), suggesting that they might drive the observed *A. gallica*-induced changes in bacterial networks. Additionally, for fungal networks, the genera *Sporothrix* (belonging to Ascomycota) and *Scopuloides* (belonging to Basidiomycota) all interacted with 12 genera, including 7 positive interaction genera and 5 negative interaction genera, implying their important roles in shaping fungal community under such complex environmental conditions. Based on previous studies, soil bacterial communities are less resistant but more resilient to disturbance than fungal communities (de Vries et al., 2018), and changes in functional taxa can alter the response of microbial communities to disturbance and their symbiotic relationships with host plants (Banerjee et al., 2018). In the current study, shifts in the relative abundance of dominant taxa (Figures 6, 7) might later facilitate the stability of networks (Figure 8), and consequently the assemblage of fungal communities in soil substrates and response of soil fungi to different *A. gallica* strains. Besides that, soil physicochemical properties or nutrient cycling were expected to be altered by symbiotic fungi (Finlay, 2004; Veresoglou et al., 2012), and correlations can be tested between environmental factors and microbial community structure. However, measurements of soil physicochemical properties were not conducted due to the slight amount of soil around *G. elata*, which is a

limitation of this study. Further study is needed to discuss the changes in soil physicochemical properties arising from *A. gallica* strains and the correlations between them.

The current study emphasizes that soil fungi rather than bacteria were more easily modified by exotic *A. gallica* strains, and that might have a potentially beneficial impact on the growth and yield of *G. elata*. Specifically, the dominant advantage of the YN strain was evidenced by its role in triggering changes in the soil fungal community, especially relative abundances of some beneficial genera such as *Mortierella*, *Agrocybe*, *Neminia*, *Armillaria*, and so on when cultivated in Guizhou Province. Although our study identified these potentially beneficial genera accompanied by a high yield of *G. elata*, further support is needed to confirm the potential functions and ecosystem services of these soil microbes by isolating and proving their roles in suppressing or fostering *A. gallica* in a co-culture experiment or mutualistic trial study with *G. elata*.

Conclusion

The current study revealed that under field conditions in *G. elata* and *A. gallica* symbiotic systems, the yield of *G. elata* significantly differed among *A. gallica* strains collected from several regions. In addition, the diversity, structure, and composition of soil microbial communities, especially fungal communities, strongly changed in response to different *A. gallica* strains, which might have a beneficial effect on the yield of *G. elata*. This comprehensive understanding of the responses that govern the selection and activity of microbial communities by *A. gallica* strains can be committed to practical management and provide us with new opportunities to increase the yields of *G. elata*.

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/, PRJNA951876; https://www.ncbi.nlm.nih.gov/, PRJNA951884.

Author contributions

YW: funding acquisition, methodology, formal analysis, writing—original draft preparation. JX: funding acquisition, methodology, and visualization. QY: funding acquisition, methodology, writing—review and editing. LG: conceptualization and supervision. CX: methodology and software. CY: formal analysis and data curation. LL: resources and data curation. WJ: conceptualization and supervision. TZ: conceptualization, funding acquisition, and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1233555/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Collection sites of *Armillaria* rhizomorphs from four main producing regions of *G. elata*.

SUPPLEMENTARY FIGURE S2
The conditions of *Armillaria* strains packets used for *G. elata* artificial cultivation. **(A)**, **(B)**, **(C)**, and **(D)** represent *Armillaria* of YN strain, GZ strain, SX strain, and AH strain respectively.

SUPPLEMENTARY FIGURE S3
The conditions of *Armillaria* strains mycelia at the harvest time. **(A)**, **(B)**, **(C)**, and **(D)** represent *Armillaria* of YN strain, GZ strain, SX strain, and AH strain respectively.

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EDITED BY
Nirmalendu Basak,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Miaochun Fan,
Northwest A&F University, China
Xinxin Liu,
Shanghai Jiao Tong University, China

*CORRESPONDENCE
Tengbing He
✉ tbhe@gzu.edu.cn

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Characteristics of rhizosphere and bulk soil microbial community of Chinese cabbage (*Brassica campestris*) grown in *Karst* area

Xiaoliao Wei[1], Tianling Fu[2], Guandi He[1], Zhuoyan Zhong[1], Mingfang Yang[1], Fei Lou[1] and Tengbing He[1,2]*

[1]College of Agriculture, Guizhou University, Guiyang, China, [2]Engineering Key Laboratory for Pollution Control and Resource Reuse Technology of Mountain Livestock Breeding, Institute of New Rural Development, Guizhou University, Guiyang, China

Understanding the rhizosphere soil microbial community and its relationship with the bulk soil microbial community is critical for maintaining soil health and fertility and improving crop yields in *Karst* regions. The microbial communities in the rhizosphere and bulk soils of a Chinese cabbage (*Brassica campestris*) plantation in a *Karst* region, as well as their relationships with soil nutrients, were examined in this study using high-throughput sequencing technologies of 16S and ITS amplicons. The aim was to provide theoretical insights into the healthy cultivation of Chinese cabbage in a *Karst* area. The findings revealed that the rhizosphere soil showed higher contents of organic matter (OM), alkaline hydrolyzable nitrogen (AN), available phosphorus (AP), total phosphorus (TP), available potassium (AK), total potassium (TK), total nitrogen (TN), catalase (CA), urease (UR), sucrase (SU), and phosphatase (PHO), in comparison with bulk soil, while the pH value showed the opposite trend. The diversity of bacterial and fungal communities in the bulk soil was higher than that in the rhizosphere soil, and their compositions differed between the two types of soil. In the rhizosphere soil, Proteobacteria, Acidobacteriota, Actinobacteriota, and Bacteroidota were the dominant bacterial phyla, while Olpidiomycota, Ascomycota, Mortierellomycota, and Basidiomycota were the predominant fungal phyla. In contrast, the bulk soil was characterized by bacterial dominance of Proteobacteria, Acidobacteriota, Chloroflexi, and Actinobacteriota and fungal dominance of Ascomycota, Olpidiomycota, Mortierellomycota, and Basidiomycota. The fungal network was simpler than the bacterial network, and both networks exhibited less complexity in the rhizosphere soil compared with the bulk soil. Moreover, the rhizosphere soil harbored a higher proportion of beneficial Rhizobiales. The rhizosphere soil network was less complicated than the network in bulk soil by building a bacterial–fungal co-occurrence network. Furthermore, a network of relationships between soil properties and network keystone taxa revealed that the rhizosphere soil keystone taxa were more strongly correlated with soil properties than those in the bulk soil; despite its lower complexity, the rhizosphere soil contains a higher abundance of bacteria which are beneficial for cabbage growth compared with the bulk soil.

KEYWORDS

microbial communities, enzymatic activity, soil properties, bacteria and fungi, co-occurrence network

Introduction

Rhizosphere microorganism has a vital function in fostering plant development and preserving plant health (Fan et al., 2017). The rhizosphere microbial diversity is greatly influenced by the bulk soil, and changes in the bulk soil microbial communities also impact the rhizosphere species diversity (Mendes et al., 2014). Edwards et al. (2015) proposed a multi-step model for the composition of soil microbes from roots in pure cultures or greenhouses, where each rhizosphere-associated compartment contains a distinct microbiome. In real agricultural systems, there is a decline in microbial diversity near the rhizosphere (Ling et al., 2022). However, most studies have focused solely on either fungal or bacterial communities (Zhang et al., 2017), and there has been limited research on the properties of microbial communities in the rhizosphere and bulk soils of crops and their response to rhizosphere effects (Fan et al., 2018). Microbial communities consist of species involved in symbiotic interactions that compete for food, energy substances, and spaces (Hibbing et al., 2010; Faust and Raes, 2012). Co-occurrence networks have been found particularly useful in studying complex relationships between numerous microbial species (Faust and Raes, 2012; Röttjers and Faust, 2018). These networks are widely used to research microbiome connections and provide valuable insights into microbial co-occurrence patterns and their critical mechanisms (Ma et al., 2020). Studies have shown that grape and soybean rhizosphere soil communities are subsets of their bulk soil communities in short-term farming systems, and the rhizosphere exhibited a simpler network of bacterial communities than bulk soil (Mendes et al., 2014). Fan et al. (2017) investigated the symbiotic patterns of three soils in the rhizosphere, such as bulk, loosely bound, and tightly bound rhizosphere in wheat and found distinct network structures and distribution patterns among them. Additionally, Wang et al. (2022) examined differences in compositional processes and bacterial community co-occurrence patterns in the rhizosphere and bulk soils with the addition of nitrogen, which were closely related to rhizosphere exudates. However, there is limited information available on topological changes in the Chinese cabbage rhizosphere bacterial and fungal symbiotic interactions in the *Karst* area compared with the bulk soil. In addition, keystone taxa play important roles in co-occurrence networks (Fan et al., 2018). Their removal may negatively impact microbiome stability and lead to significant changes in microbiome composition and function (Banerjee et al., 2018). Keystone taxa have been found in different environments (Fisher and Mehta, 2014; Fan et al., 2018), and they serve ecological functions in the rhizosphere, inhibiting fungal and bacterial pathogens (Mendes et al., 2011). Previous studies have identified a few keystone taxa and found that although they have an important role in the network, their relative abundance is relatively low (Shi et al., 2016). However, few studies have focused on the fungal and bacterial keystone taxa of *Karst* agroecosystems, let alone attempted to identify the soil properties that influence their distribution and interactions (Fan et al., 2018).

Chinese cabbage (*Brassica campestris*), a crucial cruciferous crop extensively cultivated and consumed in *Karst* areas (Nie et al., 2017), faces challenges due to the ecologically fragile nature of these landscapes (Wang et al., 2018), where agriculture plays a crucial role for livelihood (Chen et al., 2021). The cultivated land in this area features prominent exposure to carbonate rock, limited soil volume, shallow soil depth, and gradual soil development (Zhang et al., 2016), making land resources scarce. Therefore, improving crop productivity, especially Chinese cabbage, becomes crucial to meet the population's demand in this limited land (Timmusk et al., 2017). Particularly in this with restricted nutrient availability, the root system is essential to plant production (Camargo et al., 2023; Rüger et al., 2023). Previous studies have focused on the microbial community in the rhizosphere of Chinese cabbage to improve resistance and yield. For example, the biological control agent *Streptomyces alfalfae* XY25 T demonstrated promise in reducing clubroot disease, controlling rhizosphere bacterial and fungal populations in the rhizosphere of Chinese cabbage, and enhancing growth in greenhouse conditions (Hu et al., 2021); *Trichoderma harzianum LTR-2,* which works by modifying the rhizosphere soil microbial population, is discovered to be an efficient biological control agent for cabbage clubroot (Li et al., 2020). Additionally, three preceding crops were shown to increase Chinese cabbage yield and suppress clubroot disease by regulating the soil environment and microbial communities in the rhizosphere (Zhang et al., 2022). Furthermore, three arsenic-tolerant bacterial strains were isolated from the Chinese cabbage rhizosphere and bulk soils, and these strains were capable of promoting the growth of edible tissues while reducing the uptake of arsenic and cadmium by these tissues (Wang et al., 2017). Understanding microbial communities in the rhizosphere soil and their relationship with the microbial community in the bulk soil is essential for sustainable ecosystem functioning and improved crop yields in the *Karst* areas. However, there is limited research on the rhizosphere microorganisms of Chinese cabbage in *Karst* areas and their characterization in relation to the microbial community of the bulk soils and their relationship with soil nutrients. This study used high-throughput sequencing of 16S and ITS amplicons to investigate the bacterial and fungal populations in the rhizosphere and bulk soils of cabbage crops in *Karst* locations. The hypothesis was that the rhizosphere soils would exhibit a lower diversity of bacteria and fungi compared with bulk soils, and the community composition and co-occurrence networks would be simpler in the rhizosphere soils. Additionally, it was anticipated that the bacterial and fungal populations in the rhizosphere would be more impacted by the properties of the soil. The goals of this study are as follows: (1) to assess the differences in microbial community structure, potential functions, and diversity between rhizosphere and bulk soils of Chinese cabbage plantations in *Karst* regions; (2) to identify bacterial and fungal keystone taxa in rhizosphere and bulk soils of Chinese cabbage plantations in *Karst* regions. These findings will provide valuable theoretical data for promoting healthy cabbage cultivation in the *Karst* area.

Materials and methods

Study area and soil sampling

The study area covers ~0.35 square kilometers and is situated in Shiliping Town, Xingyi City, Guizhou Province, Southwest China's

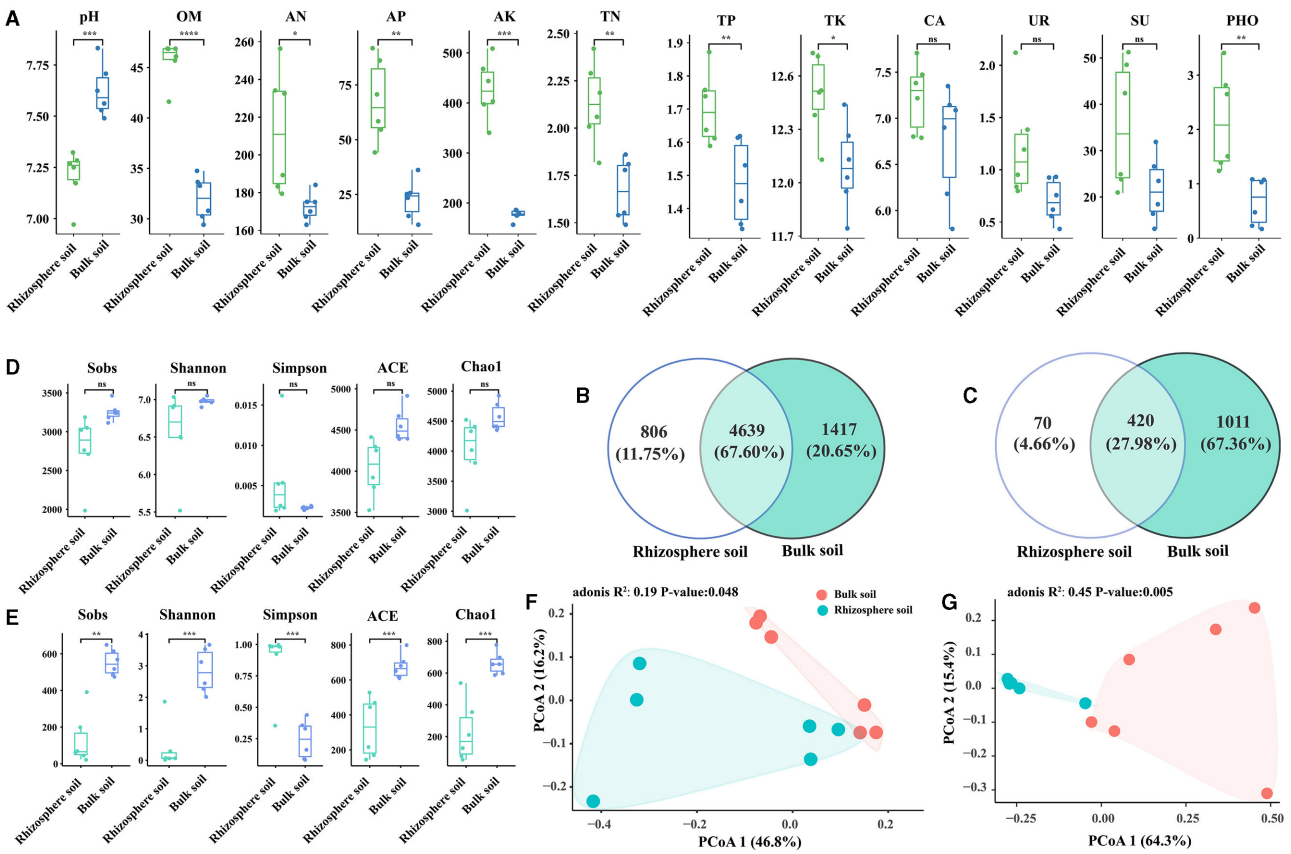

FIGURE 1
Variations in soil properties **(A)**, OTU numbers **(B, C)**, and diversity **(D–G)** between the rhizosphere soil and bulk soil of Chinese cabbage; *t*-tests were conducted for soil properties **(A)**; the number of bacterial OTUs **(B)**; the number of fungal OTUs **(C)**; bacterial alpha diversity **(D)**; fungal alpha diversity **(E)**; PCoA analysis of bacterial beta diversity **(F)**; and fungal beta diversity **(G)**. The indicators such as pH, OM, AN, AP, AK, TN, TP, TK, CA, UR, SU, and PHO represent hydrogen ion concentration, organic matter, alkali hydrolyzable nitrogen, available phosphorus, available kalium, total nitrogen, total phosphorus, total potassium, catalase, urease, sucrase, and phosphatase, respectively. "ns" shows at $p > 0.05$, "*" shows at $p \leq 0.05$, "**" shows at $p \leq 0.01$, "***" shows at $p \leq 0.001$, and "****" shows at $p \leq 0.0001$.

Karst area (24°58′ 0′′ N-25°1′ 0′′ N, 104°50′′ 0′ E-104°55′ 0′′ E) (Supplementary Figure S1). The plots measured 5 m × 5 m, with six replicates being sampled. In each plot, 25 Chinese cabbage plants were collected by the five-point sampling method. Loosely bound soil was shaken off, and tightly adhered soil was brushed off with sterilized brushes to collect the rhizosphere soil ($n = 6$). In addition to each group of Chinese cabbage plants, topsoil (0–20 cm) without plants was collected using a soil auger to serve as the bulk soil ($n = 6$). Approximately 50 g of soil was taken and placed in a sterile bag and stored in an icebox (4–10°C) in the field, and another portion was brought back to the laboratory in a sterile bag. The first part (50 g) of soil was stored in an −80°C ultra-low temperature refrigerator and used for soil microbial analysis, while the other part of the soil was placed in the room to air dry and then used for analysis of soil properties after grinding.

Analyses of soil properties

Standard soil testing techniques were employed to assess various physical and chemical properties of soil, including organic matter (OM), alkaline hydrolyzable nitrogen (AN), total nitrogen (TN), available potassium (AK), total potassium (TK), available phosphorus (AP), and total phosphorus (TP) (Bao, 2000); soil pH was determined potentiometrically with water: soil ratio of 2.5:1 (v/w). Additionally, soil enzyme activity, namely catalase (CA), urease (UR), sucrase (SU), and phosphatase (PHO) in soil; potassium permanganate titration was performed to determine soil catalase activity (CA), expressed in milliliters of 0.1 N potassium permanganate in 1 g of soil after 20 min of shaking in a shaker. The sodium phenolate colorimetric method was used to determine urease (UR). The activity of urease was expressed as the milligrams of NH_3-N in 1 g of soil present after incubation for 24 h in an incubator at 37°C. The sucrase (SU) was measured by the 3,5-dinitrosalicylic acid colorimetric method, and the activity of sucrase was expressed as the milligrams of glucose produced in 1 g of soil incubated after 24 h in an incubator at 37°C. The phosphatase (PHO) was measured by the disodium phenyl phosphate colorimetric method; after incubating in an incubator at 37°C for 24 h, the phenol mass (mg) released in 1 g of soil represented the activity of phosphatase (Zhou and Zhang, 1980).

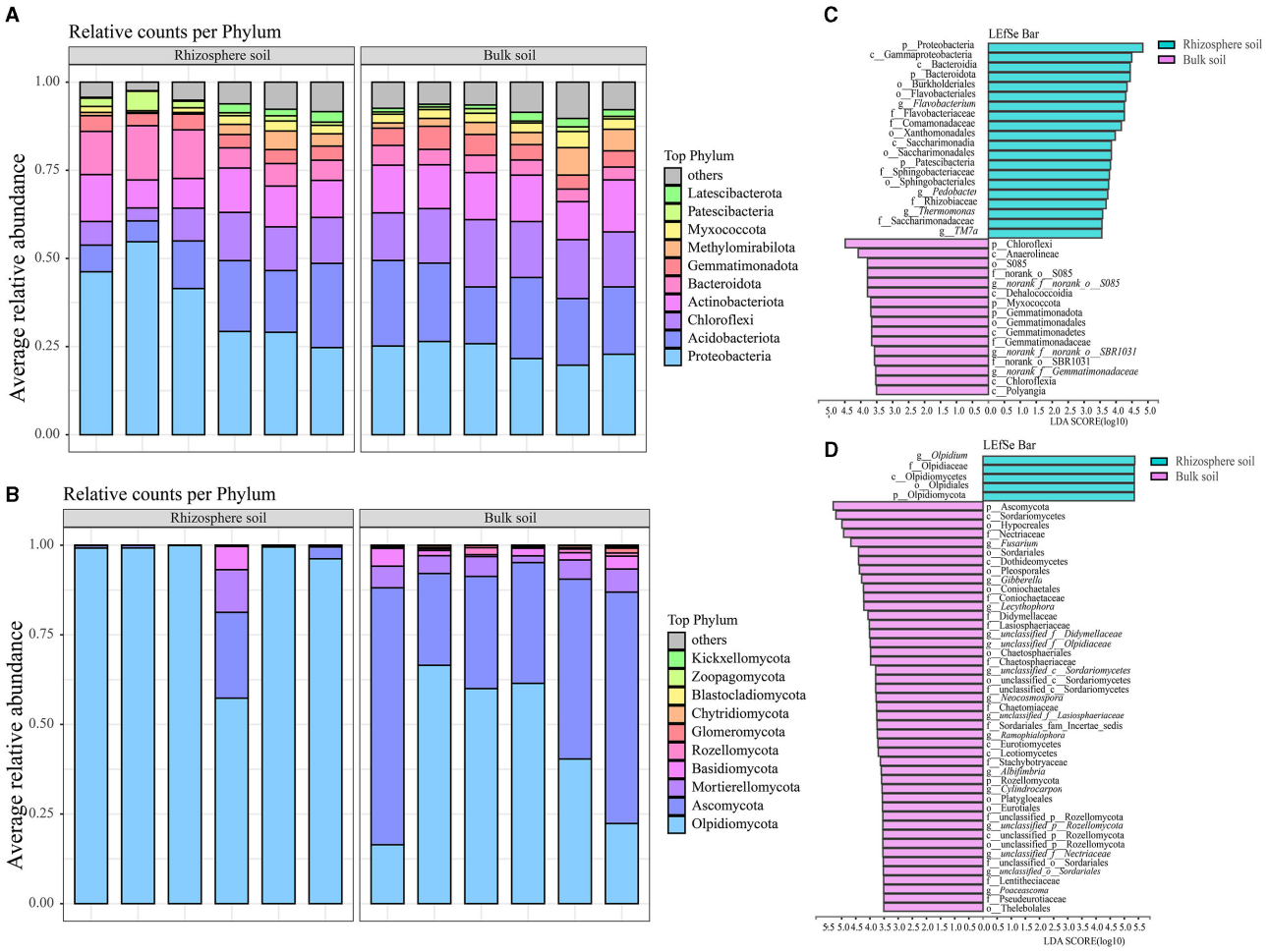

FIGURE 2
Composition and LefSe analysis of bacterial and fungal communities in both the rhizosphere soil and bulk soil of Chinese cabbage. **(A, B)** represent soil bacterial and fungal composition (at the phylum level), respectively; **(C, D)** stand for LefSe analysis results for bacteria and fungi (respectively, ranging from the phylum to genus level).

Extraction of soil DNA and sequencing of amplicon

Soil genomic DNA was extracted from 0.5 g of frozen soil (−80°C) utilizing the FastDNA® Spin Kit for soil (MP Biomedicals, USA), following the manufacturer's protocol. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a spectrophotometer of NanoDrop2000 (Thermo Scientific Inc., USA) and stored at −80°C before further use. The V3-V4 region of the bacterial 16S rRNA gene was amplified utilizing the 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers (Liu et al., 2016), and the ITS1 region of ITS rRNA gene was targeted using ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) primers (White et al., 1990; Gardes and Bruns, 1993). Each sample was subjected to triplicate amplification, and detailed steps are provided in the Supplementary Files. The PCR products were combined in equal proportions and sequenced on the Illumina MiSeq PE300 platform (Illumina, USA), following the standard protocol at Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China). The raw sequencing data were uploaded to the NCBI Sequence Read Archive database with the accession number PRJNA865816.

Bioinformatics and statistics

The raw FASTQ files were processed utilizing QIIME to demultiplex and quality-filter them based on specific conditions (Caporaso et al., 2010), and detailed steps are provided in the Supplementary Files. The operational taxonomic units (OTUs) for bacteria and fungi were clustered at a 97% sequence similarity threshold, using the UPARSE program, and chimeric sequences were removed using UCHIME (Edgar et al., 2011; Edgar, 2013). To keep the number of valid sequences consistent for each sample, the original sequence was drawn flat according to the minimum sample sequence. OTUs not belonging to soil bacterial and fungal communities were excluded, resulting in 6,862 bacterial and 1,501 fungal OTUs. The taxonomic composition of bacterial and fungal

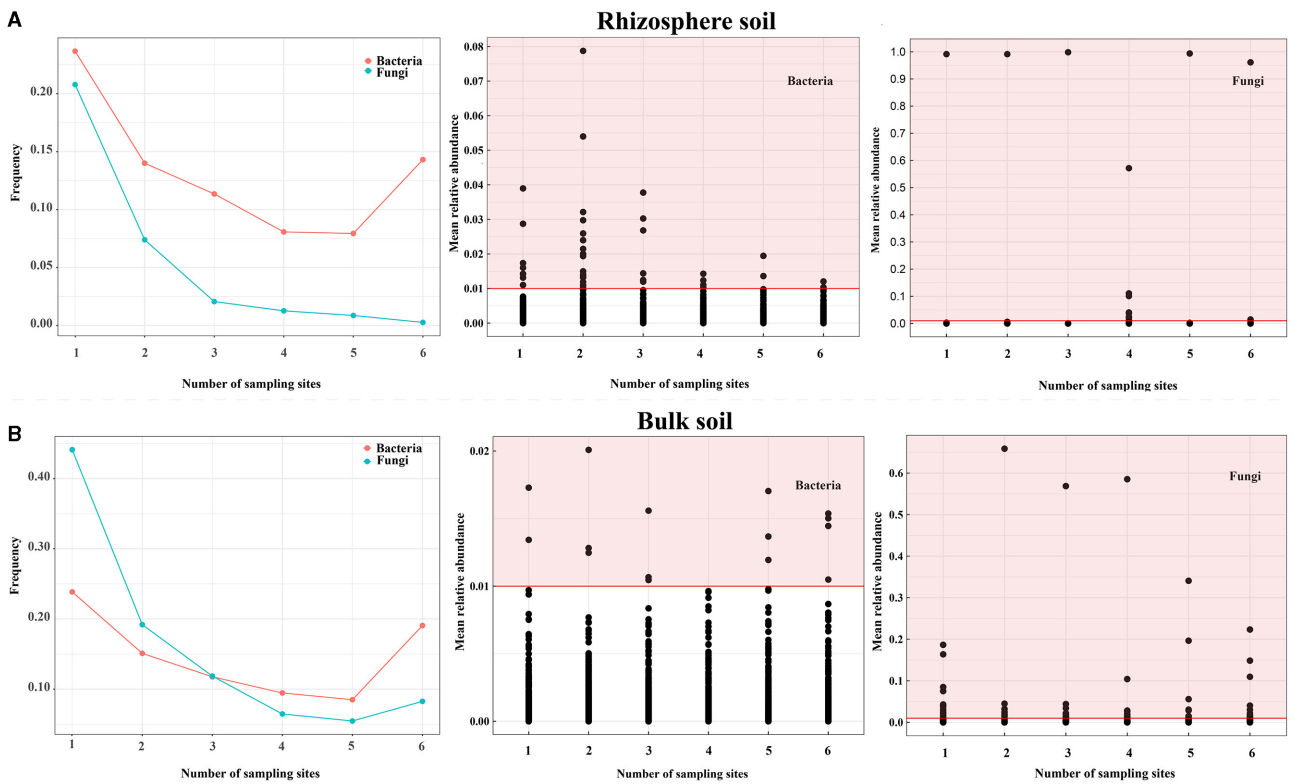

FIGURE 3
Distribution of soil bacteria and fungi of Chinese cabbage in the *Karst* area. **(A, B)** show the distribution frequency of bacterial and fungal OTUs, the OTUs that bacteria have a relative abundance of >1%, and the OTUs that fungi have a relative abundance of >1% in the rhizosphere and bulk soils, respectively. Notes: The red highlighted area indicates the number of bacterial and fungal OTUs with a relative abundance of > 1% in all sampling points for both the rhizosphere and bulk soils.

OTUs was determined using the Bayesian-based RDP classifier method with data from SILVA and UNITE databases. Functional prediction analysis of the 16S rRNA gene and ITS rRNA gene was performed utilizing FAPROTAX and FUNGuild on the Majorbio Cloud Platform. Correlation analysis and ANOVA were conducted utilizing SPSS statistical software (IBM Corporation, USA). The relative abundance of microorganisms is the proportion of specific bacteria or fungi among all bacteria or fungi, expressed as a percentage. The ggplot2, ggvenn, reshape2, d3Network, and circlize packages of R were used to create clustered heatmaps, boxplots, Venn diagrams, stacked percentage plots, Sankey diagram, and chord diagram, respectively (Wei et al., 2023). Moreover, a principal coordinate analysis (PCoA) was performed utilizing the R vegan package, and PERMANOVA, with 999 permutations, was utilized to validate the PCoA findings according to the Bray–Curtis distance. The Mantel test was achieved utilizing the vegan package in R software (Yang et al., 2017). RDA analysis was chosen based on the gradient lengths of the first axis in the DCA analysis results for bacteria and fungi (1.10 and 2.08, respectively), and its visualization was referenced from previous research (Wei et al., 2023). Network analysis was conducted according to Spearman (Spearman's r <- 0.7 or r > 0.7; P < 0.05), and other screening conditions and visualizations were followed from previous studies (Wei et al., 2023). The igraph tool in R was used to create the co-occurrence network, and Gephi was utilized for visualization (Zhang et al., 2018a). According to descriptions from earlier studies, keystone taxa were chosen (Liu et al., 2022).

Results

Analysis of soil properties and microbial diversity

In comparison with bulk soils, the rhizosphere of Chinese cabbage showed higher contents of organic matter (OM), alkaline hydrolyzable nitrogen (AN), available phosphorus (AP), total phosphorus (TP), available potassium (AK), total potassium (TK), total nitrogen (TN), catalase (CA), urease (UR), sucrase (SU), and phosphatase (PHO), while the pH value showed the opposite trend. These differences were statistically significant, except for CA, UR, and SU (Figure 1A). Bacterial species were more abundant than fungal species, and the rhizosphere soil had a lower number of OTUs than the bulk soil (Figures 1B, C). Notably, the rhizosphere soil exhibited lower bacterial and fungal alpha diversity values for indices such as Sobs, Shannon, ACE, and Chao1 compared with the bulk soil. Nevertheless, the Simpson index exhibited the contrary pattern, suggesting that the rhizosphere soil had a lower alpha diversity of bacteria and fungi than bulk soil. Additionally, there was a significant difference in the alpha diversity

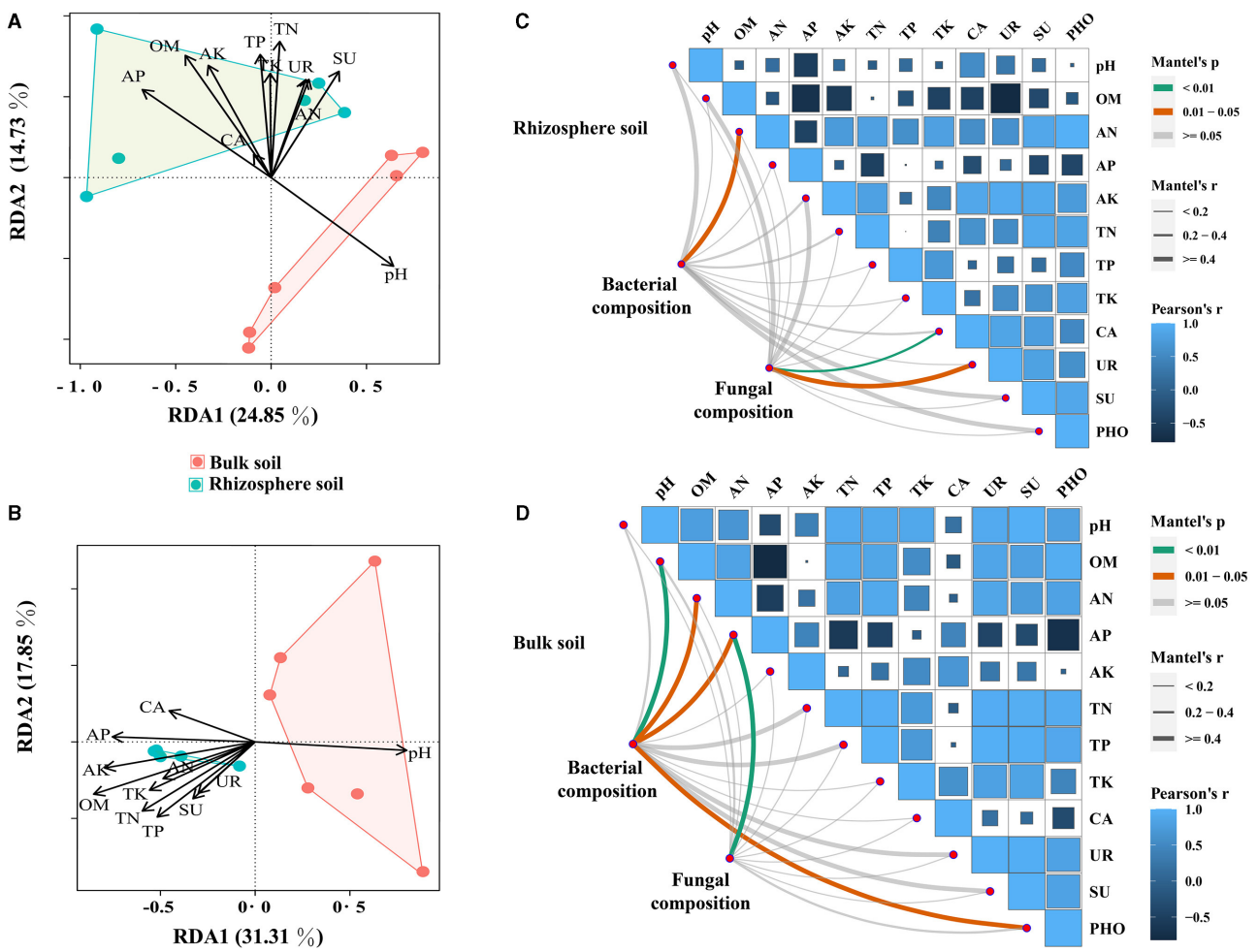

FIGURE 4
Mantel's test and RDA analysis between soil properties and bacterial and fungal communities (at the genus level). **(A, B)** represent the Mantel test results for the correlation between soil bacterial and fungal communities and soil properties, respectively. **(C, D)** represent RDA analysis results for the correlation between soil bacterial and fungal communities and soil properties, respectively.

of fungi between rhizosphere and bulk soils ($p < 0.05$), while bacterial alpha diversity did not exhibit significant variation ($p > 0.05$) (Figures 1D, E). The correlation analysis showed a positive relationship between bacterial Shannon index and fungal Shannon index, and a similar pattern was observed for other indices (except for Shannon and Simpson indices) (Supplementary Table S1). Furthermore, the PCoA results for communities of bacteria and fungi displayed clear differences between bulk and rhizosphere soils (Figures 1F, G).

Composition of microbial community

Among the 42 bacterial and 10 fungal phyla identified, 4 major phyla collectively accounted for over 70% of the total relative abundances in both rhizosphere and bulk soils of the *Karst* region, where Chinese cabbage was cultivated (Figures 2A, B and Supplementary Table S2). Proteobacteria, Acidobacteriota, Actinobacteriota, and Bacteroidota were dominant bacterial phyla in the rhizosphere soil, representing 37.59, 14.74, 10.73, and 9.87% of the total relative abundances, respectively. In contrast, Proteobacteria, Acidobacteriota, Chloroflexi, and Actinobacteriota were dominant bacterial phyla in the bulk soil, accounting for 23.60, 20.60, 16.03, and 13.00% of the total relative abundances, respectively (Figure 2A and Supplementary Table S2). The rhizosphere soil had higher relative abundances of *Olpidiomycota*, *Ascomycota*, *Mortierellomycota*, and *Basidiomycota*, which accounted for 91.89, 4.86, 2.03, and 2.03% of the total relative abundances, respectively. In comparison, the bulk soil had higher relative abundances of *Ascomycota*, *Olpidiomycota*, *Mortierellomycota*, and *Basidiomycota*, accounting for 46.16, 44.52, 5.03, and 2.44% of the total relative abundances, respectively (Figure 2B and Supplementary Table S2). Notably, there were significant differences in the fungal phyla between the bulk and rhizosphere soils. Particularly, the relative abundance of *Olpidiomycota* in the rhizosphere (91.89%) was approximately double the amount in the bulk soil (44.52%), whereas the relative abundance of *Ascomycota* was ~10 times higher in the

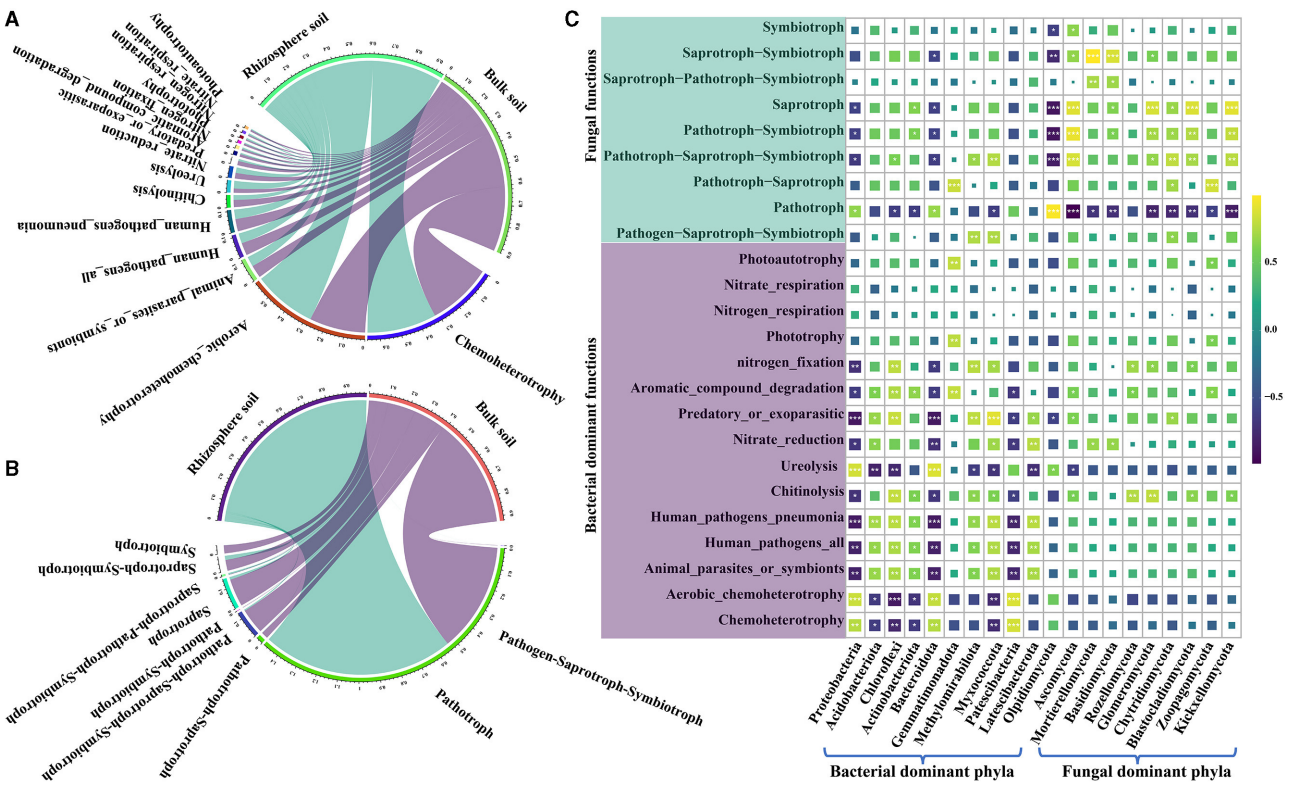

FIGURE 5
Functional prediction of bacterial FAPROTAX and fungal FUNGuild along with their correlation analysis with the dominant phyla. **(A)** Functional groups of the top 15 bacteria in the relative abundance. **(B)** functional groups of the top 15 fungi in the relative abundance. **(C)** correlation analysis between the dominant functional groups of soil bacteria and fungi and dominant bacterial phyla. "*" shows at $0.01 < p \leq 0.05$, "**" shows at $0.001 < p \leq 0.01$, and "***" shows at $p \leq 0.001$.

bulk soil (46.16%) than in the rhizosphere soil (4.86%). These findings indicate notable disparities in bacterial and fungal communities between the Chinese cabbage rhizosphere and bulk soils. LefSe analysis (LDA > 3.5) revealed remarkable variations at the taxonomy levels from phylum to genus between the bulk soils and rhizospheres (Figures 2C, D). Specifically, a greater number of bacterial taxa, including *Proteobacteria*, *Gammaproteobacteria*, and *Bacteroidia*, were enriched in the rhizosphere of Chinese cabbage compared with the bulk soils. In addition, the rhizospheres exhibited higher relative abundances of fungi such as *Olpidium*, Olpidiaceae, and *Olpidiomycetes*, compared with the bulk soils (Figure 2C). In contrast, a higher number of fungal taxa, such as *Chloroflexi*, *Anaerolineae*, and *S085*, were enriched in the Chinese cabbage bulk soils, accompanied by higher fungal relative abundances such as Ascomycota, Sordariomycetes, and Hypocreales, compared with the rhizosphere soils (Figure 2D).

Differences in the distribution of bacteria and fungi

To assess the dissimilarities in the distribution of bacterial and fungal communities between bulk and rhizosphere soil (soils at the OTU level), the OTU distributions at each sampling point were analyzed (Figure 3A and Supplementary Table S3). Bacteria were more abundant than fungi across all sites (Figure 3A and Supplementary Table S3), while fungi exhibited more OTUs with a relative abundance of >1% at all sites (0.16% for fungi versus 0.1% for bacteria) in the rhizosphere soil (Figure 3A). However, most fungi were present at ≤3 sampling point OTU (>75% of OTUs), whereas bacteria were more prevalent at >3 sites (Figure 3B and Supplementary Table S3) in the bulk soil. Moreover, fungi exhibited more OTUs with a relative abundance of >1% in all sites (0.71% for fungi vs. 0.04% for bacteria) in the bulk soil (Figure 3B). To identify core OTUs, criteria were applied for OTUs that appeared in all samples with an average relative abundance of >0.1% in each group. In the rhizosphere soil, core OTUs comprised all OTUs (0.20% for fungi and 2.67% for bacteria) and were mainly distributed across multiple bacterial and fungal phyla, such as Proteobacteria, Acidobacteriota, Bacteroidota, Olpidiomycetes, and Ascomycota. In the bulk soil, core OTUs represented all OTUs (2.89% for bacteria and 3.46% for fungi) and were typically distributed across multiple bacterial and fungal phyla, such as Acidobacteriota, Actinobacteriota, Chloroflexi, Ascomycota, and Mortierellomycota (Supplementary Table S4). These findings indicated that bacterial and fungal communities in rhizosphere and bulk soils exhibit distinct distribution patterns,

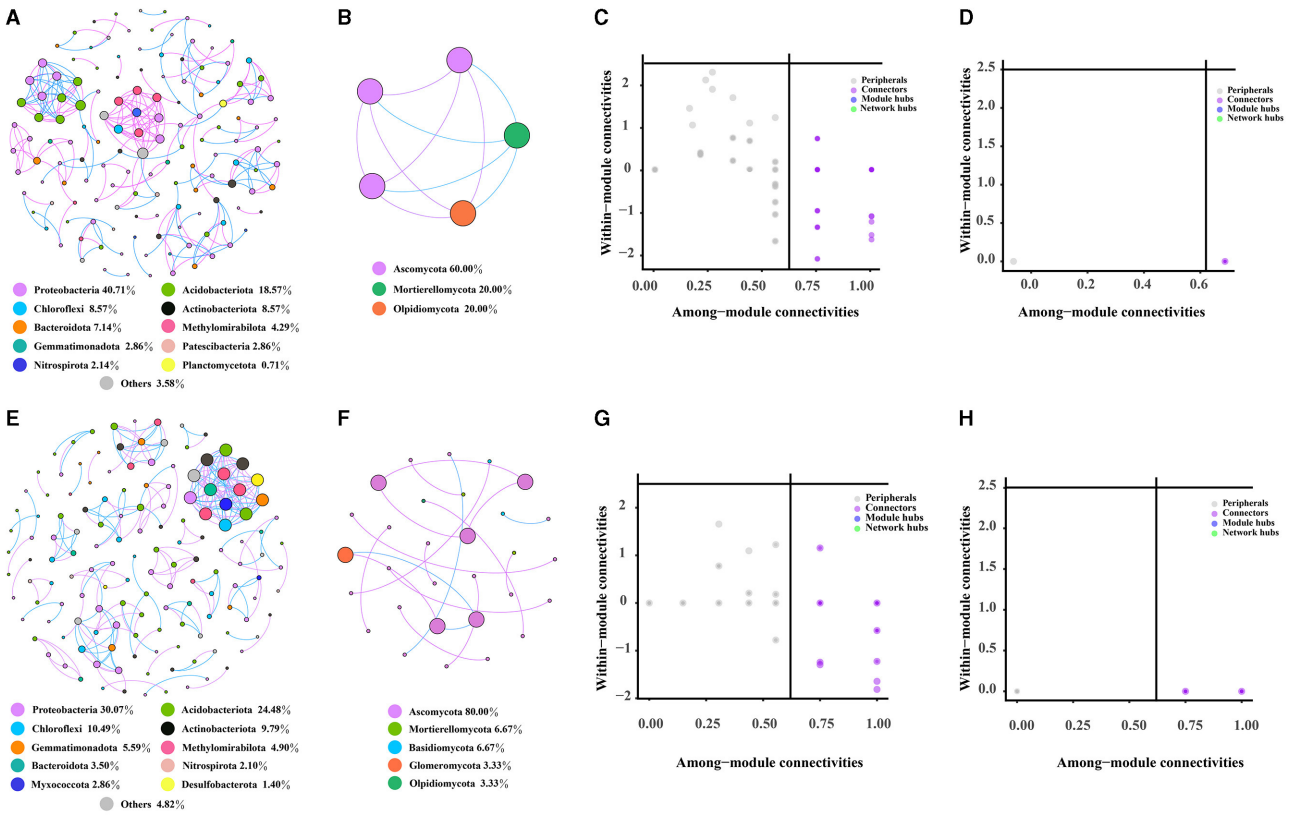

FIGURE 6
Characteristics of the rhizosphere and bulk soil networks. **(A, B)** represent the co-occurrence network of bacteria and fungi in the rhizosphere soil, respectively. **(C, D)** represent the keystone taxa of rhizosphere soil bacterial and fungal co-occurrence network, while **(E, F)**, respectively, represent the co-occurrence network of bacteria and fungi in the bulk soil. **(G, H)** show the keystone taxa of the bacterial and fungal co-occurrence network in the bulk soil.

and bacteria generally have a wider distribution range compared with fungi.

Correlation between soil properties and microbial community

Herein, the impact of soil properties on bacterial and fungal communities in relative abundance was investigated. To achieve this, Pearson's correlation analysis on the top 20 genera in relative abundance was conducted to assess their correlation with soil properties. The results revealed significant associations between several bacterial and fungal taxa and soil properties, with pH, AP, OM, and AK showing particularly strong correlations (Supplementary Figure S2). Notably, a positive correlation between pH and the dominant bacterial and fungal genera was found, while other soil properties exhibited opposite trends (Supplementary Figure S2 and Supplementary Table S5). Furthermore, the results of RDA for communities of bacteria and fungi demonstrated that OM had the most pronounced impact, displaying the highest correlation coefficients (Figures 4A, B and Supplementary Table S6). The Mantel test results suggested that soil properties had a greater impact on the microbial community in bulk soil compared with rhizosphere soils (Figures 4C, D and Supplementary Table S7). Additionally, the associations between soil properties were explored, and it was found that OM had significant positive correlations with AN, AN, AP, AK, TN, TP, and TK (Supplementary Table S8).

Bacterial and fungal functional analyses

The functional groups of bacterial communities in both rhizosphere and bulk soils of a *Karst* region were analyzed using FAPROTAX. In the rhizosphere soil, 49 functional groups were identified; in the bulk soil, 47 functional groups were found (Figure 5A and Supplementary Table S9). The major bacterial functional groups included chemoheterotrophy, aerobic chemoheterotrophy, animal parasites or symbionts, human pathogen-causing pneumonia, chitinolysis, ureolysis, nitrate reduction, predatory or ectoparasitic, and aromatic compound degradation. Notably, bacterial communities in the rhizosphere soil exhibited higher levels of chemoheterotrophic and aerobic chemoheterotrophic activities and showed superior ureolysis abilities compared with the bacteria in the bulk

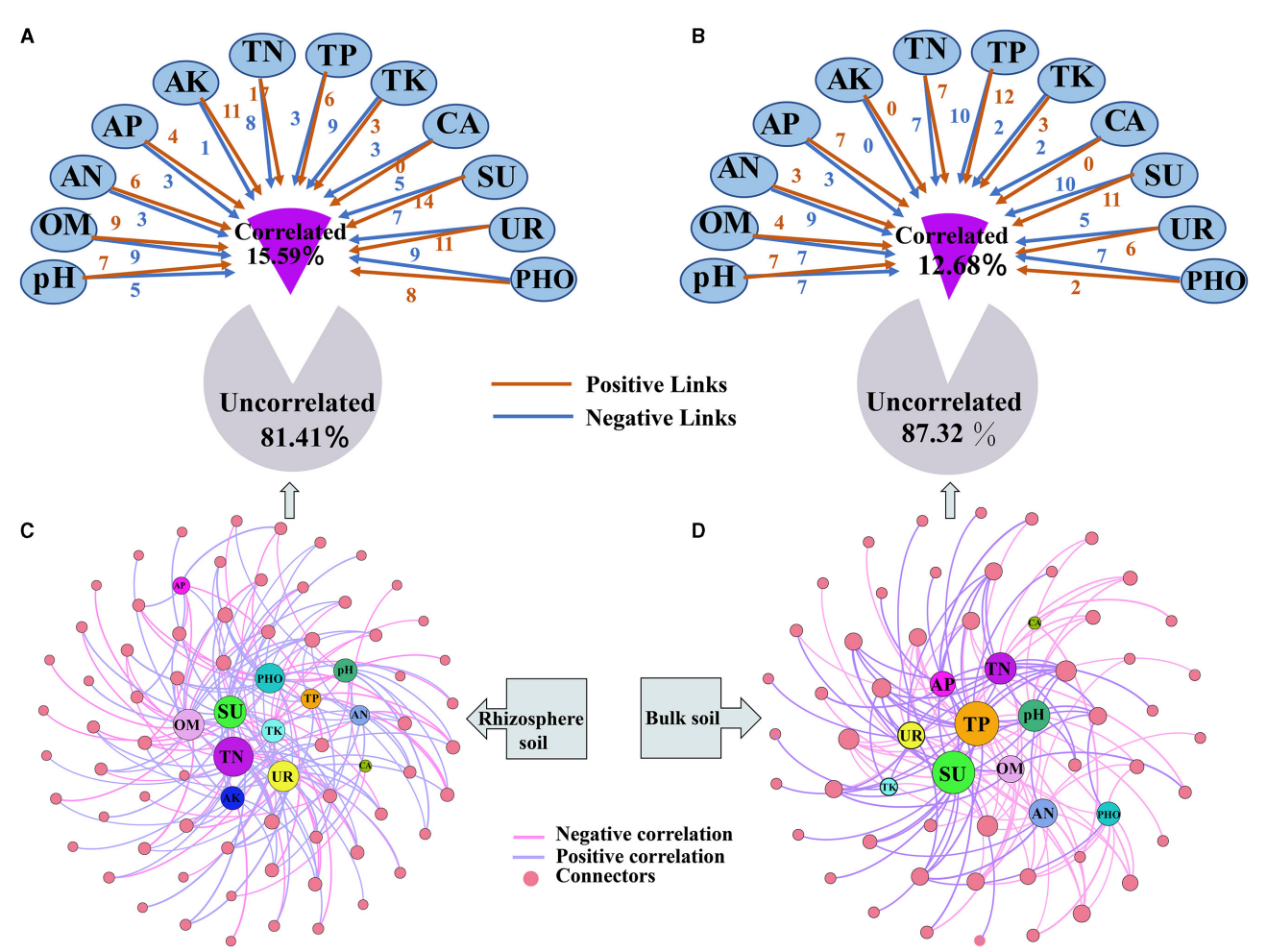

FIGURE 7
Influence of soil properties on keystone taxa in both rhizosphere and bulk soils. **(A, B)** represent the impact of soil properties on network connectors; **(C, D)** represent the correlation network between network connectors and soil properties.

soil (Supplementary Table S9). On the other hand, the bulk soil displayed a higher bacterial functional group in relative abundance associated with predatory or ectoparasitic activity and aromatic compound degradation compared with the rhizosphere soil. Moreover, bacterial pathogens were found to be less prevalent in the rhizosphere soil than in the bulk soil (Figure 5A and Supplementary Table S9). For the fungal communities, FUNGuild analysis was employed to classify their functional groups. It was found that 30.91% of the fungal OTUs were not classified into functional groups present in the FUNGuild database. Nevertheless, fungal OTUs with a confidence level of at least "probable" for analysis account for 92.43 to 99.25% of fungi in the total relative abundance (Figure 5B, Supplementary Table S9). The most prevalent trophic modes of fungi were pathotroph, saprotroph–symbiotroph, saprotroph, pathotroph–symbiotroph, and pathotroph–saprotroph–symbiotroph. Among these, pathotroph, saprotroph–symbiotroph, and saprotroph were more abundant in the rhizosphere soil, constituting 94.42, 2.04, and 1.29% of the total relative abundance, respectively. In the bulk soil, the dominant trophic modes were mainly pathotroph, saprotroph, and pathotroph–saprotroph–symbiotroph, accounting for 53.56, 13.56, and 12.41% of the total relative abundance, respectively (Supplementary Table S9). Surprisingly, the relative abundance of pathotrophs in the rhizosphere was approximately twice as high as that in the bulk soil, while the relative abundance of saprotrophs was 10 times higher in the bulk soil than in the rhizosphere (Supplementary Table S9). Furthermore, Pearson's correlation analysis was conducted to research the association between functional groups and the most dominant bacterial and fungal phyla. The results showed significant correlations between the majority of bacterial and fungal functional groups and their respective dominant phyla (Figure 5C and Supplementary Table S10). Additionally, certain functional groups of bacteria exhibited significant correlations with dominant fungal phyla, while some fungal functional groups correlated with dominant bacterial phyla (Figure 5C and Supplementary Table S10). Notably, a strong positive correlation between Ascomycota and saprotroph and a significant negative correlation between Olpidiomycota and saprotroph were found

(Supplementary Table S10). These findings indicate meaningful associations between dominant phyla and functional groups in the soil microbial communities.

Analysis of co-occurrence network

The bacterial, fungal, and bacterial–fungal co-occurrence networks in the rhizosphere and bulk soils of Chinese cabbage in a *Karst* region were investigated. Comparing the fungal networks, it was observed that bacterial networks had a greater number of nodes and edges in the rhizosphere and bulk soils (Figures 6A, E). The bulk soils were predicted to harbor a higher number of keystone OTUs belonging to bacterial and fungal categories, specifically Burkholderiales, Vicinamibacterales, Rhizobiales, and Hypocreales. Notably, Burkholderiales, Vicinamibacterales, and Rhizobiales were found to exhibit higher levels of enrichment in the rhizosphere compared with the bulk soils. The bacterial network showed a higher degree of complexity than the fungal network, as indicated by network characteristics such as nodes, edges, and keystone taxa (Supplementary Table S11). However, both networks in the rhizosphere soil were found to be less complex compared with those present in the bulk soils (Figures 6A–H). Additionally, it was observed that the proportion of bacteria (97.24%) far exceeded that of fungi (2.76%) (Supplementary Figure S3A); the majority of nodes belonged to *Proteobacteria* (39.31%), *Acidobacteriota* (17.93%), *Chloroflexi* (8.28%), and *Actinobacteriota* (8.28%) in the bacterial–fungal network in the rhizosphere soil. In the bulk soil, the bacterial–fungal network dominated most nodes, including *Proteobacteria* (23.35%), *Acidobacteriota* (19.29%), *Ascomycota* (17.77%), and *Chloroflexi* (8.12%), while the proportion of bacteria (77.16%) was considerably higher than that of fungi (22.84%) (Supplementary Figures S3B, D). Furthermore, based on various network topological characteristics, the analysis revealed that the rhizosphere soil bacterial–fungal network (with 145 nodes, 249 edges, and 35 modules) exhibited lower complexity compared with the bulk soil network (such as 197 nodes, 370 edges, and 49 modules) (Supplementary Figures S4A–C). Keystone taxa that have a significant role in the network structure of both rhizosphere and bulk soil bacterial–fungal networks are identified. Specifically, 73 OTUs were identified as keystone taxa in the rhizosphere soil network, with the majority belonging to Burkholderiales, Vicinamibacterales, and Hypocreales orders (Supplementary Figure S4E and Supplementary Table S11). In the bulk soil network, 86 OTUs were identified as keystone taxa, with the majority belonging to the Vicinamibacterales, Hypocreales, and Burkholderiales orders (Supplementary Figure S4F and Supplementary Table S12). Notably, the rhizosphere soil had a higher abundance of Burkholderiales compared to the bulk soil.

The soil properties influencing the keystone taxa

A new network that examines the relationship between keystone taxa in relative abundances and soil properties was constructed (Figure 7 and Supplementary Table S13). Soil properties were found not to significantly correlate with most keystone taxa in the rhizosphere (81.41%) and bulk soils (87.32%). However, when significant correlations were present, the strength of the correlations in the rhizosphere increased (Figures 7A, B). Upon examining the correlations, it was observed that the keystone taxa showed a higher ratio of positive correlations with soil properties compared with negative correlations. Positive correlations were more prevalent in the rhizosphere (59.63%) than in the bulk soil (47.33%) (Supplementary Table S13). Among the soil properties, the relationship correlation between TN, SU, UR, OM, PHO, and pH with keystone taxa was higher than those of other soil properties in the rhizosphere (Figure 7C). On the other hand, the correlation between TP, SU, TN, pH, and OM with keystone taxa was higher than that of other soil properties in the bulk soil (Figure 7D).

Discussion

The rhizosphere soil has higher nutrient and enzyme activity

Based on the research findings, it was observed that the rhizosphere of Chinese cabbage had a higher nutrient content and enzyme activity compared with the bulk soils in the *Karst* region. The pH level showed the opposite pattern, consistent with a previous study (Kuzyakov and Razavi, 2019). Enzyme activity typically decreases with distance from the root surface, primarily releasing organic C (Kuzyakov and Razavi, 2019). Earlier studies have shown that the ratio of sedimentary carbon in the rhizosphere, originating from roots and sediments, accounts for ∼54–63% of the total carbon in grains (Hirte et al., 2018). The unstable nature of this carbon enhances the activity of rhizosphere microbes compared with bulk soils, leading to elevated enzyme activity in the rhizosphere (Jat et al., 2021). Additionally, different enzymes are directly and significantly influenced by soil properties, such as nutrient contents, available C, biomass, and microbial activity, resulting in higher enzyme activities in the rhizosphere soil compared with the bulk soil (Kuzyakov and Razavi, 2019). Moreover, the rhizosphere releases acidic substances, which reduce the pH levels in the soil surrounding the roots (Mendes et al., 2014). Conversely, Chen et al. (2019) found that an increase in organic acid content was accompanied by a corresponding increase in organic carbon. Similar results were reported for tea and maize plants in the rhizosphere soils by Kong et al. (2020) and Yan et al. (2018). Organic matter undergoes a faster transformation, resulting in higher accumulation in the rhizosphere soil (Sokol et al., 2022). As organic matter mineralizes, it releases nitrogen, phosphorus, and potassium, resulting in increased concentrations in both the rhizosphere and bulk soils. Additionally, carbon and nitrogen tend to accumulate together in the soil, where an increase in carbon content promotes nitrogen accumulation (Tang et al., 2023). The research established a significant positive association between organic matter and essential nutrients (AN and TN, AP and TP, and AK and TK), providing insights into why the rhizosphere soil typically displays

higher nutrient levels and greater enzyme activity compared with the bulk soil.

Composition and diversity of bacterial and fungal communities

Recently conducted research has reported similar results to those found in this study, confirming that both the rhizosphere and bulk soils of Chinese cabbage in the *Karst* region exhibit lower bacterial and fungal alpha diversity and OTUs (Zhang et al., 2018b). These findings add further evidence to the notion that the plant's preference for attracting rhizosphere-colonizing microorganisms contributes to the reduced alpha diversity observed within the rhizosphere. This leads to a shift in species richness and decreased species homogeneity in the rhizosphere soil (Ling et al., 2022). As expected, bacteria are found more commonly in different environments than fungi. This study confirms this trend, with the rhizosphere soil being dominated by several bacterial phyla, including Proteobacteria (37.59%), Acidobacteriota (14.74%), Actinobacteriota (10.73%), and Bacteroidota (9.87%). In contrast, bulk soil is dominated by *Proteobacteria* (23.60%), *Acidobacteriota* (20.60%), *Chloroflexi* (16.03%), and *Actinobacteriota* (13.00%). Notably, the relative abundance of *Proteobacteria* in the rhizosphere soil is nearly double the amount in the bulk soil. *Proteobacteria* and *Bacteroidota* thrive in carbon-rich environments and are more abundant in the rhizosphere due to their high metabolic activity, rapid growth, and reproduction (Ling et al., 2022). Additionally, many anamorphic bacteria, which belong to the gram-negative phylum, play a crucial role in nitrogen fixation, leading to increased accumulation of nitrogen in the soil (Ladha and Reddy, 2003). The higher relative abundance of *Proteobacteria* and *Bacteroidota* in the rhizosphere soil suggests greater metabolic activity and higher nutrient availability compared with the bulk soil. Regarding fungi, the research showed that the dominant phyla were Olpidiomycota, Ascomycota, Mortierellomycota, and Basidiomycota in the rhizosphere and bulk soils, with varying relative abundance. *Olpidiomycota* was found to be dominant in the rhizosphere soils, whereas *Ascomycota* was dominant in the bulk soils. Fungi play a crucial role in plant and animal remains decomposition (Xiong et al., 2021), and an increase in the relative abundance of fungal *Ascomycetes* has been linked to enhanced plant debris decomposition (Wang et al., 2020). *Olpidiomycota*, represented by *Olpidium*, includes pathogens (Lay et al., 2018). Interestingly, LEfSe analysis revealed higher levels of *Olpidium*, *Olpidiaceae*, and *Olpidiomycetes* in the Chinese cabbage rhizosphere compared with adjacent bulk soils. Functional analysis further showed higher fungal pathotrophs in the rhizosphere soil, while fungal saprotrophs were considerably more prevalent in the bulk soil, nearly 10 times higher in relative abundance (Supplementary Table S9). Saprophytic fungi play an important role in breaking down plant debris, promoting decomposition of organic matter, and cycling of nutrients (Egidi et al., 2019). On the other hand, pathogenic fungi can cause damage to both plants and the soil (Chen et al., 2020). The findings suggest that the bulk soil is the primary site for plant residue decomposition, while the rhizosphere soil may harbor a higher concentration of potential pathogens. Several reasons may explain this phenomenon: First, a significant proportion of plant pathogens thrive in a saprophytic manner in the roots and thus derive energy from the rhizosphere (Larsen et al., 2015); Second, certain pathogens are more likely to colonize the rhizosphere based on the research studies by Hannula et al. (2021) and Ma et al. (2020); Third, diverse sediments found in the rhizosphere, such as carboxylic acids, sugars, amino acids, and polymeric carbohydrates, create a favorable environment for the proliferation and survival of pathogens.

Rhizosphere soil has a simpler co-occurrence network

The co-occurrence network in the rhizosphere soil of Chinese cabbage displayed a weaker structure compared with that in the bulk soil. The bacterial–fungal network in the rhizosphere soil exhibited a higher number of positive correlations and lower complexity, as expected. This aligns with the notion that the microbial community in the rhizosphere soil is a subset of the bulk soil, a common trait among various plant species (Ling et al., 2022). Previous research studies on soybean farming in different regions of China also reported a smaller and less intricate rhizosphere network compared with the bulk soil (Zhang et al., 2021). Furthermore, Ling et al. (2022) found that the rhizosphere network displayed less robustness and stability, consistent with these results. This could be attributed to increased rhizosphere resources, leading to reduced overlap and interaction among rhizosphere niches (Zhang et al., 2018a). As a result, microbial interactions in the rhizosphere decreased, enabling more microbiomes to adopt a free lifestyle leading to a simpler and weaker stable co-occurrence pattern. Furthermore, as the alpha diversity of bacteria and fungi decreased from the bulk soil to the rhizosphere soil, the complexity of the rhizosphere network also reduced (Fan et al., 2018). Keystone taxa play an irreplaceable role in shaping the co-occurrence network structure stability (Shi et al., 2016). For instance, Proteobacteria, a bacterial phylum, can rapidly build connections and utilize a majority of the carbon substrates released by roots (Philippot et al., 2013). Agaricomycetes and Dothideomycetes fungi possess connector species that enhance nutrient absorption, offer protection against pathogenic bacteria, and maintain harmonious metabolic relationships with other species (Gueidan et al., 2009). These keystone taxa exhibit a versatile, generalist metabolism that helps maintain a balanced internal environment (Fan et al., 2018). The study results indicate that the bulk soil displays greater complexity in bacterial, fungal, and bacterial–fungal networks, compared with the rhizosphere soil. Moreover, the impact of the rhizosphere soil was discovered to be more significant on fungi than bacteria. Notably, a higher number of OTUs linked to Burkholderiales and Rhizobiales are found in the rhizosphere soil, which constitutes the core root microbiome of terrestrial plants (Ji et al., 2023). Rhizobiales have been identified as endophytes in different plants and have been shown to contribute to the growth of crops such as rice and wheat (Yanni et al., 2016; Wu et al., 2018). Moreover, the keystone taxa in the bulk soil exhibited a weak correlation with soil properties. Rhizosphere soil microbial

communities are more vulnerable to disturbances under changing environmental conditions compared with bulk soil. The simpler structure of the network in the rhizosphere soil makes it challenging for the ecosystem to recover when exposed to external disruptions.

Conclusion

Through the study of the microbial communities in the rhizosphere and bulk soils of Chinese cabbage in the *Karst* regions, significant differences were observed in their composition. Specifically, the microbiome in the rhizosphere was a subset of the bulk soil, exhibiting lower alpha diversity than the latter. Moreover, the co-occurrence network in the rhizosphere soil displayed lower complexity, and keystone taxa showed a stronger correlation with soil properties compared with the bulk soil. The functional analysis revealed a higher proportion of pathogens in the relative abundance among the bacterial community in the bulk soil, while the opposite tendency was discovered for fungi in the rhizosphere soil. Despite the reduced complexity of the rhizosphere network, a higher prevalence of beneficial bacterial species that promote the growth of Chinese cabbage was observed.

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 and TF conceived the ideas and designed the methodology. GH, ZZ, MY, and FL completed validation. XW and TH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

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EDITED BY
Priyanka Chandra,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Hongmiao Wu,
Anhui Agricultural University, China
Hongchen Jiang,
China University of Geosciences Wuhan, China

*CORRESPONDENCE
Ang Zhang
✉ angzhang_henu@163.com
Qingyun Zhao
✉ qingyun_022@catas.cn

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The impact of main *Areca Catechu* root exudates on soil microbial community structure and function in coffee plantation soils

Shaoguan Zhao[1,2], Ang Zhang[1]*, Qingyun Zhao[1,3]*, Yunping Dong[1], Lanxi Su[1], Yan Sun[1], Feifei Zhu[1], Dangling Hua[2] and Wu Xiong[4]

[1]Spice and Beverage Research Institute, Chinese Academy of Tropical Agricultural Science, Wanning, China, [2]College of Agricultural Resources and Environmental Sciences, Henan Agricultural University, Zhengzhou, China, [3]Sanya Research Institute, Chinese Academy of Tropical Agricultural Science, Sanya, China, [4]Jiangsu Provincial Key Lab of Solid Organic Waste Utilization, Jiangsu Collaborative Innovation Center of Solid Organic Wastes, Educational Ministry Engineering Center of Resource-Saving Fertilizers, Nanjing Agricultural University, Nanjing, China

Coffee is an important cash crop worldwide, but it has been plagued by serious continuous planting obstacles. Intercropping with *Areca catechu* could alleviate the continuous planting obstacle of coffee due to the diverse root secretions of *Areca catechu*. However, the mechanism of *Areca catechu* root secretion in alleviating coffee continuous planting obstacle is still unclear. The changes of coffee rhizosphere soil microbial compositions and functions were explored by adding simulated root secretions of *Areca catechu*, the primary intercropping plant species (i.e., amino acids, plant hormone, organic acids, phenolic acids, flavonoids and sugars) in current study. The results showed that the addition of coffee root exudates altered soil physicochemical properties, with significantly increasing the availability of potassium and organic matter contents as well as promoting soil enzyme activity. However, the addition of plant hormone, organic acids, or phenolic acids led to a decrease in the Shannon index of bacterial communities in continuously planted coffee rhizosphere soil (RS-CP). The inclusion of phenolic acids specifically caused the decrease of fungal Shannon index. Plant hormone, flavonoids, phenolic acids, and sugars increased the relative abundance of beneficial bacteria with reduced bacterial pathogens. Flavonoids and organic acids increased the relative abundance of potential fungal pathogen *Fusarium*. The polyphenol oxidase, dehydrogenase, urease, catalase, and pH were highly linked with bacterial community structure. Moreover, catalase, pH, and soil-available potassium were the main determinants of fungal communities. In conclusion, this study highlight that the addition of plant hormone, phenolic acids, and sugars could enhance enzyme activity, and promote synergistic interactions among microorganisms by enhancing the physicochemical properties of RS-CP, maintaining the soil functions in coffee continuous planting soil, which contribute to alleviate the obstacles associated with continuous coffee cultivation.

KEYWORDS

continuous cropping obstacle, soil physic-chemical properties, soil enzyme activity, microbial community structure, functional prediction

1. Introduction

As a major cash crop worldwide, coffee plays a crucial role in international agricultural trade (Higashi, 2019). However, the sustainability of coffee production is at risk due to continuous cropping obstacles (CCO) which can cause a decline in coffee quality and production over time (Xiong et al., 2015; Zhao et al., 2018; Su et al., 2022). The CCO phenomenon is closely associated with deteriorating soil properties, the accumulation of allelopathic substances, and microbial imbalances (Xiong et al., 2018; Zhao et al., 2021; Li Q. et al., 2022). It is therefore imperative to investigate the interactions among root secretion, soil properties, and microbiome to maintaining the health of coffee rhizosphere soil of coffee rhizosphere soil and mitigate the negative effects of CCO on coffee production.

Continuous cultivation of coffee can decrease soil organic carbon content and porosity, proliferation of pests and diseases in the topsoil of coffee plantations, and reduced plant adaptability (Ajayi et al., 2021; Rezende et al., 2021; Ayalew et al., 2022). Under long-term continuous cultivation, the root exudates of coffee, such as phenolic compounds, can potentially have toxic effects on certain microorganisms, resulting in the changes of microbial community structure (Jurburg et al., 2020). Previous research has highlighted the significance of soil physic-chemical properties remediation and soil microbiome structure regulation as key factors in alleviating CCO (Xiong et al., 2015; Cheng et al., 2020; Zeng et al., 2020; Liu Q. et al., 2022; Wang et al., 2022). Root secretions also appear to play a critical role in regulating CCO, with intercropping crop groups increasing the diversity of root exudates and optimizing soil nutrients in intercropping ecosystems, thereby promoting positive effects on soil microbial diversity and stability (Zeng et al., 2020). Intercropping systems can alleviate CCO of crops through the improvement of soil bacteria metabolism, soil enzyme activity, and the alleviation of autotoxicity via root metabolite interactions (Cheng et al., 2020; Liu Q. et al., 2022). Consequently, the intercropping system is deemed to offer stable services for crop growth by regulating the soil microbiome and reducing allelochemicals (Guo et al., 2020). *Areca catechu* is a tropical cash crop that is extensively intercropping with coffee to increase the productivity per unit area in several coffee plantations (Sujatha et al., 2011). The research team were observed that coffee plants intercropped with *Areca catechu* exhibited more vigorous growth compared to those grown solely in previous study. However, further studies need to be conducted to investigate the mechanism of intercropping with *Areca catechu* as a means of alleviating coffee CCO.

It is well established that plants play a crucial role in regulating the biological and abiotic conditions of their rhizosphere soil through root secretions, which in turn can affect plant growth by modifying rhizosphere environment (Hu et al., 2018; Zhao et al., 2021; Li M. et al., 2022). Research has revealed that root secretions can act as selective agents that potentially influence the composition of the rhizosphere microbiome (Zhalnina et al., 2018; Zhou et al., 2020). Root exudates aid in nutrient acquisition by modulating the rhizosphere microbiome and facilitating plant–soil feedback to help plants fend off pathogens (Hu et al., 2018). Root exudates mediate the proliferation of specific bacterial communities primarily by targeting plant genetics and physiology to modify the composition of soil microbial communities (Fu et al., 2021; Kawasaki et al., 2021). For example, phloridzin could attract specific pathogenic microorganisms that cause sequential crop diseases (Hofmann et al., 2009); the addition of various organic acids leads to a reduction in soil microbial community richness and diversity index, ultimately leading to specific changes in microbial community structure (Ma et al., 2019); Phenolic acids can decrease soil ecosystem stability by reducing beneficial microbial communities and increasing harmful microbial communities (Huang et al., 2019). Plants are capable of adjusting the composition of their secretions at different stages of growth (Upadhyay et al., 2022). Despite the existence of various types of secretions, the effects of different secretions on soil physicochemical properties and microbial community composition remain unclear.

In this study, we conducted a field-controlled experiment to investigate the effects of primary root exudates from *Areca catechu* on the physicochemical properties, enzyme activity, microbial community, and their interactions in the rhizosphere soil of coffee plants. We aim to answer the following scientific questions: (1) Investigate which type of *Areca catechu* root exudate has a greater impact on the coffee rhizosphere microbiota; (2) Reveale the main driving factors of *Areca catechu* root exudate regulating coffee rhizosphere microorganisms.

2. Materials and methods

2.1. Experiment site

The experiment was conducted in the planting resource garden of the Spice Beverage Research Institute located in Hainan Province, China (110°6′ E, 18°24′ N). The soil in the experimental site showed a soil organic carbon (SOM) content of 164.2 g/kg, an alkali-hydrolyzed nitrogen content (AN) of 98.5 g/kg, a soil-available phosphorus content (AP) of 250.2 mg/kg, and a soil-available potassium content (AHK) of 84.1 mg/kg.

2.2. Experimental material

The soil was collected from a coffee plantation with a long history of monocropping coffee. The topsoil was excavated, and the coffee roots were dug out. The roots were gently shaken to remove non-rhizosphere soil, and then the roots with rhizosphere soil were placed in a bag and vigorously shaken to obtain the rhizosphere soil of the coffee. A sieve (<2 mm) was used to remove plant debris and fully homogenize it for the experiment.

According to previous studies, root exudates of *Areca catechu* were analyzed and determined by using liquid chromatography-mass spectrometry technology. From 6 different categories, 20 compounds with relatively high content were selected based on accessibility and relative abundance to represent the main root exudate composition of *Areca catechu* for subsequent experiments.

2.3. Experimental design

Eight treatments were added with different types of root secretions: sterile water (CK), 20% sterile methanol solution (KCK), 20% sterile methanol solution dissolved organic acids (OA), 20% sterile methanol solution dissolved amino acids (AMA), 20% sterile methanol solution dissolved phenolic acids (PA), 20% sterile methanol

solution dissolved flavonoids (FLA), 20% sterile methanol solution dissolves sugars (SUG), 20% sterile methanol solution dissolves plant hormone (AUX). Each treatment was repeated six times (Supplementary Table S1).

A randomized block group design was used in the experiment. Each treatment was replicated six times, with each replicate consisting of 600 g of soil placed in large containers. The packaged soil was placed in coffee fields to ensure uniform environmental conditions. Before each addition, the prepared exogenous secretion solutions are adjusted to neutral pH using sodium hydroxide to eliminate the strong interference of soil pH on microbial communities. Subsequently, the solutions were added to the packaged soil in the coffee fields. The daily input of carbon from other crops in sandy soil is used to estimate the amount of root exudates secreted by *Areca catechus*: 0.05–0.1 mg C/g per day (Trofymow et al., 1987; Iijima et al., 2000; Baudoin et al., 2003), as few studies have reported the daily carbon input from *Areca catechu* tree roots into the soil. The exogenous secretion added per unit soil was 0.075 mg C/g/d. In each simulated root exudate, the carbon content of each carbon source material in the solution was equal (e.g., the carbon content of citric acid, succinic acid, and tartaric acid accounted for 1/3 of the total carbon content in each added solution, excluding the carbon content in methanol). After 6 weeks of cultivation, destructive sampling was conducted, and the soil samples were stored at −80°C.

2.4. Analysis of soil physic-chemical properties and soil enzyme activities

Soil pH was measured with a pH meter (FE28, China; soil and water ratio 1:2.5). soil-available phosphorus (AP), soil-available potassium (AK), alkali-hydrolyzed nitrogen (AHN), and soil organic matter (SOM) were separately measured by the molybdenum blue, flame photometry, potassium persulfate oxidation, and dichromate oxidation method, respectively (Lu, 1999). The concentrations of soil urease (S-UE), soil dehydrogenase (S-DHA), soil cellulase (S-CL), soil catalase (S-CAT), peroxidase (S-POD), soil polyphenol oxidase (S-PPO), soil acid phosphatase (S-ACP) and soil alkaline phosphatase (S-ALP) were determined by micro method kits from Suzhou Grace Biotechnology Co., Ltd. with the use of an enzyme marker (SynergyH1, USA).

2.5. Soil DNA extraction and sequencing

Soil microbial DNA was extracted three times from 0.5 g fresh soil by using the EZNA® Soil DNA Extraction Kit (Omega, USA). The 0.8% agarose gels was used to check the purity and quality of genomic DNA. The DNA was extracted using sequences: 338F (5′-ACTCCT ACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGT WTCTAAT-3′) bacterial primers with barcode tags (Song et al., 2020). Fungal internal transcribed spacer (ITS1) genes were amplified using the ITS5F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS1R (5′- GCTGCGTTCTTCATCGATGC-3′) barcode primers, with the amplification program according to the reference description (Zhou et al., 2021). The PCR amplification products were quantified by fluorescence with the Quant-iT PicoGreen dsDNA Assay Kit and a Microplate reader (BioTek, FLx800) and were used for quantification. Each sample was mixed in the corresponding ratio based on the sequencing volume requirement of each sample, based on the results of the fluorescence quantification. Sequencing libraries were prepared using the Illumina TruSeq Nano DNA LT Library Prep Kit, which was used for sequencing, followed by double end sequencing (Paired-end) of DNA fragments from the community using the Illumina platform.

2.6. Bioinformatics analysis

Microbiome bioinformatics were performed with QIIME 22019.4 (Bolyen et al., 2019) with slight modification according to the official tutorials.[1] Briefly, raw sequence data were demultiplexed using the demux plugin following by primers cutting with cutadapt plugin (Martin, 2011). Sequences were then quality filtered, denoised, merged and chimera removed using the DADA2 plugin (Callahan et al., 2016). Non-singleton amplicon sequence variants (OTUs) were aligned with maff and used to construct a phylogeny with fasttree2 (Price et al., 2010). Taxonomy was assigned to OTUs using the classify-sklearn naïve Bayes taxonomy classifier in feature-classifier plugin (Bokulich et al., 2018) against the Greengenes 13_8 99% OTUs reference sequences (Mcdonald et al., 2012). Alpha-diversity metrics Shannon, beta diversity metrics weighted UniFrac (Lozupone et al., 2007) unweighted UniFrac (Lozupone and Knight, 2005), Jaccard distance, and Bray–Curtis dissimilarity were estimated using the diversity plugin with samples were rarefied to sequences per sample.

2.7. Statistical analysis

All statistical analyses were performed in R 4.2.0 and visualized with ggplot2. Principal coordinate analysis (PCoA) based on Bray-Curtis distance was used to explore differences of several root secretions on bacterial and fungal community structure, respectively. Soil physicochemical properties, soil enzyme activity, soil microbial diversity indices, relative abundance of specific microbial taxa, and relative abundance of soil microbial functional groups were analyzed using one-way ANOVA to determine differences among the different root secretions addition. The statistical significance ($p < 0.05$) was calculated using Tukey test. Correlations between soil properties, soil enzyme activity and soil microbial community diversity were calculated and analyzed using Spearman correlation matrices. FAPROTAX (Liang et al., 2020) and FUNGuild (Jiang et al., 2021) are commonly used to predict the functions of bacterial and fungal communities.

3. Results

3.1. Changes of soil physical and chemical properties and enzyme activities

One-way ANOVA analysis revealed significant effects of exogenous secretion addition on soil properties and enzyme activities. There were significant changes in soil-available

1 https://docs.qiime2.org/2019.4/tutorials/

potassium and phosphorus between the CK treatment and the KCK treatment. Soil-available potassium showed a significant increase of 22.61%, while soil-available phosphorus exhibited a significant decrease of 35.24%. Compared to the control treatment (KCK), the addition of AMA significantly increased soil organic matter, alkali-hydrolyzed nitrogen, and soil-available potassium by 66.44, 241.73, and 11.52%, respectively. The soil-available potassium and alkali-hydrolyzed nitrogen levels were significantly increased by 27.06 and 37.65%, respectively, under the AUX addition treatment. Treatment with FLA significantly increased organic matter by 23.79%. The addition of OA significantly increased organic matter and pH by 29.48 and 69.93%, respectively. In the PA treatment, pH and alkali-hydrolyzed nitrogen were significantly higher by 47.06 and 18.32%, respectively. The addition of SUG significantly increased organic matter, pH, and alkali-hydrolyzed nitrogen levels. A significant increase of 38.00, 42.48, and 17.85% was observed in organic matter, pH, and soil-available potassium of the SUG treatment. However, the soil-available phosphorus content was significantly reduced by 49.20 and 36.54% in the AMA and PA, treatments, respectively (all $p < 0.05$, Table 1).

There were significant differences in the activities of cellulase, urease, hydrogen peroxide enzyme, peroxidase, and acid phosphatase between the CK treatment and the KCK treatment. The activity of urease, hydrogen peroxide enzyme, and peroxidase in the KCK treatment showed a significant increase of 6.90, 9.08, and 26.39% respectively, while the activities of cellulase and acid phosphatase exhibited a significant decrease of 79.10 and 81.50%, respectively. Compared to the KCK treatment, the activities of polyphenol oxidase, dehydrogenase, and peroxidase were significantly increased by 83.90, 90.35, and 83.17%, respectively, under the AMA addition treatment. The addition of OA increased the activities of soil polyphenol oxidase, peroxidase, and dehydrogenase by 144.07, 56.12, and 97.26%, respectively. In the PA treatment, catalase, peroxidase, and dehydrogenase activities were significantly increased by 37.72, 54.66, and 61.13%, respectively. The SUG addition significantly increased polyphenol oxidase and peroxidase activities by 105.08 and 111.45%, respectively (all $p < 0.05$, Table 2).

3.2. Changes in diversity indices of bacterial and fungal communities upon addition of *Areca catechu* main root secretions

The one-way ANOVA analysis revealed significant effects of different root secretion additions on soil microbial Shannon index. There were no significant differences in Shannon index of soil bacteria and fungi communities between the CK and KCK treatments. In the bacterial community, compared to CK, the Shannon index was significantly reduced by 25.18, 26.44, and 25.50% in the AUX, OA, and PA treatments, respectively. Additionally, compared to KCK, the AUX treatment resulted in a significant reduction of 22.20% in Shannon index. In the fungal community, the PA treatment exhibited a significant reduction of 38.38% in Shannon index compared to CK, and the FLA and PA treatments exhibited significant reductions of 15.03 and 22.54%, respectively, compared to KCK (all $p < 0.05$, Figure 1).

The bacterial community Shannon index significantly and negatively correlated with S-PPO ($p < 0.05$), S-DHA ($p < 0.01$), pH ($p < 0.01$), and AHN ($p < 0.01$). Similarly, the fungal community Shannon index was significantly and negatively correlated with S-CAT ($p < 0.05$), S-DHA ($p < 0.01$), pH ($p < 0.01$), SOM ($p < 0.05$), and AHN ($p < 0.01$). Soil microbial diversity indices were also significantly and negatively correlated with soil AP ($p < 0.05$) (Supplementary Table S3).

Principal Coordinate Analysis (PCoA) was used to exploring the dissimilarity in community composition between treatments. There were no significant differences in bacterial and fungal community structure between the CK and KCK treatments. The addition of FLA, AMA, OA, AUX, and PA treatments had a significant effect on the bacterial community structure when compared to KCK. There was no significant difference in the bacterial community structure between the SUG addition treatment and KCK treatment. Similarly, the fungal community structures under the PA and FLA addition treatments were significantly different from KCK, while there was no significant difference in the fungal community structures between the AMA, AUX, OA, SUG, and KCK treatments (all $p < 0.05$, Figure 1).

TABLE 1 Effects of different root secretions on soil physic-chemical properties.

Treatments	SOM (mg/kg)	pH	AK (mg/kg)	AP (mg/kg)	AHN (mg/kg)
CK	169.17 ± 8.62^{e}	4.68 ± 0.02^{f}	75.42 ± 0.75^{f}	30.76 ± 35.34^{a}	91.93 ± 1.44^{e}
KCK	186.6 ± 9.7^{de}	4.59 ± 0.07^{f}	92.47 ± 1.3^{e}	$19.92 \pm 39.9b^{c}$	91.7 ± 1.71^{e}
AMA	310.57 ± 31.35^{a}	5.03 ± 0.2^{e}	103.12 ± 0.75^{c}	10.12 ± 25.98^{d}	313.37 ± 10.61^{a}
AUX	200.27 ± 0.42^{d}	5.39 ± 0.07^{d}	117.5 ± 1.99^{a}	22.09 ± 18.97^{b}	126.23 ± 1.75^{b}
FLA	231 ± 1.93^{c}	5.57 ± 0.04^{d}	97.79 ± 5.27^{d}	17.96 ± 38.06^{c}	100.57 ± 0.87^{d}
OA	241.6 ± 12.23^{bc}	7.8 ± 0.14^{a}	99.93 ± 1.99^{cd}	18.73 ± 19.73^{bc}	96.83 ± 3.15^{de}
PA	201.8 ± 1.3^{d}	6.75 ± 0.03^{b}	101.52 ± 4.19^{c}	12.64 ± 3.57^{d}	108.5 ± 1.71^{c}
SUG	257.5 ± 20.36^{b}	6.54 ± 0.36^{c}	108.98 ± 0.75^{b}	16.42 ± 15.47^{c}	100.57 ± 1.75^{d}

SOM (mg/kg), soil organic matter; AK (mg/kg), soil-available potassium; AP (mg/kg), soil-available phosphorus; AHN (mg/kg), alkali-hydrolyzed nitrogen. Different letters indicate significant differences between treatments ($p < 0.05$). $n = 6$. CK, control (sterile water); KCK, 20% sterile methanol solution; AMA, amino acids; AUX, auxin; FLA, flavonoids; OA, organic acids; PA, phenolic acids; SUG, sugar. Different letters indicate significant differences between treatments under the same soil physicochemical properties, $n = 6$.

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TABLE 2 Effects of different root secretions on soil enzyme activities.

Treatments	S-PPO nmol/h	S-CL μg/d/g	S-UE μg/d/g	S-CAT μmol/h/g	S-POD nmol/h/g	S-DHA μg/d/g	S-ACP nmol/h/g	S-ALP nmol/h/g
CK	212.6 ± 9.19^{c}	210.82 ± 47.72^{c}	646.97 ± 40.33^{c}	273 ± 13.03^{e}	1808.87 ± 92.82^{e}	1215.98 ± 79.91^{e}	1514.22 ± 205.09^{b}	811.56 ± 58.98^{bcd}
KCK	241.42 ± 6.74^{c}	117.74 ± 16.62^{d}	695.18 ± 7.01^{b}	300.25 ± 3.82^{d}	2457.47 ± 6.74^{cd}	1212.04 ± 171.92^{e}	834.28 ± 341.68^{c}	561.21 ± 100.76^{d}
AMA	390.96 ± 95.1^{b}	1116.3 ± 90.84^{a}	357.36 ± 31.43^{e}	351.06 ± 21.23^{c}	3313.26 ± 500.15^{b}	2314.66 ± 285.28^{a}	3079.89 ± 484.61^{a}	513.63 ± 93.89^{d}
AUX	221.6 ± 49.14^{c}	184.43 ± 31.1^{c}	432.89 ± 5.32^{d}	264.82 ± 28.78^{e}	1830.49 ± 120.89^{e}	1613.04 ± 16.48^{c}	1429.23 ± 61.01^{b}	903.61 ± 36.03^{bc}
FLA	228.81 ± 40.04^{c}	162.44 ± 49.44^{cd}	670.43 ± 19.32^{bc}	314.85 ± 27.24^{d}	2171.01 ± 49.21^{de}	1299.46 ± 317.56^{de}	1451.6 ± 178.15^{b}	1525.7 ± 143.95^{a}
OA	518.88 ± 50.9^{a}	623.8 ± 28.9^{b}	775.77 ± 22.63^{a}	426.2 ± 0.83^{a}	2599.8 ± 631.31^{cd}	2398.69 ± 210.21^{a}	1245.82 ± 329.69^{bc}	1397.63 ± 652.66^{a}
PA	279.26 ± 20.86^{c}	174.54 ± 9.08^{cd}	636.85 ± 2.19^{c}	375.98 ± 6.2^{b}	2797.63 ± 61.32^{c}	1959.34 ± 20.86^{b}	1581.32 ± 279.43^{b}	586.12 ± 127.54^{cd}
SUG	436 ± 67.84^{b}	155.48 ± 23.62^{cd}	703.18 ± 45.72^{b}	347.95 ± 1.26^{c}	3824.94 ± 475.85^{a}	1481.06 ± 4.85^{cd}	1321.87 ± 516.63^{b}	1081.03 ± 33.65^{b}

S-PPO (nmol/h/g), soil polyphenol oxidase; S-CL (μg/d/g), soil-cellulase; S-UE (μg/d/g), soil urease; S-CAT (μmol/h/g), soil catalase; S-POD (nmol/h/g), soil peroxidase; S-DHA (μg/d/g), soil dehydrogenase; S-ACP (nmol/h/g), soil acid phosphatase; S-ALP (nmol/h/g), soil alkaline phosphatase. CK, control (sterile water), KCK, 20% sterile methanol solution; AMA, amino acids; AUX, auxin; FLA, flavonoids; OA, organic acids; PA, phenolic acids; SUG, sugar. Different letters indicate significant differences between treatments under the same soil enzyme activity, $n = 6$, treatments abbreviations and indicator abbreviations see Table 1.

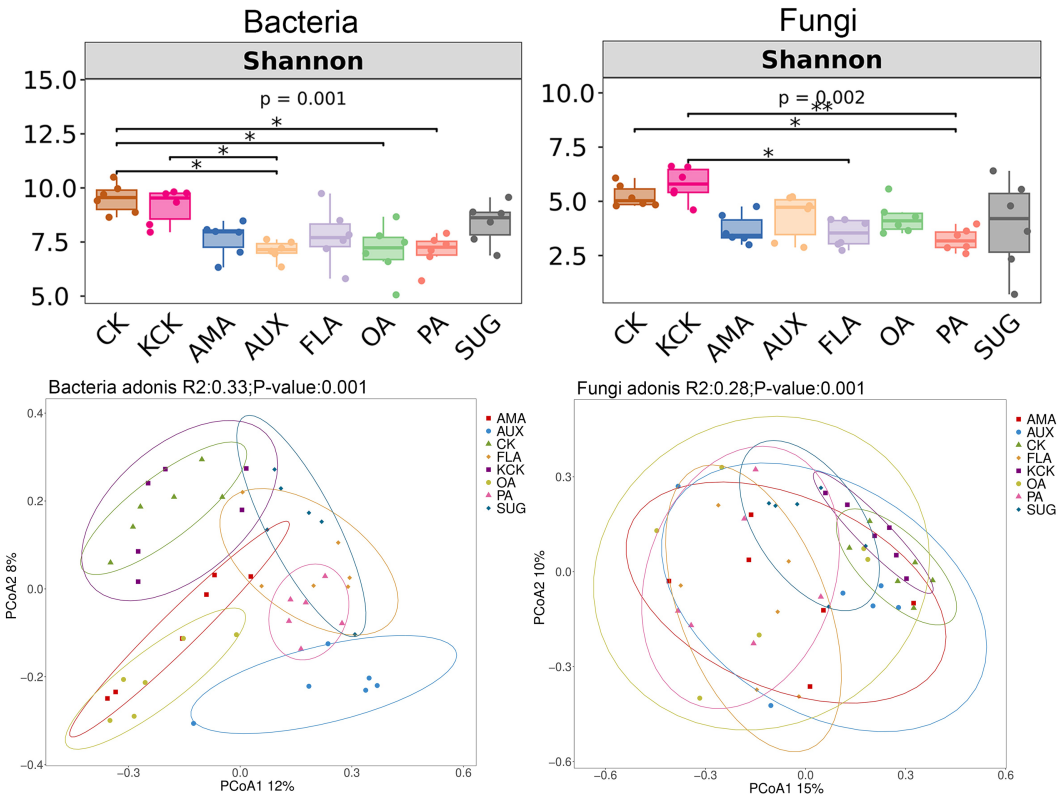

FIGURE 1
Diversity of bacterial and fungal communities in rhizosphere soil. $n = 6$. CK, control (sterile water); KCK, 20% sterile methanol solution; AMA, amino acids; AUX, auxin; FLA, flavonoids; OA, organic acids; PA, phenolic acids; SUG, sugar. * Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level; and *** Correlation is significant at the 0.001 level.

3.3. Effect of *Areca catechu* main root secretions addition on the major bacterial communities

In the CK and KCK treatments, the major bacterial communities. (relative abundance >1%) were *Mycobacterium*, *Burkholderia-Caballeronia-Paraburkholderia*, *Acidothermus*, *Sphingomonas*, *Bacillus*, *Methylobacterium*, *Bradyrhizobium*, and *JG30-KF-AS9*, and there was no significant difference in the relative abundance of these bacterial genera between the two treatments. Compared to KCK, the addition of AMA significantly reduced *Bacillus* and *Bradyrhizobium* by 72.47 and 97.00%, respectively ($p < 0.001$). The addition of AUX increased *Burkholderia-Caballeronia-Paraburkholderia* by 3866.61% ($p < 0.001$), while significantly reducing *Mycobacterium*, *Acidothermus*, *Bacillus*, and *Bradyrhizobium* by 67.22% ($p < 0.01$),

83.54% ($p < 0.01$), 56.24% ($p < 0.01$), and 90.66% ($p < 0.001$), respectively. In the FLA treatment, *Burkholderia-Caballeronia-Paraburkholderia* was significantly increased by 1513.02% ($p < 0.001$), while *Mycobacterium* was significantly decreased by 80.80% ($p < 0.01$). The addition of OA significantly reduced *Acidothermus*, *Bacillus*, *Bradyrhizobium*, and *JG30-KF-AS9* by 95.59% ($p < 0.01$), 74.91% ($p < 0.01$), 67.98% ($p < 0.05$), and 92.83% ($p < 0.05$), respectively. In the PA treatment, *Methylobacterium* was significantly increased by 131.51% ($p < 0.001$), while *Mycobacterium*, *Acidothermus*, and *Bacillus* were significantly reduced by 96.51% ($p < 0.01$), 90.91% ($p < 0.01$), and 34.68% ($p < 0.05$), respectively. The addition of SUG significantly decreased *Mycobacterium* by 85.56% ($p < 0.01$) while increasing *Sphingomonas* by 126.12% ($p < 0.001$). *Hyphomicrobium* became the dominant genus in all treatments, except the KCK treatment, and significantly increased by 91.99% ($p < 0.001$) and 104.24% ($p < 0.001$) in the PA and SUG treatments, respectively (Figure 2; Supplementary Table S2).

For the fungal community, the dominant genera were *Fusarium*, *Saitozyma*, *Mortierella*, *Trichoderma*, and *Penicillium* in CK and KCK treatments, and there was no significant difference in the relative abundance of these bacterial genera between the two treatments. Compared with KCK, the *Mortierella* treated by AMA was significantly reduced by 92.29% ($p < 0.001$); *Saitozym* and *Mortierella* were significantly reduced by 60.18% ($p < 0.05$) and 49.34% ($p < 0.01$) as a result of AUX treatment; *Fusarium* under FLA treatment was significantly increased by 298.75% ($p < 0.001$), *Trichoderma* and *Mortierella* were significantly decreased by 97.00% ($p < 0.05$) and 75.73% ($p < 0.01$); *Fusarium* treated with OA was significantly increased by 261.24% ($p < 0.001$), *Saitozyma* and *Mortierella* were significantly decreased by 83.06 and 99.40% ($p < 0.001$); *Saitozyma*, *Mortierella* was significantly reduced by 99.49 and 99.81% ($p < 0.001$) in the PA treatment group; *Mortierella* significantly reduced by 57.86% ($p < 0.001$) in treatment SUG (Figure 2; Supplementary Table S2).

Compared to the control group (KCK), the addition of *Areca catechu* main root secretions in coffee rhizosphere soil demonstrated inhibitory effects on potential pathogenic genera, such as *Mycobacterium* and *Acidothermus*, within the bacterial community. Furthermore, the inclusion of AUX, FLA, PA, and SUG led to a significant increase in the relative abundance of two potential beneficial genera, namely *Burkholderia-Caballeronia-Paraburkholderia* and *Sphingomonas*. Notably, the PA and SUG treatments resulted in the emergence of a new dominant potential beneficial genus, *Hyphomicrobium*. Within the fungal community, the FLA and OA treatments significantly elevated the relative abundance of the potential pathogenic genus *Fusarium*. Additionally, the overall relative abundance of the potential beneficial genus *Mortierella* experienced a significant reduction across all treatments (Supplementary Tables S2, S3).

3.4. Correlation of soil physicochemical properties and enzyme activities with microbial communities

Significant positive correlations were found between soil SOM and S-PPO ($R = 0.60$, $p < 0.001$), S-CAT ($R = 0.56$, $p < 0.01$), S-POD ($R = 0.56$, $p < 0.001$) and S-DHA ($R = 0.61$, $p < 0.001$), and significant negative correlations were found with S-UE ($R = 0.01$, $p < 0.05$). pH and S-PPO ($R = 0.64$, $p < 0.001$), S-CAT ($R = 0.72$, $p < 0.001$), S-POD ($R = 0.39$, $p < 0.01$) and S-DHA ($R = 0.53$, $p < 0.001$). AP exhibited significant negative correlations with several indicators including S-PPO ($R = -0.55$, $p < 0.01$), S-CL ($R = -0.23$, $p < 0.01$), S-CAT ($R = -0.62$, $p < 0.001$), S-POD ($R = -0.73$, $p < 0.001$), S-DHA ($R = -0.45$, $p < 0.01$), and S-ACP ($R = -0.28$, $p < 0.01$). The inter-root soil bacterial community genus level structure had a significant response to soil S-PPO, S-UE, S-CAT, S-DHA, and pH, and the fungal genus level structure had a significant response to soil S-CAT, pH, and AK (Figure 3). This study demonstrates that there is a close correlation between soil physicochemical factors as well as between physicochemical factors and soil microbial community structure.

There was a strong correlation between soil physicochemical factors and enzyme activities and the dominant genus of rhizosphere bacteria (Figure 4). For the relative abundance of dominant genera in the bacterial community, there was a significant positive correlation between *Mycobacterium* and S-CL ($R = 0.32$), and a significant negative correlation with pH ($R = 0.35$), AK ($R = 0.42$), and AHN ($R = 0.31$); *Burkholderia-Caballeronia-Paraburkholderia* was negatively correlated with S-PPO ($R = 0.47$), S-CAT ($R = 0.64$), S-POD ($R = 0.35$) and pH ($R = 0.31$), and positively correlated with AHN ($R = 0.29$); *Hypomicrobium* has a significant negative correlation with AK ($R = 0.45$); *Acidothermus* was negatively correlated with S-PPO ($R = 0.39$), S-CAT ($R = 0.40$), S-DHA ($R = 0.48$), pH ($R = 0.51$) and AHN ($R = 0.32$); *Sphingomonas* showed significant negative correlations with S-DHA ($R = 0.55$) and AHN ($R = 0.50$); For the relative abundance of dominant genera in fungal communities, there was a significant positive correlation between *Fusarium* and S-CAT ($R = 0.34$), SOM ($R = 0.30$), pH ($R = 0.31$), and a significant negative correlation with AP ($R = 0.30$). *Mortierella* and S-PPO ($R = 0.35$), S-CAT ($R = 0.54$), S-POD ($R = 0.35$), S-DHA ($R = 0.49$), SOM ($R = 0.45$), pH ($R = 0.57$), AK ($R = 0.31$), AHN ($R = 0.44$) were significantly negatively correlated with each other and positively correlated with AP ($R = 0.62$).

3.5. Functional predictions

The functional prediction of the soil bacterial community revealed that the main gene functional groups in each treatment were *chemoheterotrophy* and *aerobic_chemoheterotrophy*, which accounted for 66.39% of the total. There were no significant changes in the percentages of these functional groups after the addition of exogenous secretions. In the case of the fungal community, there were no differences between the functional communities of the fungi under the CK and KCK treatments. The *Undefined Saprotroph* also significantly increased by 310.21% in the PA treatment. The addition of various compounds did not significantly alter the relative abundance of the *Plant Pathogen* and *Fungal Parasite* functional groups. However, *Animal Pathogen*, *Endophyte*, *Wood Saprotroph*, and *Lichen Parasite* showed a significant increase in relative abundance in the OA treatment, by 72.90, 35.86, 64.99, and 125.78%, respectively, (all $p < 0.05$; Figure 5; Supplementary Table S4).

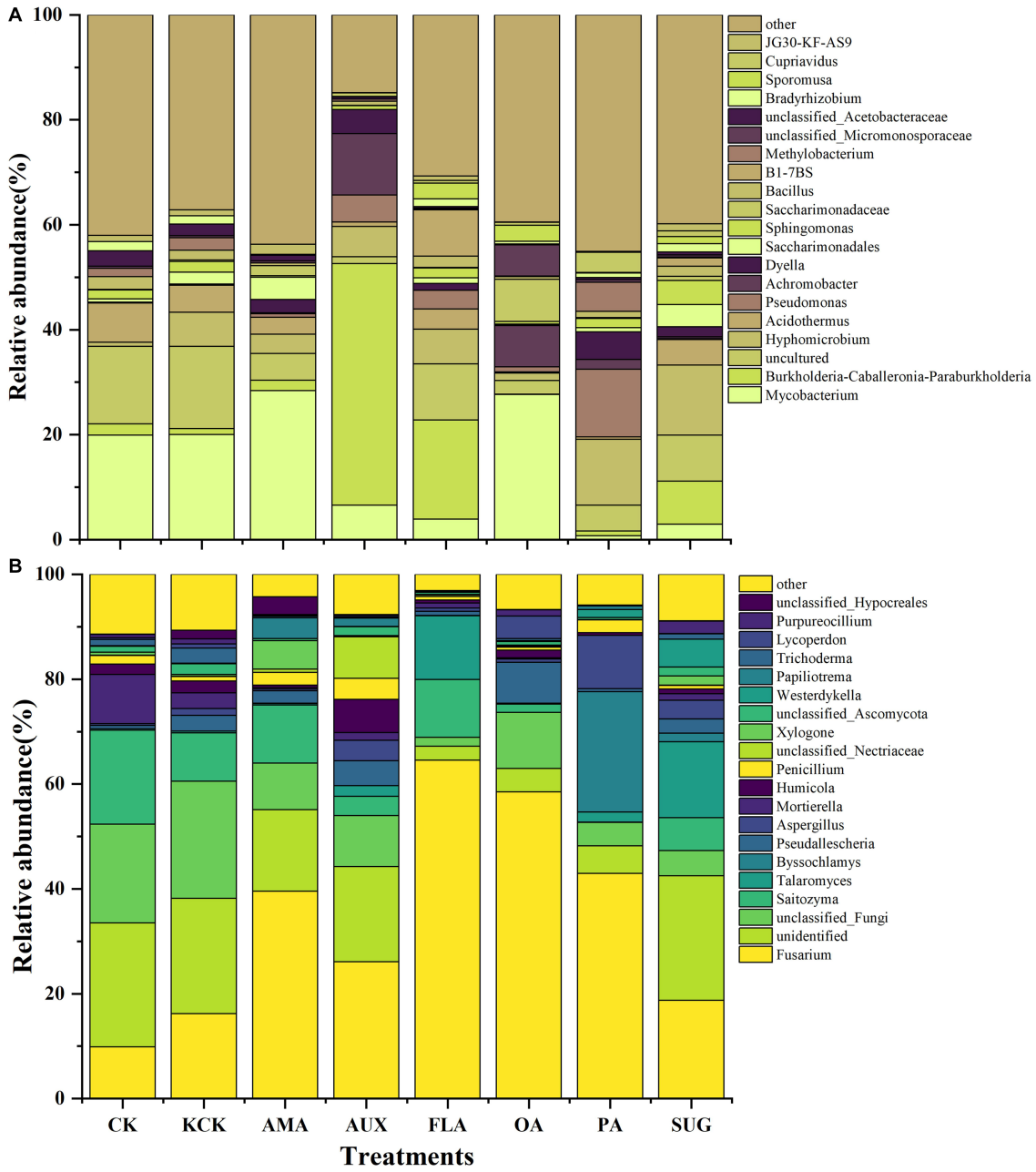

FIGURE 2
Relative abundance of top 20 genera of bacterial **(A)** and fungal **(B)** microbial communities in rhizosphere soil. *n*=6. CK, control (sterile water); KCK, 20% sterile methanol solution; AMA, amino acids; AUX, auxin; FLA, flavonoids; OA, organic acids; PA, phenolic acids; SUG, sugar.

Based on the functional annotations of the samples in the database and the abundance information, a heat map was created to show the top 20 functions in terms of abundance and their abundance information in each treatment (Figure 6). Among the bacterial functional groups, the cluster analysis revealed that CK, KCK, PA, AMA, AUX, and OA treatments had similar functional community composition, while treatments FLA and SUG were similar but had higher abundance of gene functions such as *automatic_compound_degradation*, *hydrocarbon_degradation*, and *automatic_hydrocarbon_degradation*. *Nitrate_reduction*, *methylotrophy*, and *methanol_oxidation* genes had higher relative abundance in other treatments. In the fungal functional groups, the functional community composition of treatments CK, KCK, and SUG were grouped together, while the other treatments were grouped together.

4. Discussion

4.1. Effects of different root secretions on soil physic-chemical properties and enzyme activities

Soil enzymes play an important role in the degradation and transformation of soil nutrients such as alkali-hydrolyzed nitrogen, and

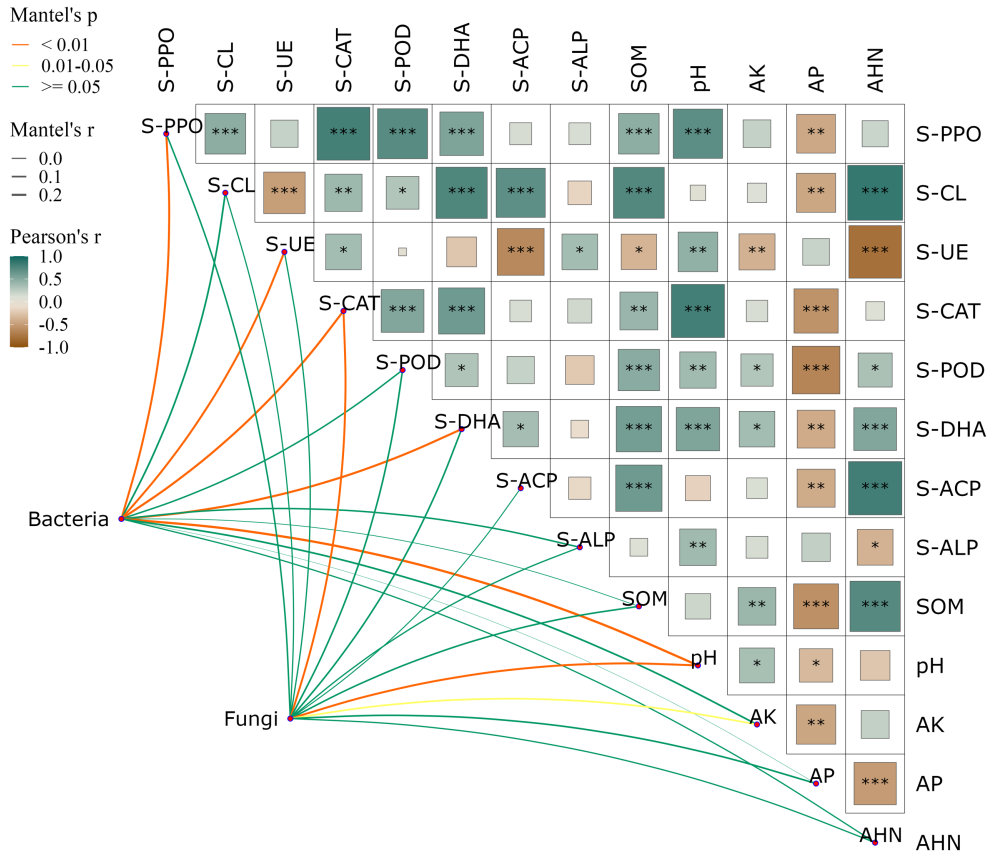

FIGURE 3
Effects of soil physic-chemical and enzyme activities on relative abundance of bacterial **(A)** and fungi **(B)** communities at the genus level. Heatmap showing the correlation between soil properties, network diagram shows the correlation between soil microbial community and soil properties. Red represents positive correlation, blue represents negative correlation, the darker the color, the stronger the correlation. The symbol "*" indicates statistical significance at $p < 0.05$, "**" indicates $p < 0.01$, and "***" indicates $p < 0.001$.

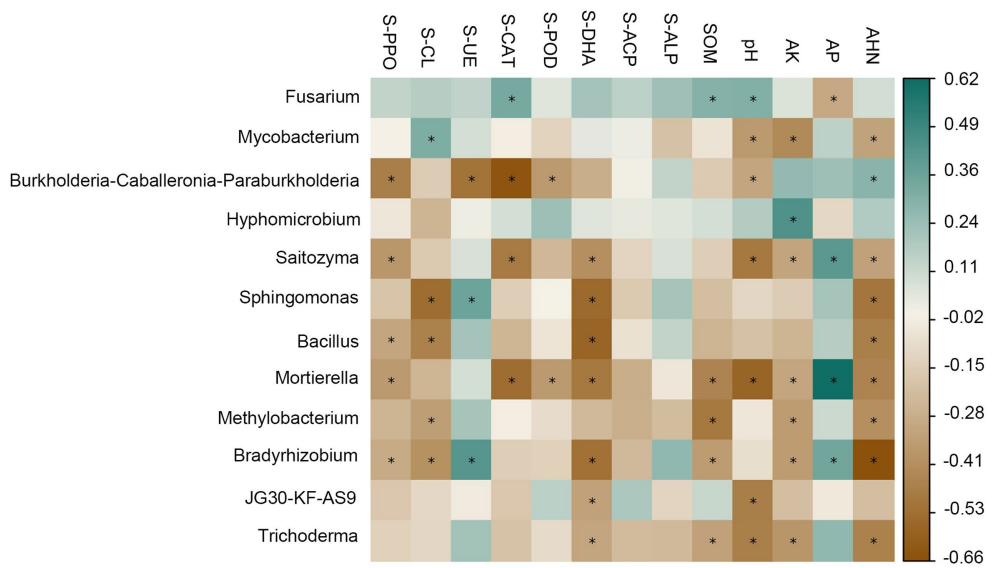

FIGURE 4
Relationships among soil physic-chemical, enzyme activities and dominant genera relative abundance of bacterial and fungal communities at the genus level. Cyan represents positive correlation, brown represents negative correlation, the darker the color, the stronger the correlation. The symbol "*" indicates statistical significance at $p < 0.05$.

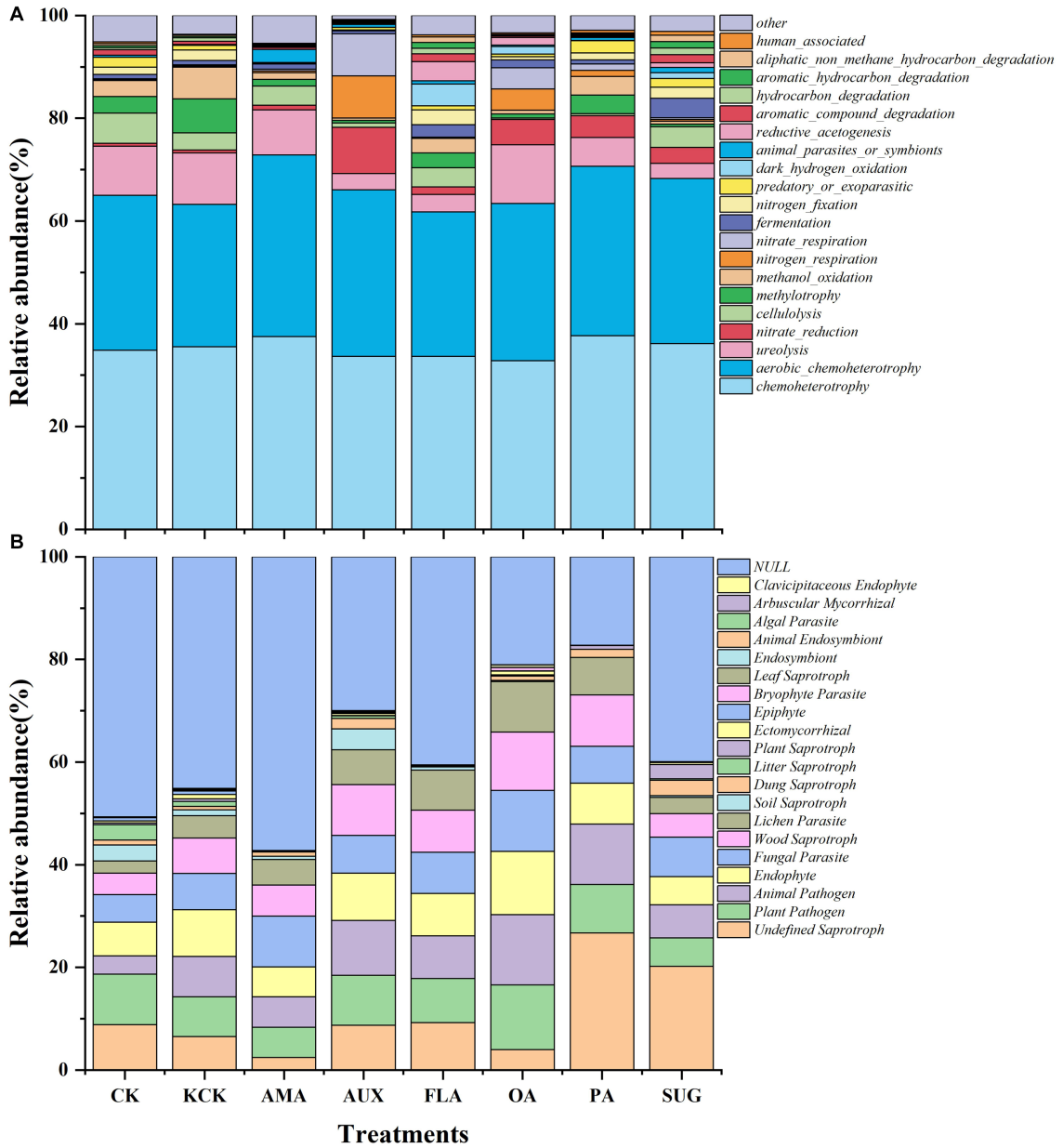

FIGURE 5
Effects of different root secretions on relative abundance of the top 20 functional genes in soil bacteria **(A)** and fungi **(B)** communities. $n = 6$. CK, control (sterile water); KCK, 20% sterile methanol solution; AMA, amino acids; AUX, auxin; FLA, flavonoids; OA, organic acids; PA, phenolic acids; SUG, sugar.

also participate in other physiological and biochemical processes. The activity and diversity of soil enzymes can have a significant impact on the soil physico-chemical properties, such as nutrient cycling, soil structure, and organic matter decomposition (Jing et al., 2020). On one hand, the findings of this study indicate that the addition of compounds significantly increased the activities of S-PPO, S-DHA, S-POD, and S-CAT, suggesting that the root secretions of *Areca catechu* mainly affect redox enzymes (Table 2). Specifically, S-PPO can accelerate the soil humification process, while S-DHA, S-POD, and S-CAT have been found to play crucial roles in soil bio-oxidation (Tan et al., 2017; Telesiński et al., 2019; Li Q. et al., 2022). The four soil enzyme activities mentioned above exhibited a significant positive correlation with soil organic matter (SOM) and pH in this study (Figure 3) supporting the notion that oxidoreductases in lichen can catalyze the formation of alkali-hydrolyzed nitrogen (Beckett et al., 2013). The present study demonstrates that crop root secretions, an important driver of microbial activity and a steady source of soil organic carbon, exert a robust regulatory effect on both soil physicochemical and enzymatic activities (Yan et al., 2023). Through a series of biochemical reactions, various types of secretions are transformed into the nutrients necessary for organisms, providing diverse sources of carbon for the soil microbiome. In the present study, the addition of different root secretions led to significant increases in pH, soil organic matter (SOM), available potassium (AK), and alkali-hydrolyzable nitrogen (AHN), as

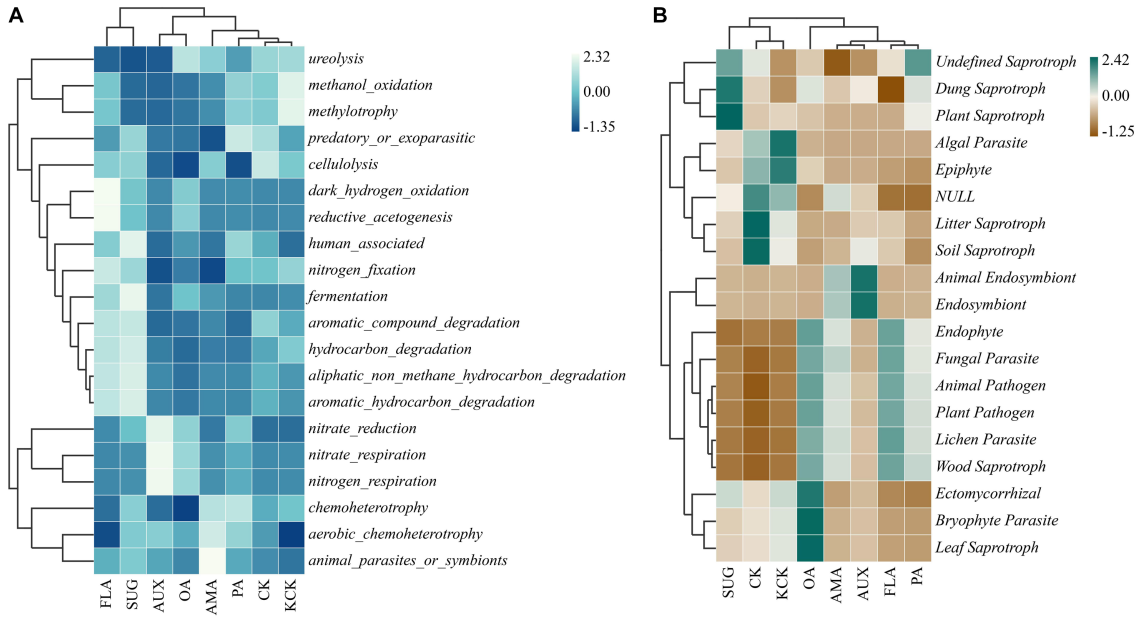

FIGURE 6
Clustering heat map of the functional diversity of rhizosphere microbial genes, bacterial **(A)** and fungal **(B)**. $n = 6$. CK, control (sterile water); KCK, 20% sterile methanol solution; AMA, amino acids; AUX, auxin; FLA, flavonoids; OA, organic acids; PA, phenolic acids; SUG, sugar.

confirmed by the results presented in Table 1. Interestingly, the noticeable decrease in available phosphorus (AP) observed in this study could be related to the increased activity of catalase. Prior research has indicated that catalase is a key factor that inhibits the conversion of soil organic phosphorus to effective phosphorus (Strickland et al., 2015). Under the absence of exogenous phosphorus interference, the increase in catalase activity led to a significant reduction in soil effective phosphorus content, as demonstrated in this study (Wang et al., 2012). The results of this study suggest that acid phosphatase activity was not affected significantly by the addition of root secretions, which may correspond to a low rate of available phosphorus (AP) accumulation in the soil (Liu et al., 2023).

4.2. Effect of different root secretions on the diversity of soil microbial communities

As a crucial mediator of interaction between crop and soil microbial communities, the alterations in both the type and quantity of root secretions could markedly impact the diversity of soil microbial communities (Zhalnina et al., 2018). Root secretions have complex and diverse effects on microbial diversity, potentially simultaneously influencing multiple microbial groups. However, specific compounds in root secretions, such as plant hormones, have been indicated to directly impact microbial growth and metabolism, thereby influencing microbial diversity (Vives-Peris et al., 2020; Zhao et al., 2021). Some flavonoid and organic acid compounds found in root secretions are only mineralized by specific microorganisms, while most soil microorganisms are unable to utilize them, thus affecting soil microbial diversity (Qu and Wang, 2008; Eilers et al., 2010).

The results from Supplementary Table S4 in the Appendix demonstrated that soil microbial diversity was inversely correlated with soil properties, following the application of AUX, OA, and PA. This suggests that the reduction in bacterial and fungal diversity could be attributed to changes in soil properties resulting from these treatments. While the addition of AUX, OA, and PA had a beneficial effect on AK, AHN, and pH in the rhizosphere soil of this study, the ensuing increase in nitrogen and pH levels may have exceeded the tolerable range of certain microbial communities, thereby inducing adverse changes in their living environment that inhibited their growth. Specifically, the pronounced alterations in the soil environment could hinder the growth of microbial populations that had adapted to the original living conditions (Zheng et al., 2022). The rise in soil nitrogen content accelerated the loss of rare species and led to a decline in microbial diversity. While an increase in other nutrients may help certain microorganisms withstand the negative impacts caused by changes in soil nutrient conditions, the overall effect was a decrease in microbial diversity (Wang et al., 2018).

Root secretions exhibit notable spatiotemporal variability during various physiological stages of crops, and the impacts of distinct components on the assembly of microbial structures may differ significantly (Zhalnina et al., 2018). Previous research has demonstrated that the composition of the rhizosphere microbial community is influenced by the composition of root secretions. The significant differences observed in microbial community structure among FLA, PA, and CK in this study were in line with previous findings (Cadot et al., 2021; Figure 1). The selectivity of microorganisms for the consumption of root secretions influences the aggregation of rhizosphere microorganisms. Certain secretions encourage the colonization of specific microorganisms, while others inhibit microbial growth. As such, different types of secretions can have varying impacts on the diversity of rhizosphere microorganisms (Sasse et al., 2018; Siczek et al., 2018; Bao et al., 2022). In this study, FLA and PA may have divergent impacts on the abundance of dominant species, consequently leading to alterations in microbial diversity (Figures 1, 2).

4.3. Effect of root secretions on the abundance of dominant microbiome

The reduction in microbial community abundance and diversity observed in response to compounds addition may be attributed to the inhibition of harmful microorganisms and stabilization of microbial structure (Salem et al., 2022). This study found that the addition of compounds leads to alterations in both the diversity and relative abundance of dominant species of soil microbial communities. The interaction between plant root exudates and specific microbial communities is complex and diverse. They can attract or repel microorganisms, modulate symbiotic and antagonistic relationships, thereby influencing the health and growth of plants (Zhang et al., 2022). Specific root secretions can attract beneficial bacteria and reduce the proliferation of pathogenic bacteria, contributing to these changes (Liu A. et al., 2022). Terpenoids and polyketides present in root secretions have been found to suppress phytopathogenic mycobacteria while facilitating the recruitment of specific bacteria to rhizosphere soils (Strickland et al., 2015). Chemical substances in plant root exudates can serve as signaling molecules to attract beneficial microorganisms and establish symbiotic relationships. Among them, phenolic and flavonoid compounds have been shown to enhance the expression of nodulation genes in nitrogen-fixing bacteria (Hirsch et al., 2003).These signaling molecules can trigger chemical reactions in rhizobial bacteria, leading to the formation of root nodules and the establishment of symbiotic relationships with plant roots (Xie et al., 2019).

Therefore, the addition of compounds is more likely to impact bacterial communities rather than fungal communities, as demonstrated in Figure 3 where fungal communities were found to be insensitive to the addition of compounds. Our analysis revealed that the relative abundance of dominant bacterial genera had significant negative correlations with soil physicochemical properties such as S-PPO, S-UE, S-CAT, S-DHA, pH, and AK. This finding suggests that the microbial community in soil that has undergone continuous coffee cropping exhibits sensitivity to changes in soil physicochemical properties and enzyme activities brought about by root secretion addition, which is consistent with the results of previous studies (Liu et al., 2019; Figure 4). The addition of root secretions promotes the interaction between microorganisms, and regulates the structure of soil microbial community by reducing the intensity of competition between microbial communities (Eldridge et al., 2017). In addition, the addition of root secretions also enhances the recruitment of microorganisms compatible with root secretions and reduces the survival space of pre-existing dominant species. This may explain the observed reduction in the abundance of dominant genera (Santoyo, 2022). The results observed in this study demonstrate how the addition of primary exudates from *Areca catechu* roots promotes the transition of soil microbial community structure from imbalance to stability.

4.4. Effect of different compounds on functional genes

The structure and function of soil microorganisms are considered to be inseparable, thus the difference of soil microbial communities under compounds addition may change the functional type of these microbial communities (Xie et al., 2022). Soil microbial community function may be influenced by the addition of simulated compounds, and microbial substrate preferences for root metabolites (amino acids and organic acids) can positively aid in the selection of specific functional taxa (Mundra et al., 2022). *Chemoheterotrophy* and *Aerobic chemoheterotrophy* can participate in the carbon cycle and accelerate the decomposition of alkali-hydrolyzed nitrogen (Yu et al., 2021). However, The exogenous secretions did not significantly affect the percentage of *chemoheterotrophic* functional genes in this study (Figure 5).

In this study, the CK and KCK treatments showed a higher proportion of potential pathogenic functional groups such as *Plant Pathogen* and *Fungal Parasite*. These findings suggest that a high relative abundance of pathotrophic fungal functional groups in coffee rhizosphere soils may be the primary cause of CCO (Liu et al., 2019). It is worth noting that the relative abundance of functional groups such as *Plant Pathogen*, *Fungal Parasite*, *Animal Pathogen*, *Endophyte*, W*ood Saprotroph*, and *Lichen Parasite* did not show significant changes after the addition of various compounds (except for organic acid compounds). This indicates that prior to the addition of compounds, the fungal functional groups present in coffee rhizosphere soils were more complex, and the addition of compound compounds led to a decrease in fungal functional group complexity (Figure 5). These results suggest that the potential risk of coffee disease can be mitigated by the addition of secretions from *Areca catechu*.

5. Conclusion

This study reveals that adding primary exudates from *Areca catechu* roots can enhance soil fertility and enzyme activity in coffee rhizosphere soils, resulting in significant changes in the structure and function of soil microbial communities. Specifically, the addition of flavonoids, plant hormone, phenolic acids, and sugars can inhibit the growth of bacterial pathogens in continuously cultivated soils by promoting soil nutrient enrichment and increasing soil enzyme activity, thus reducing the risk of coffee diseases. The addition of organic acids and flavonoids may increase the relative abundance of pathogenic genera like *Fusarium* and lead to a significant increase in the relative abundance of pathogenic fungal functional groups. The inclusion of plant hormone, phenolic acids, and sugars could promote the proliferation and relative dynamic balance of non-pathogenic bacteria in continuously cropped coffee rhizosphere soils, which ultimately contributes to overcoming challenges associated with continuous cropping in coffee rhizosphere soils.

Data availability statement

The data presented in the study are deposited in the Mendeley Data repository, accession number doi: 10.17632/tgfz6h2bxh.1.

Author contributions

SZ: Conceptualization, Data curation, Formal analysis, Software, Validation, Visualization, Writing – original draft, Writing – review &

editing. AZ: Methodology, Writing – review & editing. QZ: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. YD: Formal analysis, Methodology, Project administration, Supervision, Writing – review & editing. LS: Formal analysis, Supervision, Writing – review & editing. YS: Formal analysis, Project administration, Supervision, Writing – review & editing. FZ: Supervision, Writing – review & editing. DH: Supervision, Writing – review & editing. WX: Formal analysis, Supervision, Visualization, 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.2023.1257164/full#supplementary-material

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EDITED BY
Priyanka Chandra,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Hanuman Singh Jatav,
Sri Karan Narendra Agriculture University, India
Hayssam M. Ali,
King Saud University, Saudi Arabia

*CORRESPONDENCE
Mingzhou Qin
✉ mzqin@henu.edu.cn
Asim Shahzad
✉ agron12@yahoo.com

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Heavy metals mitigation and growth promoting effect of endophytic *Agrococcus terreus* (MW 979614) in maize plants under zinc and nickel contaminated soil

Asim Shahzad[1,2]*, Anam Siddique[2], Shazia Ferdous[2], Muhammad Ahmar Amin[3], Mingzhou Qin[1]*, Uzma Aslam[2], Muhammad Naeem[4], Tasmia Bashir[5] and Abdul Shakoor[1]

[1]The College of Geography and Environment, Henan University, Kaifeng, China, [2]Department of Botany, Mohi-Ud-Din Islamic University, AJ&K, Pakistan, [3]Rawalpindi Women University, Rawalpindi, Pakistan, [4]Department of Plant Science, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China, [5]Department of Botany, Rawalpindi Women University Rawalpindi, Rawalpindi, Pakistan

Introduction: Heavy metals such as iron, copper, manganese, cobalt, silver, zinc, nickel, and arsenic have accumulated in soils for a long time due to the dumping of industrial waste and sewage. Various techniques have been adapted to overcome metal toxicity in agricultural land but utilizing a biological application using potential microorganisms in heavy metals contaminated soil may be a successful approach to decontaminate heavy metals soil. Therefore, the current study aimed to isolate endophytic bacteria from a medicinal plant (*Viburnum grandiflorum*) and to investigate the growth-promoting and heavy metal detoxification potential of the isolated endophytic bacteria Agrococus tereus (GenBank accession number MW 979614) under nickel and zinc contamination.

Methods: Zinc sulfate and nickel sulfate solutions were prepared at the rate of 100 mg/kg and 50 mg/kg in sterilized distilled water. The experiment was conducted using a completely random design (CRD) with three replicates for each treatment.

Results and Discussion: Inoculation of seeds with *A. tereus* significantly increased the plant growth, nutrient uptake, and defense system. Treatment T4 (inoculated seeds), T5 (inoculated seeds + Zn100 mg/kg), and T6 (inoculated seeds + Ni 100 mg/kg) were effective, but T5 (inoculated seeds + Zn100 mg/kg) was the most pronounced and increased shoot length, root length, leaf width, plant height, fresh weight, moisture content, and proline by 49%, 38%, 89%, 31%, 113%, and 146%, respectively. Moreover the antioxidant enzymes peroxidase and super oxidase dismutase were accelerated by 211 and 68% in contaminated soil when plants were inoculated by *A. tereus* respectively. Similarly the inoculation of *A. tereus* also enhanced maize plants' absorption of Cu, Mn, Ni, Na, Cr, Fe, Ca, Mg, and K significantly. Results of the findings concluded that 100 mg/kg of Zn and Ni were toxic to maize growth, but seed inoculation with *A. tereus* helped the plants significantly in reducing zinc and nickel stress. The *A. tereus* strain may be employed as a potential strain for the detoxification of heavy metals

KEYWORDS

endophytic bacterial, *Agrococus terreus*, *Zea mays*, heavy metals stress, zinc contamination nickel contamination, antioxidant enzymes

1. Introduction

Food security necessitates; an increase in global food production. The primary emphasis of agricultural studies is on crop cultivation. These analyzes overlooked the variability and unreliability of the annual grain output- as well as the inherent instability of production (Knapp and Van Der Heijden, 2018). Research on foods that may contain heavy metals has been prompted by increasing emphasis on food safety (Adamson et al., 2018). Pollution of farmlands with hazardous metals is a major ecological threat. These compounds are widespread and have negative short- and long-term effects on plant development, making them prime examples of soil pollutants (Zhang et al., 2023). All living forms are endangered by heavy metals in the soil (Ding et al., 2022). Heavy metals are chemically metallic elements with relatively high density and low toxicity. Nevertheless, this physical characteristic is very harmful to plants and other creatures (multiple metabolic systems may be disrupted by the toxicity of heavy metals. Regardless of the exact symptoms and signs associated with each heavy metal, even minimal exposure to these compounds has a negative influence on plant growth (Dutta et al., 2018). Multiple pathways exist for the transport of heavy metals, which may have devastating health consequences (Balali-Mood et al., 2021).

Oxidative stress and heavy metal toxicity pose severe threats to agricultural systems (Hou et al., 2020). In addition to other environmental pressures, the presence of hazardous metals causes plants to increase their enzymatic defense system (Haider et al., 2021). By replacing metal ions with metal-enzymes, these heavy metals inhibit a range of plant activities, including fertilization, as well as a number of plant morphological changes (Morkunas et al., 2018), Alterations in the physiological and biochemical cycles of plants reduce plant growth (Ali et al., 2020). Metals never degrade; however, when their concentrations in a facility surpass safe levels, they have a negative impact on plant health and the environment. Inhibition of cytoplasmic molecules and breakdown of the cell structure due to oxidative pressure are two direct detrimental effects of excessive metal concentrations (Mishra et al., 2017). Metals that may be absorbed by plants are dissolved in the soil solution or expelled by roots (Sarwar et al., 2017). Even when plants require certain heavy metals for development and sustenance, excessive amounts of these elements may be harmful to plant life.

Inhibition of cytoplasmic enzymes and oxidative damage to the cellular structure are direct adverse consequences at higher dosages (Arif et al., 2016) and the activities of soil microorganisms and the detrimental impacts of heavy metals may also inhibit plant development and growth. For example, owing to the high concentration of minerals, the breakdown of organic matter in the soil is hindered, and the number of beneficial bacteria decreases, causing a loss of nutrients in the soil (Urra et al., 2019). As a consequence of heavy metal interference with the function of soil microorganisms, the activity of enzymes crucial to plant metabolism is also inhibited. These detrimental effects result in stunted plant development, which may ultimately lead to plant death (Shi et al., 2022). Another unfavorable consequence of heavy metal accumulation is the release of reactive oxygen species (ROS), the composition of which is determined by the equilibrium between ROS production and ROS elimination in plants, which is influenced by factors such as the presence of heavy metals, temperature, light intensity, etc (Zandi and Schnug, 2022). Carbon dioxide (CO_2) fixation in chloroplasts contributes considerably to ROS formation owing to the severe reduction in the photosynthetic electron transport chain (Sachdev et al., 2021). Highly reactive oxygen species (ROS) may interact with macromolecules such as deoxyribonucleic acid, oils, enzymes, and lipids, as well as other important biological components such as chemical interactions within the cellular system and redox potential (De Almeida et al., 2022). Heavy metal ions, such as Copper (Cu), zinc (Zn), manganese (Mn), and iron (Fe), are important for plant metabolism, however, their excess is very toxic. For example, when Zn and Mn are abundant, they impact plant development and reduce the effects of Fe (Ferrol et al., 2016). Maize (*Zea mays* L.), which ranks third among cereals after wheat and rice and serves as a staple food, is a major cereal crop grown worldwide as a food and feed crop (Chen et al., 2022). Maize, is naturally rich in carbohydrate, which is consumed as a major food source in developing countries, However because of the contamination of soil with heavy metals, the accumulation of heavy metals in maize plants reaches beyond the permissible limits (Vongdala et al., 2019) and these heavy metals taken up by maize plants, alter their physiological features, and molecular and cellular attributes even at low concentrations in soil (Rizvi et al., 2022).

By using a biological concentration of microorganisms, contaminated soil can be successfully decontaminated from heavy metals (Anastopoulos et al., 2019). Bacteria, Firmicutes, and Actinobacteria have been proposed as alternatives for the decontamination of metal-polluted soils with high concentrations of Mn, Pb, and As (Xiao, 2017). Bacteria are vital microorganisms for the removal of heavy metal-contaminated soils (Bacteria have the ability to reduce and detoxify pollutants such as heavy metals in soil by the process of immobilization, activation, absorption, and transformation; Hassan et al., 2017). The presence of free-living and specific plant growth promoting rhizobacteria (PGPR) in the rhizosphere soil significantly helps plant root development and plant survival by altering the availability of nutrients, thereby increasing plant growth (Nadeem et al., 2014). At different stages of a plant's life, all its tissues interact with microorganisms; nonetheless, this interaction seldom causes harm to the plant. Bacteria are found in the roots and surfaces of plants. Plants emit chemicals and nutrients that maintain and attract microbes (Rajkumar and Kurinjimalar, 2021). Microorganisms produce compounds that promote plant development (Schirawski and Perlin, 2018). The primary carbon source for soil bacteria is carbon from photosynthesis or plant residue (Wang et al., 2020).

Endophytic microorganisms and plants develop symbiotic interactions because both parties gain from these interactions. The

host plant absorbs compounds that boost nutrient absorption and growth in return for room and protection (Riaz et al., 2021). Roots often contain more bacteria than dirt. This indicates that plant roots reject 5–30% of the microscopic organic acids, amino acid, and carbohydrate molecules that bacteria require for survival (Liu et al., 2017). Endophtic bacteria can penetrate plant parts such as roots, flowers, leaves, and branches. These microorganisms may have developed in the seeds (Halwas, 2017). Endophytic bacteria were isolated from several monocot, dicot, woody, and herbaceous plants, including oak (*Quercus* L.), pear (*Pyrus* L.), sugar beet (*Beta vulgaris* L.), and corn (*Zea mays* L.) (Chebotar et al., 2015). Among the endophytic bacteria of maize are *Pantoea*, *Rhanella*, *Rhizobium*, *Herbaspirillum*, *Pseudomonas*, *Brevundimonas*, *Enterobacter*, and *Burkholderia*. *Pseudomonas fluorescence* produces a siderophores and phosphate solubilizers. *Vibernum grandiflorum* is a medicinal plant that includes approximately 230 species of deciduous Adoxaceae shrubs. Most members of the genus *Viburnum* are indigenous to tropical and temperate regions. Pakistan is home to six species of *Viburnum*, including *Viburnum contonifolium*, *Viburnum tinus*, *Viburnum cylindricum*, and *Viburnum grandiflorum*. *Viburnum* root and stem extracts exhibit antifungal, insecticidal, and phototoxic effects (Raoof and Siddiqui, 2013). It is used in traditional medicine as a blood cleanser, laxative, and a diuretic. It is calming and antispasmodic, and helps in liver conditions. Its branches are utilized to create toothbrush bristles. *Viburnum* branches are both a source of fencing materials and fuel (Kumar et al., 2020). Information on endophytic bacteria from medicinal plants and their use as plant promoters is rare, and a detailed study is required on the *Agrococcus terreus* strain, on which very few reports are available to date; therefore, the current study was designed to isolate the bacterial endophytes from the roots of a medicinal plant, *Viburnum grandiflorum*, and to investigate the growth-promoting potential of isolated endophytic bacteria (*Agrococcus terreus*) in maize plants grown in nickel- and Zn-contaminated soil.

2. Methodology

2.1. Sample collection

The roots of *V. grandiflorum* were collected from District Poonch (Davi Gali) at an elevation of 5,000 ft. Healthy and mature plants were selected and collected for the isolation of bacteria. The roots of the selected plant *Viburnum grandiflorum* were carefully cut and packed in zip–lock plastic bags. The samples were then brought to the laboratory for further research (Figure 1).

2.2. Isolation of bacterial endophytes

Bacterial endophytes were isolated from the roots of *Viburnum grandiflorum* in nutrient agar medium using the method described by Rekha et al. (2015). Small pieces of root were made using a sterilized knife. Surface sterilization was performed by sequential cleaning with ethanol (70%) for 5 min, 1% HgCl2 for 1–2 min and double-distilled water 5–6 times for 2–5 min. Sterile root pieces were macerated in phosphate buffer (PB) at PH 7.0.After maceration, the

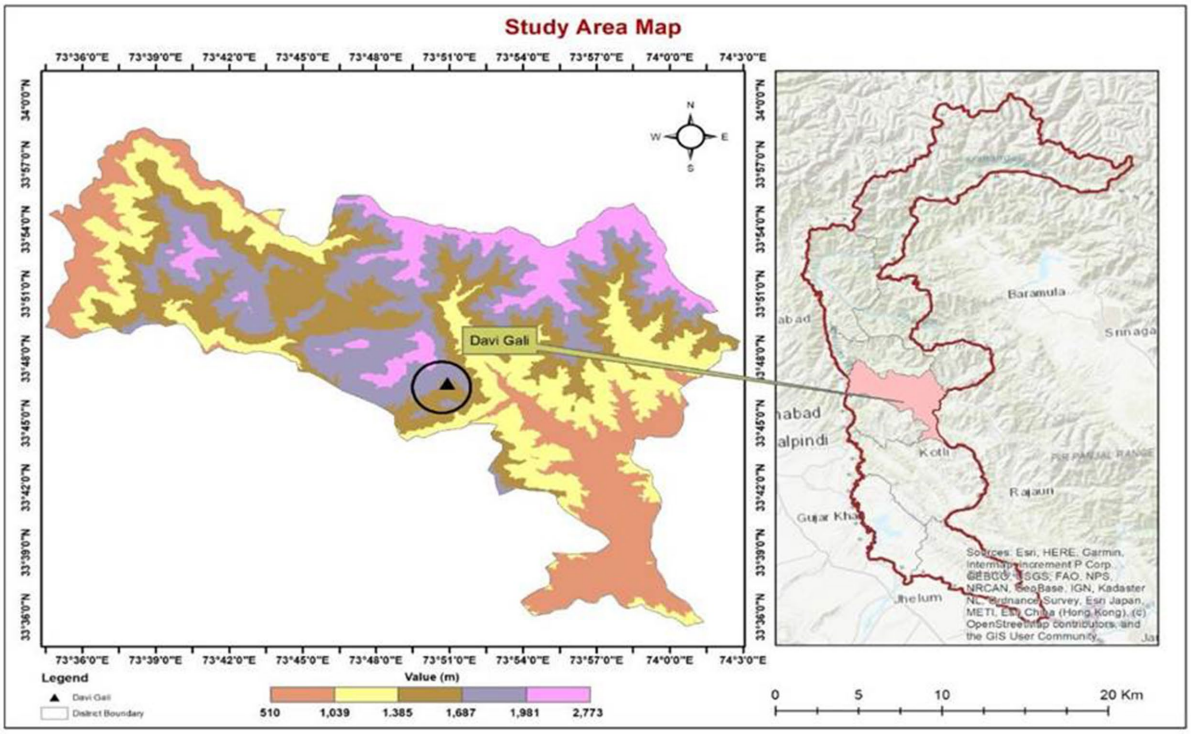

FIGURE 1
Study area map.

samples were grinded with a disinfected pestle and mortar in 9.5 mL of the final buffer wash with distilled water grinded. From grinded sample 10 mL was taken into falcon tube. Two replicate samples were prepared and centrifuged at 3000 rpm for 15 min. Afloat was collected, and serial dilutions from this extract were prepared in phosphate buffer (10–5, 10–6 and 10–7). From each dilution a 0.1 mL extract was inoculated on separate Petri plates containing nutrient agar media. The plates were incubated at 37C° observation was taken after 48 to 72 h. Bacterial isolates were picked from the plates and purified using streaking techniques. This isolation process was repeated until pure colonies were obtained. Based on morphology and color, different colonies were obtained. Gram staining was performed to identify Gram-positive and Gram-negative bacteria (Rekha et al., 2015).

2.3. Identification, and 16S rRNA sequencing of endophytic bacteria

Genomic DNA was extracted from the bacterial strains using the Bacterial Genomic DNA Kit (Smith et al., 2003). The bacterial strain was grown in nutrient broth and incubated overnight to extract the genomic DNA. About 1.5 mL of saturated bacterial culture was transferred to an Eppendorf tube and harvested by centrifugation at 10000 rpm for 2 min. The pellets were collected and harvested again with 1.5 mL of cell culture. Thereafter, the pellets were drained using a paper towel. The pellets were re-suspended and lysed in 450ul µL transcription buffer (TF) with dynamic pipetting. To remove proteins and cell remains 45ul of 10% sodium dodecyl sulfate (SDS) solution and 5ul of 20 mg/mL proteinase K were added, mixed well, and incubated for an hour at 37oC. Thereafter, the clear supernatant was transferred to a clean vial and approximately 500ul phenol-chloroform was added and mixed energetically by inverting the tube until the phases were completely mixed. The vial was centrifuged at10000rpm for 2 min. The upper aqueous phase was transferred to a new Eppendorf tube, mixed with a phenol-chloroform mixture again, and centrifuged at 10000 rpm for 5 min. The obtained aqueous phase was transferred to a new tube and 50ul of sodium acetate was added and mixed well by hand. Thereafter, 300ul of isopropanol was added and mixed gently to precipitate the DNA, which was centrifuged at 10000 rpm for 2–5 min. The DNA was washed with 1 mL of 70% ethanol for 30 s and centrifuged. DNA was drained with ethanol, dried, and re-suspended in 50-100ul. T.E buffer and DNA were stored at -20oC. The similarity index of the sequences of the isolated endophytic bacterial strains was determined using BLAST in the online tool NCBI nucleotide BLAST. By using sequence match application and BLAST to show the resemblance of new sequences with the reference sequences in the databases (Auld et al., 2013). The phylogenetic association of the isolated bacterial strains was resolved using the neighbor-joining technique (Saitou and Nei, 1987). A phylogenetic tree was constructed using molecular evolutionary genetics (Stefanova et al., 2015).

2.4. Culturing of *Agorcocus tereus*

The culture of the isolated *A. tereus* bacteria was identified as (Acc. No. MW 979614) was spread over nutrient agar medium. Nutrient agar was prepared by dissolving 28 gm/1000 mL of nutrient agar medium in distilled water.

2.5. Preparation of inoculums

For inoculum preparation, 250 mL nutrient broth medium was sterilized. The medium was autoclaved for 45 min at 121°C. The broth was inoculated with a 24 h old culture and incubated at 32°C and 150 rpm in a shaker incubator (NB-205LF) for 3–5 days.

2.6. Seed inoculation

Maize (*Zea mays*) seeds were obtained from the National Agricultural Research Center Islamabad (Pakistan). The surface of the seeds was sterilized by washing with 95% ethanol and then immersing them in 10% Clorox for 2–3 min. The seeds were then washed in sterile filtered H_2O 2–3 times. Seeds were gently stirred to remove any loose dirt collected from seedlings (Omid et al., 2010). Plant height, shoot length, root length, leaf length, number of leaves, fresh plant weight, moisture content, leaf proline, leaf protein, leaf sugar estimate, peroxidase dismutase (POD), and super oxide dismutase (SOD) were measured and analyzed (Liu et al., 2010).

2.7. Preparation of zinc and nickle solution and treatment application

Analytical grade zinc sulfate and nickel sulfate were used during this study, and Ni and Zn solutions were prepared for different treatments in autoclaved distilled water. Approximately 100 mg of zinc sulfate and nickel sulfate were dissolved in 100 mL of water. Both zinc and nickel solutions were added to 1 kg of soil (100 mg/100 mL/ 1 kg) (Ye et al., 2017). Eight different treatments were used (Table 1), and the plants were harvested after 28 days of germination.

2.8. Plant parameters studied

Root and shoot lengths were measured in centimeters from the apex to the tip of the shoot and root, respectively (Prajapati et al.,

TABLE 1 Treatments made in experiment.

Treatments	Conditions
T1	Control (No Heavy Metals)
T2	Seed + Zinc (Zn)100 mg/kg
T3	Seed + Nickel (Ni)100 mg/kg
T4	Inoculated seeds
T5	Inoculated Seed +Zn100mg/kg
T6	Inoculated Seed +Ni100mg/kg
T7	Inoculated Seed + Ni 50 mg/kg + Zn 50 mg/kg
T8	Seed +Ni50mg/kg + Zn50mg/kg

2008). The seedlings were removed from their containers, the excess dirt was washed off, and the final weight in grams was determined (Banerjee et al., 2016). To determine the dry weight, the seedling roots and shoots were oven-dried overnight at 70 0C, and the weight was measured in grams (Ricigliano, 2015).

2.9. Plant nutrient analysis

The per-chloric acid digestion method was used to assess the nutrient content of the maize plants (McLaren et al., 2012). This technique was used to determine the presence of nutrients in plant components. Plant leaf material (0.25 g) was used for the nutrient analysis.

Cations in plants = (ppm in extract - blank) × A × dilution factor W A = Total volume of extract (mL) W=Weight of the dry plant.

2.10. Leaf protein contents

The protein extract of fresh maize plant leaves was tested using the most effective technique (Rahu et al., 2021). About 0.1 g and fresh maize leaves were used to determine the protein content. Maize plant leaves (0.1 g) were placed in a phosphate buffer. The mixture was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with purified water, and the volume was increased to 1 mL. The resulting solution was added to 1 mL of alkaline CuSO4 reagent and shaken for 10 min, after which folline reagent was added to the solution and incubated for 30 min at 28 ± 2°C. The absorbance was measured at 650 nm using a spectrophotometer.

2.11. Leaf proline content

The proline content of the maize leaf extracts was evaluated (Sairam and Srivastava, 2000). About 0.1 g of fresh maize plant leaves were used to examine proline content. Maize plant leaves (0.1 g) were placed in 10 mL of methanol. After that the mixture was centrifuged at 3000 rpm for 10 min. After 24 h, the mixture was refrigerated. After 24 h, the mixture was again centrifuged.1 mL and 5% phenol was added and incubated at room temperature for at least 1 h. Then, sulfuric acid (2.5 mL of sulfuric was added and the absorbance was measured at 490 nm.

$$\text{Proline} = K^{*}\text{Dilution Factor}^{*}\text{Optical density / weight of sample}$$

The K value is 9.6.

2.12. Leaf sugar content estimation

Fresh maize leaves (0.5 g) were homogenized in 10 mL distilled water. The extract was centrifuged at 3000 rpm for 5 min. The supernatant was collected and incubated for 1 h at room temperature with 1 mL of 80% phenol. Following the incubation period, 5 mL sulfuric acid was added. The samples were incubated for 4 h at room temperature. Readings were recorded at 420 nm. The sugar value was determined using the given formula.

$$\text{Total sugar}\left(\mu g / g\right) = \frac{\text{k value of sugar} * \text{Dilution factor} * \text{absorbance value}\left(\text{mg}\right)}{\text{Weight of sample}\left(g\right)}$$

2.13. Peroxidase (POD) analysis

The peroxidase dismutase activity of maize plant leaves was investigated using a standard procedure (Van Assche et al., 1988). Approximately 1 g of cold maize leaves was used to examine the POD antioxidant enzyme.1 g of frozen maize leaves was placed in an icy mortar with 0.5 M calcium chloride solution. The extract was centrifuged at 1000 rpm for 10 min, and the supernatant was transferred to clean test tubes and stored in a freezer. The pellet of the cell wall extract remaining in the centrifuge tube was 2.5 mL of ice-cold mixed calcium chloride solution 0.5 M and centrifuged.

The supernatants were collected twice.

The reaction mixture was prepared as follows:

0.1 mL extract+1.5mlMES + 0.5 mL p-Phenylenediamine+0.45ml Hydrogen peroxide.

Add MES Buffer in blank cuvette. At 510 nm the reading was recorded. In two times The readings were recorded twice, at 0 min and 3 min.

2.14. Superoxide dismutase analysis

The superoxide dismutase assay for maize plant tissue was assessed (Mushtaq et al., 2022) Mix (a) about 0.2 g of frozen maize leaves were used to examined the SOD enzyme. Plant tissue (b) 0.2 g of plant tissue was placed in 1 g polyvinylpyrrolidone (PVP polyvinylpyrolidone) +0.0278gNaEDTA solutions in a cooled pestle and mortar. Mix (c): The mixture was centrifuged at 4°C for 10 min. The supernatant was collected and raised up to 8 mL by adding phosphate buffer of pH 7.(Mix d) 0.0278 g NaEDTA +1.5 g Methionine +0.04 g Nitro blue tetrazolium chloride (NBT) was dissolved in 100 mL of phosphate buffer (pH 7.8). (Mix e)Take 10 mL from mix (d) and raise its volume up to 50 mL with pH 7.8 Phosphate buffer.(Mix f)Dissolve the 0.0013 g of riboflavin in 100 mL of phosphate buffer (pH 7.8).(Mix g)Take 20 mL from mix (f) and raise its volume to 50 mL with distilled water.

Three types of assays were performed: 1st Reference, 2nd Blank and 3rd Reaction mixture.

The reference sample was mixed well and kept in the dark, and the reaction mixture was kept in the light chamber for approximately 20 min. Absorbance was measured at 560 nm using a spectrophotometer.

2.15. Statistical analysis

The study was carried out in pots using a complete randomized design (CRD) with Statistix 8.1. The data for various parameters were

obtained after 28 d of seedling growth. Triplicate values were recorded for each treatment (Zhang et al., 2021).

3. Results

The present investigation was performed for the isolation and Characterization of endophytic bacteria from the roots of *Viburnum grandiflorum* (guch) and its application on maize plant. Total of four bacterial colonies were isolated from the roots of *Viburnum grandiflorum*.

3.1. Identification and 16 s rRNA sequencing of endophytic bacteria

Two endophytic bacterial strains were isolated from *Viburnum grandiflorum* out of which one bacterial strain 16S rRNA sequence showed 93% similarity with *Agrococcus terreus*.

3.2. Phylogenetic relation of the isolated bacteria from *Viburnum grandiflorum*

Figure 2 shows the phylogenetic relationship of the strain isolated from *Viburnum grandiflorum*. The isolated *A. terreus* strain branched off from a group of clades. The *A. terreus* group during evolution did not show much similarity with any clades, but with the ancestor of the clades. There were 10 positions in the final dataset. In the first clade, one species from France was closely related to a species from India, and these two species were next related to Canadian species. In the next clade, one species from Sweden was closely related to the species from China. In the next clade, one species from Egypt was closely related to the species from Switzerland, and these two species were closely related to species from China.

3.3. Effect of zinc (Zn) and nickel (Ni) on plant parameters maize seeds germination %

The germination percentage of maize seeds varied between the inoculated and uninoculated seeds, as illustrated in Figure 3A. There was no significant change in seed germination% among treatments, except for T5 (inoculated seed + Zn) and T8 (Seed + Zn + Ni). The combined application of Ni and Zn (T8) hindered germination and resulted in a 40% drop in germination% when compared to the control; however, the strength of inhibition was reduced when seeds were infected with *A. terrus* (T7), and germination% increased by 33% when compared to T8. Zn substantially inhibited seed germination, with a 40% reduction in infected seeds at T5 (inoculated seed + Zn). Shoot length was affected by several treatments Figure 3B. The T5 (inoculated seed + Zn) plant had the longest shoots, which increased by 49.19% above the control. *A. tereus* (T7) considerably enhanced the shoot length in the presence of Zn and Ni, and a 22% increase in shoot length was found compared to T8. The combined application of Zn and Ni (T8) reduced shoot length. Figure 3C demonstrates that inoculation with *A. tereus* had a substantial effect on root length compared to uninoculated seeds. In the presence of Ni and Zn (T2 and T3), root length was suppressed; however, inoculation of seeds with *A. tereus* greatly enhanced root length (T5) and T6, and the % increase was 38.82 and 37.02% greater than that of T2 and T3, respectively. Similarly, the combined treatment of Ni and Zn (T8) decreased root growth, but inoculation of seeds with *A. tereus* (T7) significantly improved root length compared to T8 by 25.00%. The number of leaves varied between inoculated and uninoculated plants (Figure 3D). There was no significant difference in the number of leaves between any of the treatments, with the exception of T3 (Seed + Ni),

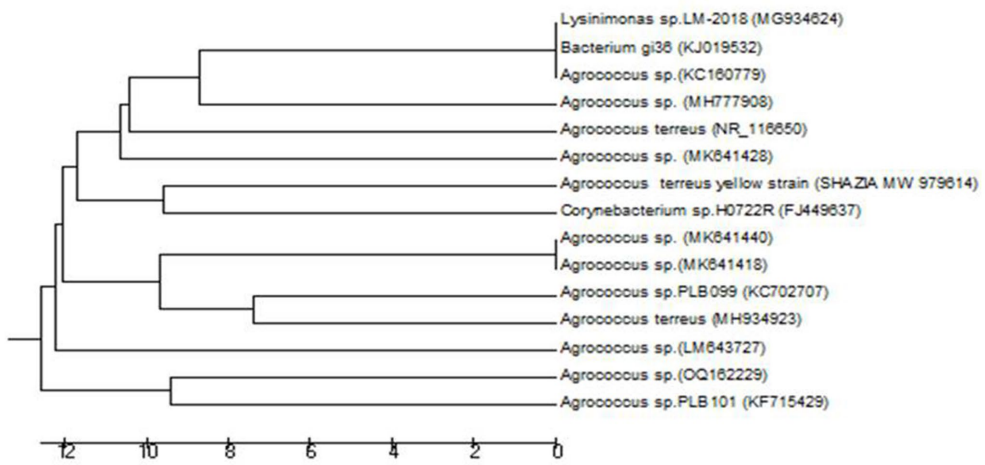

FIGURE 2
Neighbor–Joining Tree of *Agrococcus terreus* Isolated from *Viburnum grandiflorum*. The evolutionary history was inferred using the UPGMA method [1]. The optimal tree with the sum of branch length = 126.99494267 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [2] and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 706 positions in the final dataset. Evolutionary analyzes were conducted in MEGA6 [3].

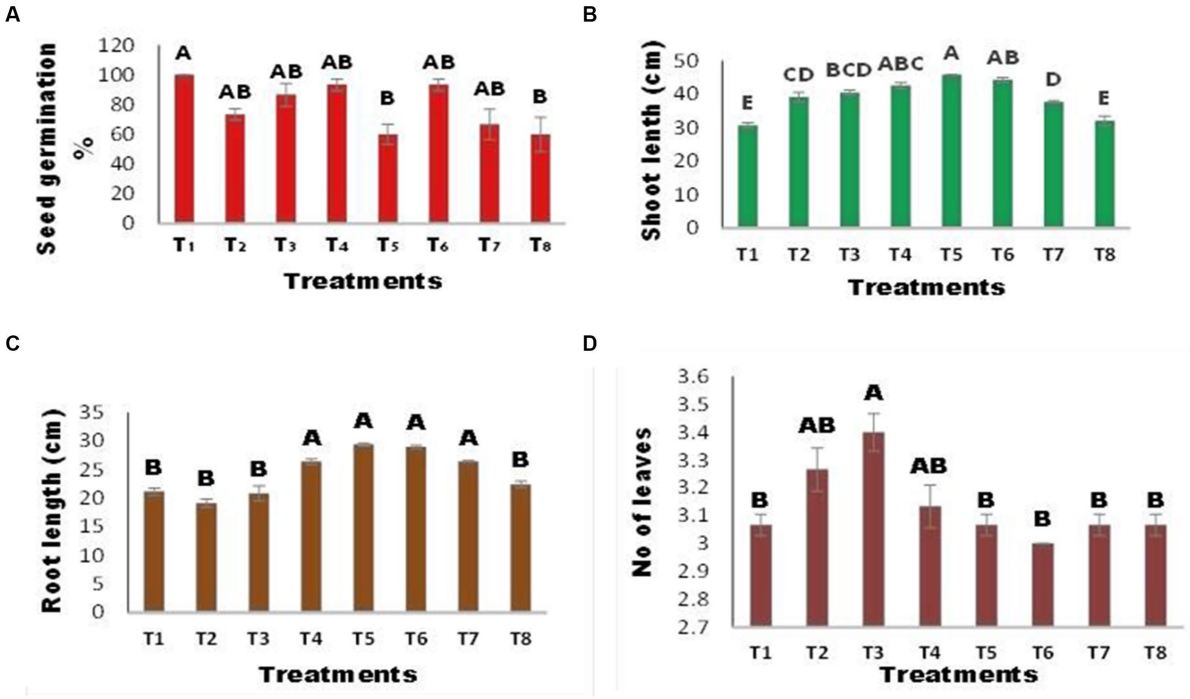

FIGURE 3
Effect of Zinc (Zn) and Nickel (Ni) on **(A)** Germination % **(B)** Maize Shoot Length **(C)** Root length **(D)** No of leaves The bars sharing common letter are non-significantly different otherwise vary significantly at $p > 0.05$. T1 = control, T2 = seed+Zn100mg/kg, T3 = seed+Ni100mg/kg, T4 = inoculated seed, T5 = inoculated seed+Zn100mg/kg, T6 = inoculated seed+Ni100mg/kg, T7 = inoculated seed+Ni 50 mg/kg + Zn50mg/kg, T8 = seed+Ni 50 mg/kg + Zn 50 mg/kg.

where the increase was 10.86% and the number of leaves was much greater than that in T2 and T4. The leaf width of maize plants differed between the inoculated and uninoculated seeds, as shown in Figure 4A. Leaf width was reduced in the presence of Ni and Zn (T2 and T3); however, inoculation of seeds with *A. tereus* led to an 89% increase in leaf width compared to the control. Similarly, the combined application of Zn and Ni decreased the leaf width of T8 (Seed + Ni + Zn), while inoculation with *A. tereus* (T7) diminished the impact of Ni and Zn, resulting in a leaf width increase of 51.87% relative to T8. Inoculation with *A. tereus* showed a notable difference in plant height relative to uninoculated seeds (Figure 4B). In the presence of Ni and Zn (T2 and T3), plant height was impeded, whereas inoculation of seeds with *A. tereus* significantly increased plant height in T5 and T4, with increases 31.50 and 27.26% greater than those in T2 and T3, respectively. Similarly, the combined application of Ni and Zn (T8) decreased plant height, but inoculation of seeds with *A. tereus* (T7) tended to result in a 13.03% increase in plant height compared to T8. The inoculation of *A. tereus* had a substantial effect on leaf fresh weight compared to uninoculated seeds (Figure 4C). The leaf fresh weight was suppressed in the presence of Ni and Zn (T2 and T3), but inoculation of seeds with *A. tereus* considerably boosted the leaf fresh weight in T6 and T5, with increases of 113 and 104% more than those of T2 and T3, respectively. Likewise, the combined treatment T8 decreased leaf fresh weight, but the treatment T7 substantially raised leaf fresh weight compared to T8 by 56.41%. The results revealed a variance in dry weight in the inoculated and uninoculated treatments at 4 (d). Except for the T2 treatment, which showed a 64.83% increase in dry weight, no significant differences in dry weight were identified between the treatments. In contrast, seed inoculation with *A. tereus* increased the dry weights of T6 and T5 by 16.48 and 13.18%, respectively. Similarly, the combined treatment of Ni and Zn (T8) decreased the dry weight; however, the T7 treatment considerably increased the dry weight compared to T8 by approximately 20.87%. The results demonstrated that the inoculation of *A. tereus* significantly changed the moisture content of seeds compared to uninoculated seeds (Figure 4D). The moisture content decreased in treatments T2 and T3, whereas inoculation of seeds with *A. tereus* enhanced the moisture content in treatments T5 and T6 by 136.53 and 146.92%, respectively, compared with T2 and T3. Similarly, the T8 treatment restricted the moisture content; however, the T7 treatment enhanced the moisture content by about 68.84% compared to the T8 treatment.

3.4. Effect of zinc (Zn) and nickel (Ni) on maize proline protein and sugar content

There were variations in the proline content of the inoculated and uninoculated maize seedlings (Figure 5A). The proline content was decreased in T3 and T4; however, inoculation of seeds with *A. tereus* enhanced the proline content in T6 by approximately 80.95% compared to the control. Similarly, T8 showed a non-significant increase of 51.08% compared with T7. Protein content revealed that *A. tereus* inoculation considerably influenced protein content compared to uninoculated seeds (Figure 5B). Treatments T2 and T3 had lower protein levels. However, inoculation of seeds with *A. tereus* considerably increased the protein content of T4 and T5, with

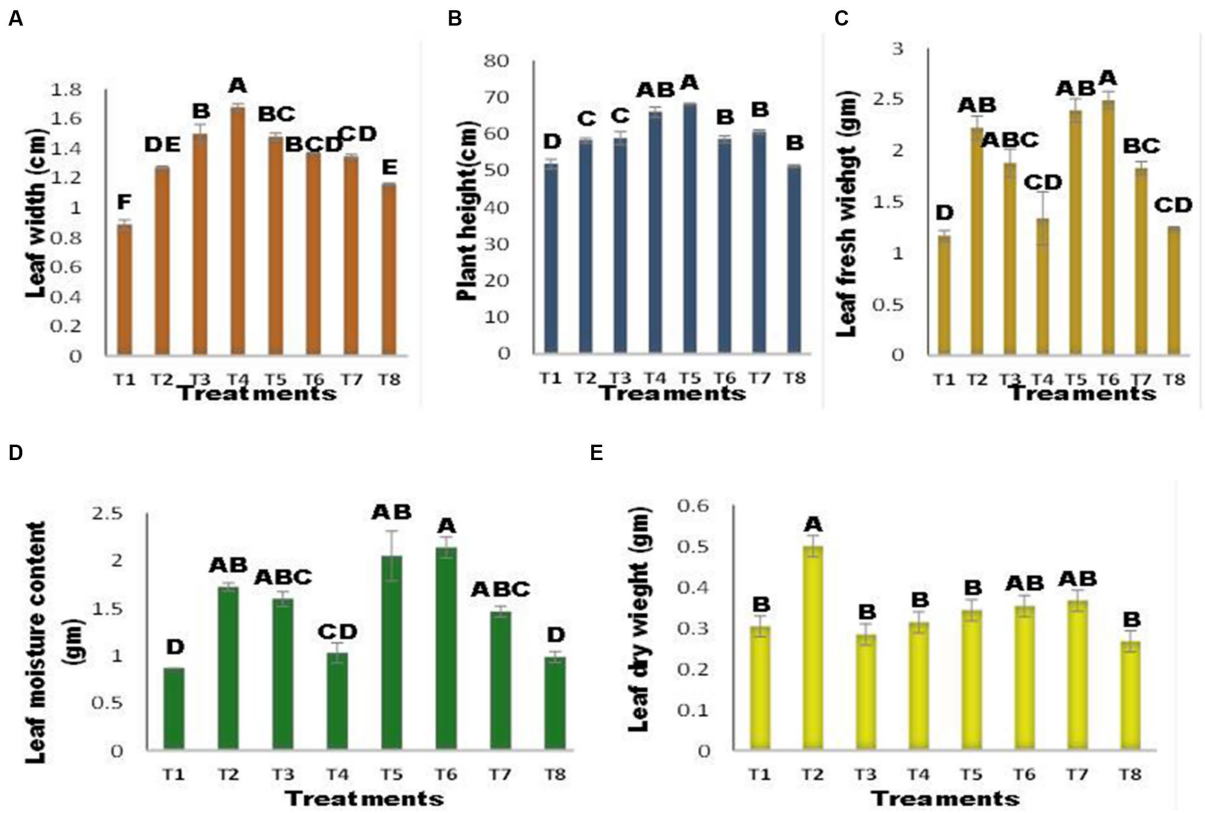

FIGURE 4
Effect of Zinc (Zn) and Nickel (Ni) on **(A)** Leaf width**(B)** Plant height **(C)** Leaf fresh weight **(D)** Leaf Moisture content **(E)** Leaf dry weight. The bars sharing common letter are non-significantly different otherwise vary significantly at $p > 0.05$. T1 = control, T2 = seed+Zn100mg/kg, T3 = seed+Ni100mg/kg, T4 = inoculated seed, T5 = inoculated seed+Zn100mg/kg, T6 = inoculated seed+ Ni100mg/kg, T7 = inoculated seed+Ni 50 mg/kg + Zn 50 mg/kg, T8 = seed+Ni 50 mg/kg + Zn 50 mg/kg.

increases of 46.17 and 27.90% greater than those of T2 and T3, respectively. The results indicated that seed inoculation with *A. tereus* significantly improved the sugar content of T4 treatment plants by 12.64% compared to that of control plants (Figure 5C). *A. tereus* increased the sugar content, but Ni and Zn lowered the sugar content in T7 by 40.42% against the control.

3.5. Effect of zinc (Zn) and nickel (Ni) on peroxidase enzyme and superoxide dismutase enzyme

Inoculated and uninoculated seeds varied in POD of the maize plants (Figure 6A). The POD concentrations in T1 and T8 decreased in the presence of Ni and Zn. While *Agrococus tereus* inoculation increased the POD content, T7 and a 211.47% increase over the control were observed. The results demonstrated the difference between the inoculated and uninoculated superoxide dismutase values (Figure 6B). The addition of Zn (T2, T5) resulted in insignificant changes compared with the control treatment. While the addition of Ni increased the SOD content by 16.78% (T3), inoculation of seeds with *A. tereus* in the presence of Ni (T6) decreased the SOD content by 50.68 and 57.76% relative to T3 and the control, respectively. Similarly, inoculation with *A. tereus* (T7) resulted in greater SOD content in the presence of Ni and Zn (T8), and 68.21% of the combined Ni and Zn treatments resulted in a higher SOD content than T8. In the presence of Ni, the SOD content was greater than that in all other treatments, but *A. tereus* inoculation (T6) lowered the SOD concentration by up to 50.68% relative to T3. The addition of Zn to *A. tereus* did not significantly reduce the SOD content in comparison with T2.

3.6. Accumulation of Zn and Ni by maize seedlings

The addition of Zn (T2, T5) resulted in significant changes compared with the control (Figure 7A). *A. tereus* inoculation of seeds in the presence of Ni (T6) considerably enhanced Zn accumulation in maize seedlings, with an increase of 66.80% compared with T3, whereas seeds with Ni lowered Zn content and decreased it by 6.39%. The accumulation of Ni in maize plants differed between inoculated and uninoculated seedlings (Figure 7B). The addition of Ni to the soil considerably increased the Ni content of maize seedlings (T3). This rise was tenfold greater than that of the other treatments. In all other treatments, the Ni content was not markedly different. Ni accumulation in T6 decreased by up to 43.60% with the addition of *A. tereus*.

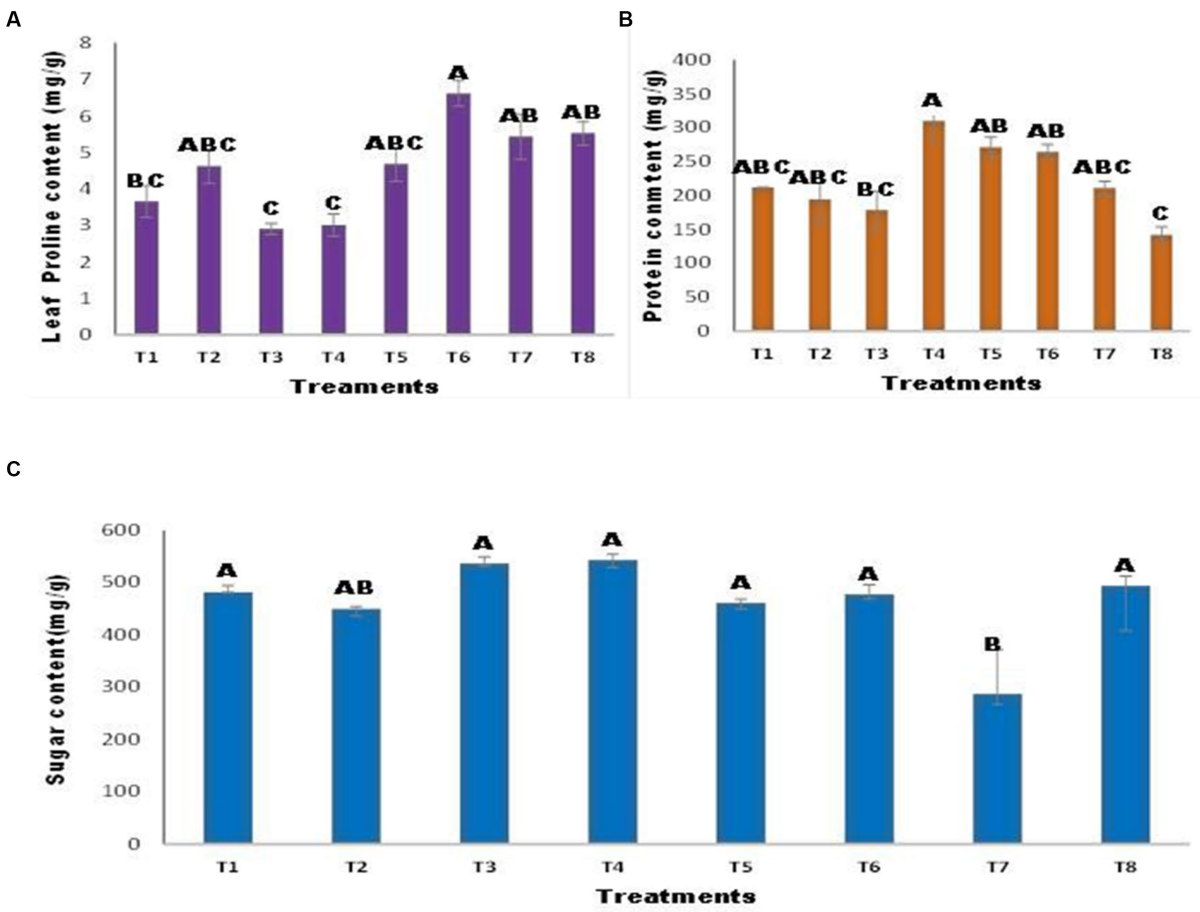

FIGURE 5
Effect of Zinc (Zn) and Nickel (Ni) on **(A)** Proline **(B)** Protein **(C)** Sugar content. The bars sharing common letter are non-significantly different otherwise vary significantly at $p > 0.05$. T1 = control, T2 = seed+Zn100mg/kg, T3 = seed+Ni100mg/kg, T4 = inoculated seed, T5 = inoculated seed+Zn100mg/kg, T6 = inoculated seed+ Ni100mg/kg, T7 = inoculated seed+Ni 50 mg/kg + Zn 50 mg/kg, T8 = seed+Ni 50 mg/kg + Zn 50 mg/kg.

3.7. Accumulation of micro and macro nutrients by maize seedlings

Accumulation of micro-and macronutrients in maize seedlings has been documented (Table 2). Copper (Cu) content varied in both inoculated and uninoculated seeds, T7 with having the highest Cu content (21.31%). The manganese (Mn) content of the inoculated seeds was greater than that of the uninoculated seeds. Maximum Mn content was found in T4 (6.27%). The inoculation of *A. tereus* increased the sodium (Na) content. T7 had the highest Na content (206.58%) and lowest Na content (16.90%). The potassium (K) content of maize seeds increased when *A. tereus* was inoculated. T7 had the highest K concentration (42.03%), whereas T2 had the lowest K concentration (35.68%). In comparison to other treatments, *A. tereus* inoculation increased the iron (Fe) content of seedlings. T7 had the highest Fe amount (48.38%), whereas T3 had the lowest (50.34%). Inoculation with *A. tereus* increased Ca content in maize seedlings. T7 had the highest calcium (Ca) content (60.85%), whereas T2 had the lowest content (27.21). Inoculation with *A. tereus* increased the amount of magnesium (Mg) in the plant, but the uninoculated plants had less Mg. T7 (92.07%) had the highest Mg level, whereas T3 had the lowest Mg content (50.76%).

4. Discussion

Endophytic bacteria are plant-beneficial bacteria that prevail inside plants and can facilitate plant growth under normal and stressed conditions (Lumactud and Fulthorpe, 2018) Endophytic bacteria can be beneficial to plants in several ways. Endophytic bacteria have a wide range of host (Ibáñez et al., 2017). Every plant is a host of one or more endophytes but most of the flora is unexplored. Endophytic bacteria can be isolated from these plants and used as biostimulators to develop a secure and sustainable agricultural system (Afzal et al., 2019). During the present investigation, similar findings were reported in which the isolated strain from the roots of the medicinal plant *Viburnum grandiflorum* showed variation in morphology and colony forming units (CFU). These colonies were morphologically different from one another and these results were supported by the findings of Padder et al. (2017) who demonstrated that endophytic bacterial strains with different sizes, shape textures and populations range from the roots of (*Brassica rapa L.*). In the present study, the bacterial counts were different from each other and these results are in close agreement with those of Yang et al. (2013) who demonstrated that endophytic bacteria isolated from many crops such as, cotton and sweet corn were different from each other. Jasim et al. (2014) described that this variation in the endophytic bacterial

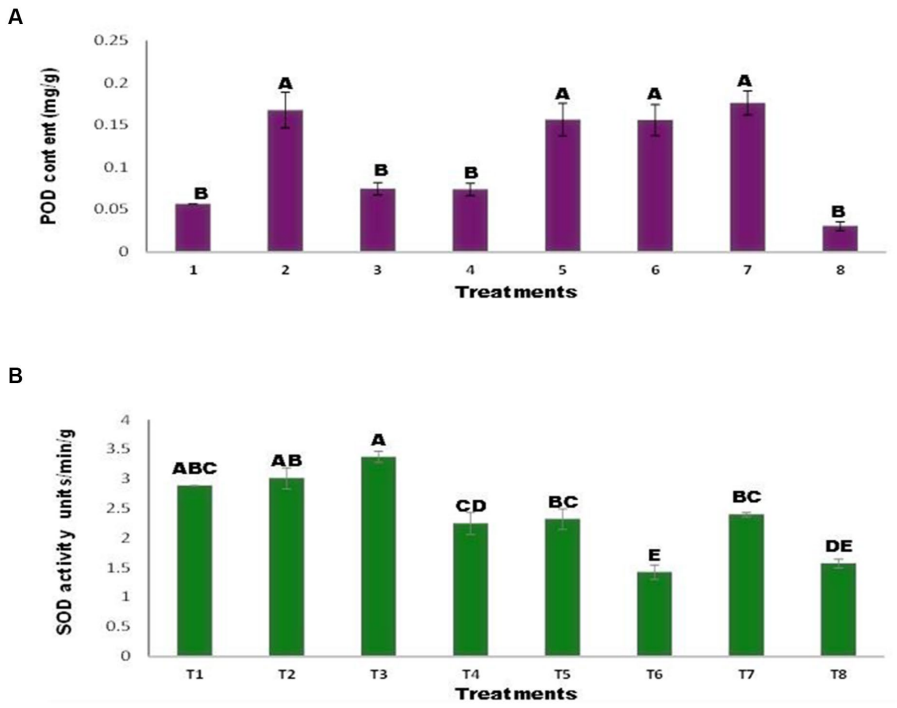

FIGURE 6
Effect of Zinc (Zn) and Nickel (Ni) on **(A)** POD **(B)** SOD. The bars sharing common letter are non-significantly different otherwise vary significantly at $p > 0.05$. T1 = control, T2 = seed+Zn100mg/kg, T3 = seed+Ni100mg/kg, T4 = inoculated seed, T5 = inoculated seed+Zn100mg/kg, T6 = inoculated seed+Ni100mg/kg, T7 = inoculated seed+Ni 50 mg/kg + Zn 50 mg/kg, T8 = seed+Ni 50 mg/kg + Zn 50 mg/kg.

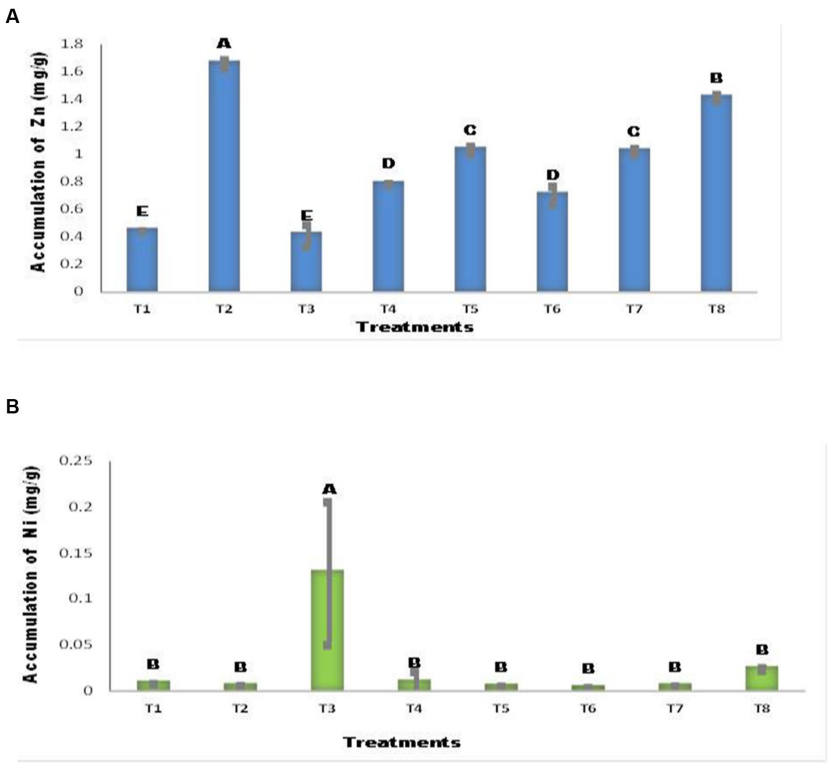

FIGURE 7
Accumulation of **(A)** Zn **(B)** Ni by Maize plant. The bars sharing common letter are non-significantly different otherwise vary significantly at $p > 0.05$. T1 = control, T2 = seed+Zn100mg/kg T3 = seed+Ni100mg/kg, T4 = inoculated seed, T5 = inoculated seed+Zn100mg/kg, T6 = inoculated seed+Ni100mg/kg, T7 = inoculated seed+Ni 50 mg/kg + Zn 50 mg/kg, T8 = seed+Ni 50 mg/kg + Zn 50 mg/kg.

TABLE 2 Accumulation of micro and macro nutrients by maize plants.

Nutrients	Nutrient concentration							
	T1	T2	T3	T4	T5	T6	T7	T8
Cu (mg/g)	0.30 ± 0.023B	0.35 ± 0.003A	0.08 ± 0.004D	0.15 ± 0.003D	0.27 ± 0.005B	0.27 ± 0.002B	0.36 ± 0.009D	0.11 ± 0.002CD
Mn (mg/g)	0.92 ± 0.004A	0.77 ± 0.03B	0.33 ± 0.001C	0.98 ± 0.0005A	0.43 ± 0.06C	0.12 ± 0.003D	0.69 ± 0.03B	0.12 ± 0.01D
Na (g/Kg)	5.77 ± 0.14D	3.81 ± 0.15E	4.03 ± 0.08E	15.20 ± 0.51B	8.99 ± 0.33C	9.14 ± 0.14C	17.70 ± 0.184A	4.79 ± 0.51DE
K (mg/g)	32.43 ± 0.49C	20.86 ± 0.52E	17.51 ± 0.30E	43.51 ± 1.117A	38.87 ± 1.00B	28.47 ± 0.61D	46.07 ± 0.78A	17.92 ± 0.56 E
Fe (mg/g)	17.36 ± 0.15C	10.65 ± 0.48D	8.62 ± 0.25C	24.40 ± 0.55AB	22.10 ± 1.05B	15.21 ± 0.39B	25.77 ± 0.58A	10.80 ± 0.17D
Ca (g/Kg)	16.43 ± 0.20B	11.96 ± 0.31D	13.81 ± 0.33D	24.48 ± 0.40A	19.59 ± 0.22B	15.92 ± 0.11C	26.43 ± 0.72A	12.66 ± 0.38D
Mg (g/Kg)	9.62 ± 0.18D	4.85 ± 0.169E	4.73 ± 0.24E	17.30 ± 0.26 BC	16.22 ± 0.55C	18.48 ± 0.51B	21.77 ± 0.13A	5.38 ± 0.51E

Treatments sharing common letter are non-significantly different otherwise vary significantly at $p > 0.05$. T1 = control, T2 = seed + Zn100mg/kg T3 = seed + Ni100mg/kg, T4 = inoculated seed, T5 = inoculated seed + Zn100mg/kg, T6 = inoculated seed + Ni100mg/kg, T7 = inoculated seed + Ni 50 mg/kg + Zn50mg/kg, T8 = seed + Ni50mg/kg + Zn50mg/kg.

population could be due to physical, chemical, genetic, and climatic factors. Moreover, the method of isolation may also play a major role in the bacterial count. Similarly, Liaqat and Eltem (2016) isolated seven morphologically distinct bacterial strains from peach and pear roots. Approximately five out of seven strains were gram-positive, short, and rod-shaped, and two isolates were gram- and rod-shaped, demonstrating that roots are rich sources of microbial diversity. Hu et al. (2023) also reported that the abundance of bacteria in roots is due to root exudation and the digestion of a diverse class of multifarious compounds.

Janda and Abbott (2007) have reported that the use of 16S rRNA gene sequencing for identification is stunning. It is an important bioinformatics tool that provides information on genus and species identification. Several studies have shown a variety of endophytic bacteria in medicinal plants by Jalgaonwala and Mahajan (2014) and Muthukumar et al. (2017), used a similar tool (16S rRNA sequencing) and identified endophytic bacteria *Stenotrophomonas maltophilia* and *Bacillus subtilis* from the roots of the medicinal plant *Prosopis cineraria*, (Wang et al., 2016) have been identified as endophytic bacteria *Sphingomonas* and *Pseudomonas* were identified using a 16S rRNA sequencing tool from the roots of rice (*Oryza sativa* L.). El-Deeb et al. (2013) reported the presence of endophytic bacteria *Bacillus megaterium*, *Bacillus pumilus*, and *Bacillus licheniformis* from the roots of the medicinal plant *Plectranthus tenuiflorus*.

Micronutrients are needed for plant development, and their deficiency results in reduced crop output; thus, the absence of these micronutrients in staple foods such as grains may have detrimental impacts on human health (Moreno-Lora et al., 2022). Various micronutrients perform distinct functions during plant development. Ojeda-Barrios et al. (2021) found that zinc deficiency is persistent in calcareous soils and that bioavailable zinc levels are poor in almost half of the cereal-growing soils. Along with other nutrients Ni is a micronutrient required for plant metabolic activity (Tariq et al., 2022). Germination is hindered by zinc toxicity (Ozturk et al., 2006). The increased toxicity of heavy metals reduces plant biomass and growth (Lin et al., 2016). However, nickel and zinc in appropriate amounts of Ni and Zn play a crucial role in a wide range of physical processes in maize, from seed germination to yield, and without an adequate supply of these metals, plants cannot complete their life cycle. In contrast, large quantities of heavy metals can disrupt many biological processes in plants. In addition to causing toxicity in plants at high soil concentrations, Ni may also interfere with plant metabolism when combined with other minerals (Yusuf et al., 2011).

In the present study, the effects of Zn and Ni on plant height, leaf length, number of leaves, root length, fresh weight nutrient content, moisture content, and leaf width were observed, which indicated a significant decrease in germination percentage with increasing levels of zinc (Zn) and nickel (Ni) contamination. These findings are consistent with those of Jócsák et al. (2022), who observed that an increase in heavy metal content leads to a decrease in plant biomass and growth. Moreover, in the current study, seed inoculation with *A. tereus* increased plant growth. These findings were in agreement with those of Broadley et al. (2007), who stated that under normal soil conditions, plant growth improved when inoculated with PGPR due to increased carbon and nutrient content, aeration, and solubility of some essential nutrients, such as N and P (Maurya and Singh, 2010), and increased nutrient availability (Pedram, 2017). Ni and Zn inhibited root length, which is similar to the finding of Ansari et al. (2021) who found a reduction in root length in the presence of cadmium (Cd), which may have been attributable to Cd-induced damage to the protein structure. Previous studies have reported that the endophytic bacteria *Burkholderia* sp. and *Methylobacterium oryzae* enhance the growth of *Lycopersicon esculentum* under Ni and Cd stress (Madhaiyan et al., 2007). During the current investigation, similar results were observed in which the inoculation of *A. tereus* enabled the plant to overcome Ni and Zn stress and increase the root length. Weiner et al. (2012) observed that the presence of Cd hindered the growth of maize shoots. Higher concentrations of heavy metals, including Cd, Zn, and Ni, are toxic to cell membranes, causing a reduction in plant growth parameters due to cell membrane damage and the removal of ions from the damage site. Similarly, our research revealed that inoculated seeds had increased leaf length and number (Dimkpa et al., 2009; Naeem et al., 2023). Plant growth and shoot length have been reported to decrease in the presence of Ni and Zn (Sharma et al., 2020). Changes in the germination process and growth of root stems and leaves as a consequence of the adverse effects of chromium (Cr) on plant growth and development. The total dry biomass production and yield of plants were influenced by Cr exposure, and these results are in close agreement with our findings that Ni and Zn lowered the dry biomass. In the current study, however, inoculation of seeds with *A. tereus* significantly aided the plant in terms of dry weight and reduced the repercussions of heavy metals on maize plants. Ni and Zn decreased the fresh weight of maize plants, whereas *A. tereus* significantly affected the leaf fresh weight, as reported in the present study and consistent with

previous findings. The inoculation of seeds with a bacterial strain in contaminated soil has the potential to increase the production of hormones in plants, which can increase their fresh weight (Iqbal et al., 2017; Zhang et al., 2023). The results of this study showed that proline content in maize plant leaves was decreased in the presence of zinc (Zn) and nickel (Ni) and these findings were comparable with those results Qiao et al. (2015), who suggested that the addition of zinc (Zn) and cadmium (Cd) to soil affected plant growth by lowering proline content. Moreover, in the current study, inoculation with *A. tereus* significantly enhanced proline content in maize plants. Similarly, Mittal et al. (2008) reported that inoculation of plants with beneficial bacteria enhanced proline activity. The increase in proline content may be due to the excretion of organic acid microbes in the soil, which helped the plant overcome metal stress. A higher amount of organic acids may be responsible for the reduction in the pH of the soil and is helpful in increasing the activity of soil enzymes, and a lower concentration of these metals could be helpful in proline activity (Shen et al., 2020). The protein content was reduced by the effects of Zn and Ni in the current study, but when seeds were inoculated with *A. tereus*, the protein content increased. These findings are supported by (Oleńska et al. (2020) and Zhao et al. (2023), who discovered that when seeds were inoculated with PGPR, there was an improvement in protein content and plant growth under normal soil conditions, which could be due to increased carbon and nutrient content, aeration, and solubility of some essential nutrients. The sugar concentration increased in rice and bean cotyledons under Cd stress (Huybrechts et al., 2019). In the current study, the sugar content was significantly reduced when plants were grown in Ni- and Zn-contaminated soil, but *A. tereus* promoted resistance in plants, and sugar content was enhanced in plants inoculated with *A. tereus*. During this study, the proline content decreased in the presence of Ni and Zn; however, *A. tereus* helped the plant withstand contaminated soil and significantly enhanced the proline content, indicating a favorable relationship between proline accumulation and plant stress. Similar outcomes have been published (Hayat et al., 2012), indicating that proline content, when subjected to stressed conditions, responded as an excellent osmolyte and conducted a diverse range of crucial functions. The current findings further demonstrate that the antioxidant enzymes SOD and POD vary considerably (Rajput et al., 2021). The variation in antioxidant enzymes was dependent on the concentration, type of heavy metal, plant species, and exposure of the plant to the contaminant in the soil. Similarly, in the current study, the presence of Ni and Zn metals in soil, even at low concentrations, had a significant effect on antioxidant enzymes, such as SOD, in maize. However, bacterial inoculation detoxifies heavy metal toxicity and reduces the SOD content in plants, which may have been caused by the action of a bacterial strain that minimized toxic effects in the soil environment for plants. Similar findings were reported by Sies et al. (2022), in which the plants under stress conditions showed reduced activity of superoxide dismutase (SOD), which may lead to a decrease in the production of glutathione (GSH) and ascorbate (ASC), resulting in a neurotic oxidative defense mechanism in plants. Dragišić Maksimović et al. (2012) found that Si can mitigate Mn toxicity in cucumber plants by increasing POD content; similarly, inoculation with *A. tereus* augmented POD content and reduced heavy metal toxicity. Microorganisms create 1-aminocyclopropane-1-carboxylate (ACC), which regulates the levels of ethylene by metabolizing it and promoting plant development when plants are under stress (Singh et al., 2011). However, according to our study, aggregation-enhanced development of maize plants may be the result of an increase in plant hormone levels caused by *A. tereus* inoculation. The production of Fe-chelating agents by microorganisms lowers the bioavailability of heavy metals. Bacteria transform the bioavailable form of Zn into an inaccessible form (Chibuike and Obiora, 2014). *A. tereus* improves the absorption of Cu, Mn, Ni, Na, Cr, Fe, Ca, Mg, and K of maize plants in comparison to uninoculated plants based on the accumulation of macro and micronutrients. Fan et al. (2021) found that A. species may increase the phytoavailability of Fe, Cd, Pb, Cr, Ni, and Cu. Siderophore complexation with metals increases metal uptake, which is why siderophores limit the phytoavailability of heavy metals and make bacterial cells more resistant to plant stress by forming complexes with metals (Ma et al., 2016). The study of the accumulation of heavy metals indicated variations in their composition, such as an increase in the concentration of Cu. Similarly, PGPR, which aided in the development of the plant shoots and roots, ultimately exhibited the same outcomes. The inoculation with *A. tereus* showed substantial potential for the absorption of micro-and macronutrients in maize plants. Additionally, previous studies have established the mechanism of PGPBs and plant pathways that contribute to the phytobiome and ultimately promote plant development. The indirect influence of micro- and macro-nutrient intake on plant growth and production has also been determined through research.

5. Conclusion

The current research concluded that 100 mg/kg of zinc (Zn) and nickel (Ni) was toxic for plant development; leading to a substantial reduction in plant biomass and inoculating maize seeds with *A. tereus* was efficient in lowering Zn and Ni toxicity in various plant parameters. Seed germination, shoot length, plant height, and moisture content were all significantly higher in treatment T5 (*A. tereus* + seeds + Zn 100 mg/kg) than in the uncontaminated soil. Additionally, T4 (*A. tereus* + Seeds) decreased plant defense enzymes (SOD, POD) in Ni and Zn polluted soil, but increased protein, proline, and sugar content. Moreover, *A. tereus* inoculation showed resistance against the combined stress of Ni and Zn, and boosted POD and SOD in maize. *A. tereus* inoculation aided maize plants in effectively absorbing nutrients including Cu, Mn, Ni, Na, Cr, P, Mg, K, and *Ca. A. tereus* can, thus, be suggested for future application as a possible plant growth-promoting bacterial inoculum to assist plants grown in heavy metal polluted soil and can be employed as bio-fertilizer to stimulate the plant development.

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/, MW 979614.

Author contributions

AsS: Formal analysis, Investigation, Software, Supervision, Writing – original draft, Writing – review & editing. AnS: Formal analysis, Investigation, Methodology, Writing – original draft. SF: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. MA: Validation, Writing

– review & editing. MQ: Resources, Validation, Visualization, Writing – review & editing. UA: Writing – review & editing. Formal analysis, Investigation, Methodology, Writing – original draft. MN: Conceptualization, Software, Writing – review & editing. TB: Investigation, Methodology, Validation, Writing – review & editing. AbS: Writing – review & 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.2023.1255921/full#supplementary-material

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EDITED BY
Nirmalendu Basak,
Central Soil Salinity Research Institute (ICAR), India

REVIEWED BY
Yongjie Yu,
Nanjing University of Information Science and Technology, China
Bholanath Saha,
Bihar Agricultural University, India
Parul Sundha,
Central Soil Salinity Research Institute (ICAR), India

*CORRESPONDENCE
Jianhong Wang
✉ Wangjh@zaas.ac.cn

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Various green manure-fertilizer combinations affect the soil microbial community and function in immature red soil

Jing Xu, Linlin Si, Xian Zhang, Kai Cao and Jianhong Wang*

Institute of Environment, Resource, Soil and Fertilizer, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

Green manure application is a common practice to improve soil fertility in China. However, the impact of different green manure-fertilizer combinations on the soil microbial communities in the low-fertility immature red soil in southern China remains unclear. In this study, we conducted a pot experiment using two common green manure crops, ryegrass (*Lolium perenne* L.) and Chinese milk vetch (*Astragalus sinicus* L.), along with a fallow treatment. We also considered three combined fertilizer management strategies, including mineral, humic acid, and organic manure fertilizers. We evaluated the soil microbial biomass, activity, communities, functional prediction and their correlation with soil properties during green manure growth and incorporation periods, to assess the potential alterations caused by different green manure and fertilizer combinations. Our findings indicate that green manure application, particularly in combination with organic fertilizers, increased the alpha diversity of the soil bacterial community, while the opposite trend was observed in the fungal community. The application of green manure altered the soil microbial communities during both growth and incorporation periods, especially the taxa that participate in carbon, nitrogen and sulfur cycles. Notably, ryegrass significantly increased the relative abundance of bacterial phylum *Firmicutes* and fungal phylum *Ascomycota*, whereas Chinese milk vetch significantly stimulated the bacterial phylum *Acidobacteria* and fungal phylum *Glomeromycota*. Compared with fallow treatments, green manure application significantly increased the soil pH by 4.1%–12.4%, and microbial biomass carbon by 29.8%–72.9%, regardless of the types of combined fertilizer. Additionally, the application of green manure resulted in a 35.6%–142.6% increase in urease activity and a 65.9%–172.9% increase in β-glucosidase activity compared to fallow treatments, while led to a 22.5%–55.6% decrease in catalase activity. Further analysis revealed that the changes in both bacterial and fungal communities positively correlated with soil pH, soil organic matter, total nitrogen and alkali hydrolyzed nitrogen contents. Moreover, the relationship between the soil microbial community and soil enzyme activities was regulated by the specific green manure species. In conclusion, our results provide insight into the effects of different green manure-fertilizer combinations on soil microorganisms and their underlying mechanisms in improving soil fertility in the low-fertility immature red soil.

KEYWORDS

enzyme activity, fertilizer, green manure, immature red soil, soil microbial community

1 Introduction

As large-scale farming expands in the red soil of hilly regions in southern China, some immature red soil has been reclaimed as farmed topsoil. However, the low levels of organic matter, nutrients, and biological activity in immature red soil limit crop yields (Xu et al., 2003; Wilson et al., 2004; Bao et al., 2019). Thus, improving soil fertility is crucial in immature red soil fields. Green manure and fertilization can affect soil carbon sequestration, plant growth, and microbial community structure (Elfstrand et al., 2007; Chavarría et al., 2016; Maitra et al., 2018). Green manure application is a traditional farming practice in China, which assists in creating sustainable agricultural systems and plays an important role in maintaining fertility and crop productivity (Tejada et al., 2008a; Pittelkow et al., 2015). Previous studies have demonstrated that green manure crops can improve soil physical and chemical properties by forming and stabilizing soil macroaggregates and increasing soil organic matter and nutrient availability (Jama et al., 2000; Hwang et al., 2015; Adetunji et al., 2020). Furthermore, the application of green manure can influence soil biological properties, such as the microbial activity, diversity and communities in agricultural soils (Caban et al., 2018; Gao et al., 2020; He et al., 2020). Thus, the application of green manure as one major organic material to increase organic inputs to rice production systems is considered as the most practical option to enhance the organic matter dynamics and nutrient cycling in low-fertility immature red soil.

Soil microorganisms are the key to ecological processes such as soil organic matter decomposition, nutrient cycling, and other critical agroecosystem processes that promote plant growth and soil quality (Bardgett and van der Putten, 2014; Fierer, 2017). Green manure plants can regulate their rhizosphere microbiome through rhizodeposition and the nutrient trade-off between soil and plants during the growth period (Gleixner et al., 2002; Pausch and Kuzyakov, 2018; Saleem et al., 2020). Moreover, the incorporation of green manure crops into the soil provides fresh organic matter for microorganisms, which benefits soil by increasing the soil microbial biomass and enhancing enzyme activities in agricultural systems (Tejada et al., 2008b; Hwang et al., 2015; Khan et al., 2019; Brtnicky et al., 2021). As soil enzymes are mainly released from soil microorganisms, enzyme activities can indicate potential changes in the soil microbial communities and biochemical processes (Sinsabaugh et al., 2008). The activities of soil enzymes such as catalase, β-glucosidase, and urease are associated with the degradation of organic matter and the cycling of carbon and nitrogen (Stott et al., 2010; Dharmakeerthi and Thenabadu, 2013; Kolesnikov et al., 2019). Zheng et al. (2018a) found that green manure incorporation significantly enhanced the activities of β-glucosidase, β-xylosidase, and cellobiohydrolase, which can accelerate green manure degradation, and this was correlated with relative increases in the families *Ruminococcaceae* and *Lachnospiraceae*. The effects of green manure on different enzyme activities are reported to vary with the species of green manure, suggesting that specific dominant microbes are established when different green manure species are applied to the soil (Taylor et al., 2002; Gao et al., 2016; Khan et al., 2019). Recently, high-throughput sequencing has been used to assess the effects of green manures on soil microbes, and these studies have reported that the microbial community composition changes according to the types (e.g., leguminous and non-leguminous) of green manure crops used (Kataoka et al., 2017; Tao et al., 2017; Zhang et al., 2017, 2020; Silva et al., 2021). With regard to newly cultivated red soil with low fertility, however, research on the effects of different types of green manure on soil biological properties, especially microbial communities, during both green manure growth and incorporation, is limited.

Because of the low levels of organic matter and available nutrients in immature red soil, other fertilizers such as mineral fertilizer, biochar, pig manure, and humic compounds are usually applied together with green manure to accelerate soil quality improvement (Marschner et al., 2003; Katterer et al., 2019; Han et al., 2021). Previous studies showed that the application of green manure combined with different fertilizers has various effects on soil properties, altering the microbial biomass, activity, and community structure (Baumann et al., 2009; Lori et al., 2017). Green manure in combination with mineral fertilizer has been suggested to be more effective at increasing soil carbon stability and grain yields than using either mineral fertilizer or green manure alone (Gao et al., 2016; Das et al., 2019; Kamran et al., 2021). A recent study showed that green manure (*Arachis pintoi*) combined with natural phosphate promoted enzyme activities and affected the soil microbial community structure (de Medeiros et al., 2019). In addition, humic compounds, such as humic acid, play a role in a variety of soil processes and are related to soil physical and chemical properties, influencing the soil nutrient cycle and nutrient bioavailability (Moran et al., 2005; Olk et al., 2019). Also, organic manure, produced by composting and fermenting livestock manure, is usually used to improve soil fertility in agricultural systems (Lu et al., 2011). In a previous study, we found that both humic acid and organic manure improved soil fertility, and the combined application of organic amendments and mineral fertilizer maximized economic benefits by increasing rice grain yield and enhancing soil quality, resulting in a profit increase of 17%–33% (Si et al., 2023). Therefore, these two organic fertilizers are commonly utilized in practice. Previous studies have indicated the positive effects of combining green manure with organic fertilizer on soil humus and soil organic carbon accumulation in long-term agricultural systems (Ghosh et al., 2018; Wen et al., 2018; Masilionyte et al., 2021). However, the impact of various combinations of green manure and fertilizers on the characteristics and function of microbial community at the initial stage of low-fertility soil cultivation remains unclear. Understanding these factors is crucial for gaining further insights into how green manure practices influence soil fertility and health in the immature red soil.

Ryegrass (*Lolium perenne* L.) and Chinese milk vetch (*Astragalus sinicus* L.) emerge as the primary green manure variations in southern China, extensively cultivated in green manure-rice rotation systems in preference to winter fallow (Lei et al., 2022). Ryegrass, belonging to the *Poaceae* family, exhibits a robust root system and displays strong adaptability to low-quality soils, resulting in substantial biomass production (He et al., 2020). Chinese milk vetch is considered as the most popular green manure in southern China since its higher nitrogen fixing capability. Consequently, the residues of ryegrass have a higher C:N ratio than Chinese milk vetch. The differences in plant composition and ecological functions of the two green manure species may result in varying impacts on soil properties during both growth and incorporation periods. Overall, the goal of the study was to estimate the effects of two types of green manure, ryegrass and Chinese milk vetch, in combination with mineral fertilizer, humic acid, or organic manure on the characteristics of the soil microbial

community. This included evaluating the growth, activity, composition, and function of the microbial community in immature red soil. To control the environmental conditions, a greenhouse pot experiment was conducted to test the following hypotheses: (a) the growth and incorporation of green manure have the potential to stimulate specific functional groups of the soil microbial community that are involved in nutrient cycling; (b) the application of various green manure-fertilizer combinations may improve the soil physicochemical and biological properties of low-fertility immature red soil by altering the microbial community; and (c) these effects on the soil microbial community are contingent upon the specific green manure plants species utilized.

2 Materials and methods

2.1 Study site and soil characteristics

The experiment was carried out in a greenhouse in western Zhejiang Province, China (119° 3′ 26″ E, 28° 56′ 06″ N), where the soil parent material comprises calcareous red sandstone, sand shale, and red glutenite and has a weak structure. The immature red soil used for the experiment was collected randomly from red subsoil in a hilly area in Quzhou, Zhejiang Province. The basic physical and chemical properties of the soil were as follows: pH (1:5, soil:water) 5.30, soil organic matter 2.51 g kg^{-1}, total nitrogen (N) 0.28 g kg^{-1}, total phosphorus 0.68 g kg^{-1}, total potassium 3.6 g kg^{-1}, alkali hydrolyzable nitrogen 21.2 mg kg^{-1}, available phosphorus 3.05 mg kg^{-1}, and available potassium 55 mg kg^{-1}.

2.2 Experimental design and sampling

Two types of green manure application, ryegrass or Chinese milk vetch, were compared to a fallow treatment, in combination with three types of fertilizers, including mineral fertilizer, humic acid fertilizer, or organic manure. A control treatment without green manure plants or fertilizer was also included. As a result, a total of 10 treatments were designed as follows: control treatment (CK); fallow soil with mineral fertilizer (CF), fallow soil with humic acid fertilizer (HAF), fallow soil with organic manure (OMF), ryegrass combined with mineral fertilizer (RG), ryegrass combined with humic acid fertilizer (RH), ryegrass combined with organic manure (RO), Chinese milk vetch combined with mineral fertilizer (MG), Chinese milk vetch combined with humic acid fertilizer (MH), and Chinese milk vetch combined with organic manure (MO). Each treatment was replicated three times (Figure 1). The organic matter contents of the humic acid fertilizer (Ruiyu Trading Co. Ltd., Shandong, China) and the organic manure (Fengji Biotech Co., Ltd., Jiangxi, China) are 75.3% and 29.2%, respectively. The nutritional content details are shown in Supplementary Table S1.

Each pot (volume 10.1 L, height 31 cm) held 10.0 kg of red soil (DW) screened through 5 mm meshes. Except for the CK, all treatments received mineral fertilizer (N: 0.05 g kg^{-1} urea, P: 0.12 g kg^{-1} $Ca(H_2PO_4)_2$ and K: 0.04 g kg^{-1} KCl) prior to green manure plants seeding. The humic acid or organic manure was thoroughly mixed with the potted soil of HAF, RH and MH or OMF, RO and MO, respectively, to achieve a concentration of 20 g kg^{-1} (WW). Before sowing, seeds were disinfected and rinsed with 5% sodium hypochlorite. Seedlings were thinned to 15 seedlings per pot after germination. Supplementary water was added as needed during plant growth.

The study began in November 2018. The average temperatures during the growth period ranged from 7.2°C to 15.6°C and during the incorporation period from 16.5°C to 25.0°C. After 5 months of growth, the green manure crops were harvested at the beginning of the reproductive growth stage. For the six treatments that involved planting ryegrass and Chinese milk vetch, the shoots of the green manure crops were cut and the roots carefully separated from the soil. To collect the rhizosphere soil adhering to the roots of the green manure crops, we gently shook the soil from the roots located in the topsoil layer (2–10 cm; Nazih et al., 2001; Wang et al., 2009). In order to maintain consistent sampling depth, for the three fallow treatments and CK, we sampled the topsoil (2–10 cm) using a 38 mm diameter soil core sampler. The soil samples were collected in sterile tubes, transported to the laboratory under liquid nitrogen, and stored at −80°C until further analysis.

The fresh shoots of ryegrass and Chinese milk vetch in each treatment were cut chopped into approximately 3 cm lengths and thoroughly mixed within each set of three replicates. Then, precisely weighed 200.0 g fresh residues of each treatment and evenly incorporated into the soil of the respective pot. During the incorporation period, the soil was maintained in a flooded state for 8 weeks, according to field practices, and the soil was sampled in May 2019. Throughout the study, no chemical plant protection agents (such as fungicides, pesticides, or herbicides) were used. From all 10 treatments, approximately 500 g soil samples were removed using a 38 mm diameter soil core sampler at the depth of 0–15 cm from each pot and divided into three parts. The first subsample was collected in sterile tubes (50 mL) and stored at −80°C prior to total soil DNA extraction. The second sub-sample was sieved through a 2 mm sieve and stored at 4°C to determine the soil microbial biomass carbon and enzyme activity within 7 days. The last subsample was air-dried and finely ground to pass through 2 and 0.20 mm sieves prior to analyzing its physical and chemical properties.

2.3 Samples analyses

To determine the soil microbial biomass carbon, chloroform fumigation and K_2SO_4 extraction were performed, and the extract was analyzed with a carbon and nitrogen analyzer (Vance et al., 1987). The activity of β-glucosidase was determined using the *p*-nitrophenol colorimetric method (Eivazi and Tabatabai, 1988). Urease activity and catalase activity were measured using the phenol-sodium hypochlorite colorimetric method and the potassium permanganate titration method, respectively, following the protocols described by Guan et al. (1986). Soil pH was measured using a pH meter (Mettler-Toledo FE20, Shanghai, China) in a soil and distilled water suspension (1:5, w/v). Soil organic matter (SOM), total nitrogen content (TN), total phosphorus content (TP), Olsen-phosphorus content (OP), and alkali hydrolyzale nitrogen content (AN) were measured according to the methods described by Lu (1999) with some modifications. Specifically, SOM was measured using the potassium dichromate external heating method. TN was analyzed using the SKD-100 automated Kjeldahl nitrogen analyzer (PEIOU, Shanghai, China). AN was determined

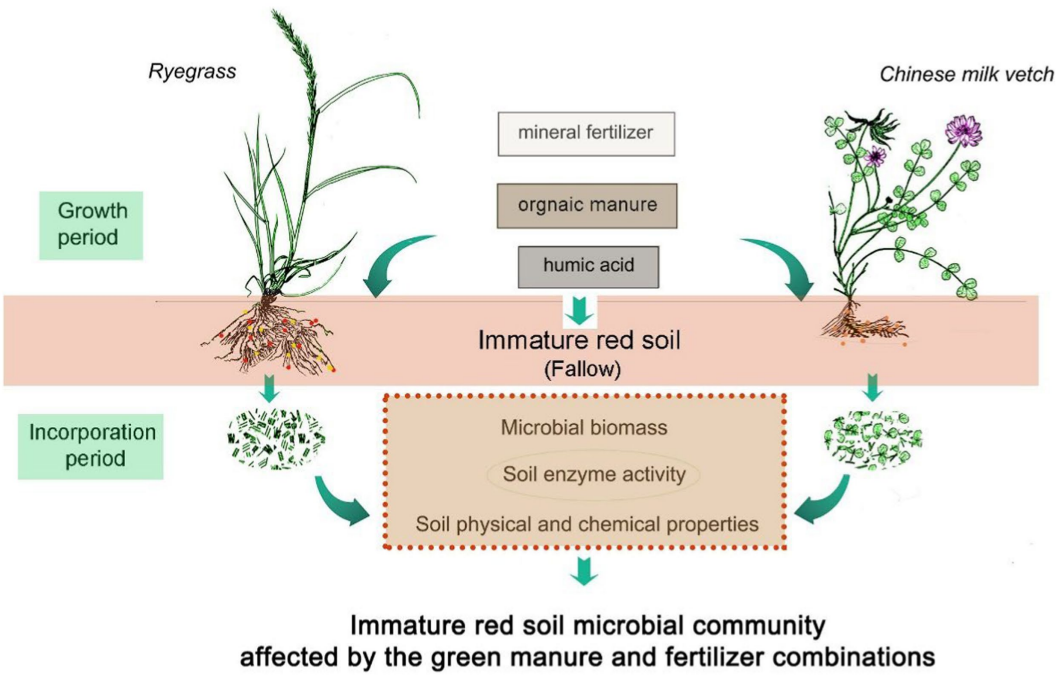

FIGURE 1
Diagram of the experimental design. The fallow treatment was the soil without green manure application, while the ryegrass and Chinese milk vetch application treatments including growth period and incorporation period. All the three treatments were combined with three fertilizers, including mineral fertilizer, organic manure and humic acid fertilizer. Also, a control without green manure and fertilizer was set up.

using a reduction diffusing method after alkaline hydrolysis. TP and OP were measured using extraction and colorimetric methods.

A kit (MP FastDNA® Spin Kit for Soil) was used to extract total DNA from the soil. The quality of DNA was assessed by 1% agarose gel electrophoresis. The V3-V4 hypervariable region of the 16S rRNA gene was selected for PCR amplification with primers 338F (5′-ACT CCT ACG GGG AGG CAG CAG-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). The ITS1-ITS2 region of the fungal gene was amplified with primers ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS2 (5′-GCT GCG TTC TTC ATC GAT GC-3′). The PCR reactions were conducted by using the programs: 3 min of denaturation at 95°C; followed by 27 cycles of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C; and a final extension at 72°C for 10 min with a thermocycler PCR system (GeneAmp 9700, ABI, United States). Sequencing for bacterial and fungal communities was accomplished using an Illumina MiSeq PE300 platform by the Majorbio Company (Shanghai, China), following the manufacturer's instructions. All the raw sequences tested in the study were uploaded to the NCBI SRA database (accession nos., PRJNA992634 and PRJNA992506).

2.4 Statistical analysis

SPSS software (v.21.0) was used for statistical analyses to test the effects of green manure and fertilizer types on soil properties, microbial biomass carbon, and enzyme activities with two-way ANOVA and to compare the differences between treatments with one-way ANOVA. The data were compared by the least significant difference (LSD) method at the 5% confidence level. Levene's test was used to check the homogeneity of variance.

The sequencing data of soil bacterial and fungal communities from green manure growth and incorporation periods were analyzed as described by Xu et al. (2018). Briefly, the original sequencing data were quality-filtered, merged, and optimized; then, the Operational Taxonomic Units (OTUs) with 97% similarity were clustered by using the UPARSE pipeline after removing the chimeric sequences (Edgar, 2013). The taxonomy of the bacterial and fungal OTUs was analyzed against the Sliva (SSU138) 16S rRNA database and Unite (Release 8.0) database, respectively.

The alpha diversity was assessed according to the Chao1 and Shannon indices, which indicated the richness and diversity of the microbial community, respectively. Principal coordination analysis (PCoA) based on the OTU level was used to determine the degree of dissimilarity among bacterial and fungal communities in the 10 treatments. Redundancy analysis (RDA) or canonical correspondence analysis (CCA) at the genus level were performed to investigate the relationships between the bacterial and fungal communities of soil samples from each treatment and soil enzyme activities, respectively. And the distance-based RDA (db-RDA) at the bacterial and fungal genus level were performed to investigate the relationships between the soil samples from each treatment and the soil physical and chemical properties. Analysis of similarities (ANOSIM) was used to assess the statistical significance between different treatments. Comparisons of two groups of bacterial or fungal taxa were analyzed with Welch's *t*-test. A multiple groups comparison of bacterial or fungal taxa and linear discriminant analysis (LDA) effect size (LEfSe)[1]

1 http://huttenhower.sph.harvard.edu/lefse

were used to elucidate the significant differences in bacterial or fungal taxa among the green manure treatments (ryegrass and Chinese milk vetch) and fallow treatment, regardless the combined fertilizers. The functional groups of the bacterial community were predicted using the functional annotation of prokaryotic taxa (FAPROTAX) database[2] based on the 16S rRNA gene data (Louca et al., 2016). And functional properties of the fungal community were assigned using the FUNGuild database[3] based on the ITS gene data (Nguyen et al., 2016). The data were analyzed using a combination of R packages, the Galaxy web application, and the functional prediction tools mentioned above, all of which were accessed through the Majorbio Cloud Platform (Ren et al., 2022).

3 Results

3.1 Effects of green manure growth and fertilizer combinations on the rhizosphere soil microbial community

3.1.1 Soil microbial community composition

From the soil samples taken during the green manure growth period, approximately 1,472,105 effective bacterial sequences and 1,830,116 fungal ITS region sequences were obtained, respectively. A total of 4,564 bacterial OTUs and 3,017 fungal OTUs were obtained at 97% similarity. Among 38 bacterial phyla, the most abundant phylum in all treatments except for the CK was *Proteobacteria*, accounting for 30.32%–49.41% of the total abundance, followed by *Chloroflexi* (15.16%–25.90%), *Actinobacteria* (7.52%–20.78%), *Acidobacteria* (5.37%–11.38%), and *Firmicutes* (0.74%–20.99%; Figure 2A). Both ryegrass and Chinese milk vetch stimulated the relative abundance of *Acidobacteria*. The relative abundance of *Firmicutes* in ryegrass rhizosphere soil was 3.6- and 3.0-fold higher than in Chinese milk vetch rhizosphere soil and fallow soil, respectively (Supplementary Figure S1). With respect to the different fertilizer types, the relative abundance of *Firmicutes* was more enhanced in RH than in RG ($p < 0.01$) and RO ($p < 0.05$), while there were no significant differences at the phylum level between the three types of fertilizers in the rhizosphere soil of Chinese milk vetch ($p > 0.05$). Among the 14 fungal phyla, the most abundant phylum was *Ascomycota* (51.82%–84.43%), in which the relative abundance of the classes *Sordariomycetes* and *Dothideomycetes* were significantly lower in the ryegrass rhizosphere than in fallow soil when combined with mineral fertilizer and organic manure, respectively (Figure 2B; Supplementary Figure S2).

In addition, LEfSe (LDA > 3.5) detected bacterial and fungal biomarkers in the rhizosphere soil of green manure. The bacterial phylum *Nitrospirae* (LDA = 3.94, $p < 0.05$) was significantly affected by planting ryegrass, and the order *Rhizobiales* (LDA = 4.02, $p < 0.01$) was significantly stimulated by Chinese milk vetch (Figure 2C). The fungal orders *Eurotiales* and *Sordaridles* in the ryegrass rhizosphere soil were significantly different from those in the Chinese milk vetch rhizosphere soil and fallow soil (Figure 2D).

3.1.2 Soil microbial alpha diversity

The soil bacterial richness and diversity, as indicated by the Chao1 and the Shannon indices, were enhanced in the rhizosphere soil of both types of green manure when combined with the mineral fertilizer (Table 1). Compared with the *CF* treatment, the Chao 1 exhibited a 39.7% increase in the RG treatment and a 60.0% increase in the MG treatment. Similarly, the Shannon indices showed a 21.4% increase in the RG treatment and a 28.2% increase in the MG treatment. When combined with humic acid fertilizer, the Shannon indices in the RH and MH treatments were 8.8% and 9.6% higher, respectively, than those in the HAF treatment. Although the Chao1 and the Shannon indices were highest in the MO treatment among all treatments, there were no significant differences among the green manure treatments and fallow treatments when combined with organic manure (OMF vs. RO and MO).

In terms of the soil fungal community, both the Chao1 and the Shannon indices were significantly lower in the RG treatment compared to the MG treatment (Table 1). In addition, the Chao1 in the RO treatment and the MO treatment were 30.6% and 30.7% lower than that in the OMF treatment, respectively. Furthermore, the Shannon index in the MO showed a 33.2% reduction compared to the OMF treatment.

3.1.3 Soil microbial community structure

According to the PCoA based on the OTU level, the bacterial communities in the rhizosphere soils were clustered according to the green manure species (ANOSIM: $R = 0.71$, $p = 0.001$) more than combined fertilizer types (ANOSIM: $R = 0.35$, $p = 0.001$; Figure 3A). While, the fungal communities in the rhizosphere soil of the green manure crops showed weakly significant differences from those in the fallow treatments (ANOSIM: $R = 0.208$, $p = 0.001$), so was located among the three fertilizer types (ANOSIM: $R = 0.120$, $p = 0.009$; Figure 3B).

3.1.4 Functional prediction of the microbial community in rhizosphere soil

Through FAPROTAX functional prediction, 66 functions were annotated in bacterial communities, and the prominent functions were related to carbon (C), nitrogen (N) and sulfur (S) cycling and were shown to be significantly affected by the growth of green manure plants (Figure 3C). Among the functional groups involved in C cycling, the relative abundance of aerobic chemoheterotrophy predicted functions was significantly higher in rhizosphere soil of Chinese milk vetch than ryegrass, while the opposite was found for fermentation. The abundant bacterial groups involved in the N cycle such as nitrification, nitrogen fixation, aerobic ammonia oxidation, nitrate reduction, nitrate respiration, nitrogen respiration and ureolysis were significantly higher in rhizosphere soil of Chinese milk vetch than ryegrass and fallow treatments. The abundances of functional groups involved in the S cycle, such as respiration of sulfur compounds, sulfur respiration and sulfate respiration, however, were significantly higher during ryegrass growth than Chinese milk vetch.

FUNGuild analysis predicted 9 trophic mode groups of fungal community, with saprotroph being the major function (Fallow: 22.5%, ryegrass: 38.1% and Chinese milk vetch: 24.2%). The relative abundance of dung saprotroph was significantly higher in rhizosphere soil of ryegrass than Chinese milk vetch and fallow treatments (Figure 3D).

2 http://www.loucalab.com/archive/FAPROTAX/

3 https://github.com/UMNFuN/FUNGuild

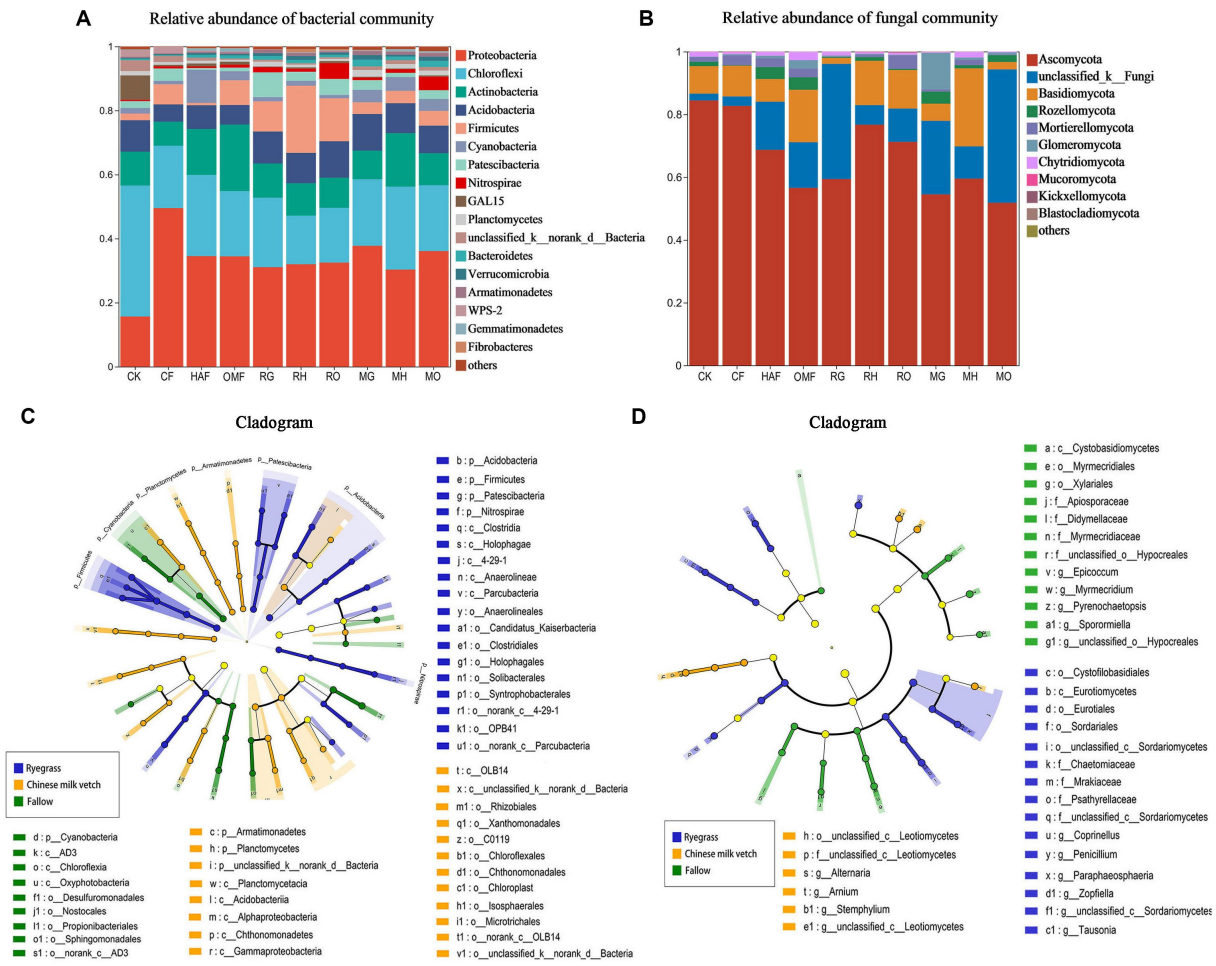

FIGURE 2
The relative abundance of **(A)** bacterial community composition and **(B)** fungal community composition at phylum level in bulk soil of fallow treatments and rhizosphere soil during green manure growth period. Regardless of the combined fertilizers, the LEfSe of **(C)** bacterial community and **(D)** fungal community in bulk soil of fallow treatments and rhizosphere soil during green manure growth period. Cladograms indicate the phylogenetic distribution of microbial lineages associated with the fallow, ryegrass and Chinese milk vetch treatments. The most different bacteria are represented in the different color. Each circle's diameter is proportional to the taxon's abundance. The blue, yellow, and green taxa indicate the biomarkers in ryegrass, Chinese milk vetch and fallow treatments, respectively.

3.2 Effects of green manure incorporation and fertilizer combinations on soil properties

3.2.1 Soil physicochemical properties

The application of green manure combined with fertilizers had a significant impact on soil pH, SOM, TN, and AN (Table 2; Supplementary Table S2). Compared to the fallow treatments, the application of ryegrass led to a significant increase in soil pH ranging from 9.1% to 12.4%. Whereas the application of Chinese milk vetch increased soil pH by 4.1% in MG and 5.0% in MH, but no significant increase was observed in MO. Also, the TN contents in both the RG and MG treatments were 14% higher than the *CF* treatment. Additionally, the contents of AN were significantly increased in RG and RO treatments, whereas no statistically significant improvement was observed in the Chinese milk vetch application treatments. In addition, the SOM content showed a remarkable increase when combined with humic acid fertilizer, surpassing that of organic manure in both green manure application and fallow treatments.

3.2.2 Soil microbial biomass carbon content

Both green manure incorporation ($F_{2,27}=31.98$, $p<0.001$), combined fertilizer application ($F_{2,27}=243.16$, $p<0.001$), and their interaction ($F_{4,27}=24.19$, $p<0.001$) significantly affected the soil microbial biomass carbon content (SMBC). Compared with CF, the SMBC showed a 40.6% increase in RG, but not in MG. While the SMBC in MO treatment exhibited the highest value among the 10 treatments. The SMBC in RH and MH treatments were 44.7% and 72.9% higher, respectively, compared to the HAF treatments. However, the SMBC was significantly decreased by humic acid fertilizer, compared to the organic manure (Figure 4A).

3.2.3 Soil enzyme activities

Compared with the fallow treatments, green manure application significantly increased soil urease and β-glucosidase activities but inhibited catalase activity when combined with the mineral fertilizer and organic manure (Figures 4B–D). Under the humic acid fertilizer treatments, however, no significant differences in the activity of these three enzymes were seen between the fallow and green manure

TABLE 1 Bacterial and fungal diversity indices in rhizosphere soil of green manure treatments and bulk soil of fallow treatments during growth period, and in the bulk soil of green manure treatments and fallow treatments during incorporation period.

Treatment combination	Growth period				Incorporation period			
	Bacterial community		Fungal community		Bacterial community		Fungal community	
	Chao 1	Shannon	Chao 1	Shannon	Chao 1	Shannon	Chao 1	Shannon
CK	1177.63 ± 14.21a	4.73 ± 0.07a	562.88 ± 43.02abc	3.16 ± 0.19ab	1276.86 ± 23.83ab	4.67 ± 0.12a	259.70 ± 21.09ab	3.59 ± 0.22bc
CF	1100.39 ± 72.99a	4.44 ± 0.07a	387.74 ± 41.96abc	3.40 ± 0.45abc	1159.02 ± 82.84a	4.83 ± 0.18ab	218.23 ± 60.60a	1.86 ± 0.60a
HAF	1745.05 ± 138.59bc	5.22 ± 0.23b	714.37 ± 83.31cd	3.94 ± 0.23c	1498.00 ± 140.14bc	4.99 ± 0.18ab	397.50 ± 76.75b	3.51 ± 0.52bc
OMF	2016.07 ± 90.93de	5.56 ± 0.03cd	761.41 ± 54.29d	3.98 ± 0.20c	1666.10 ± 70.43cd	5.05 ± 0.05bc	365.09 ± 106.31ab	3.75 ± 0.17c
RG	1537.64 ± 73.17b	5.39 ± 0.11bc	360.02 ± 49.90a	2.80 ± 0.34a	1609.67 ± 53.09cd	5.34 ± 0.10cd	386.20 ± 37.92b	2.63 ± 0.38ab
RH	1837.31 ± 42.01cd	5.68 ± 0.07cd	405.73 ± 78.98ab	3.66 ± 0.32bc	1651.04 ± 63.79cd	5.34 ± 0.12cd	362.60 ± 36.34ab	2.84 ± 0.16abc
RO	2005.49 ± 129.75cde	5.59 ± 0.18cd	528.23 ± 104.03abc	3.73 ± 0.09bc	2061.47 ± 59.38e	5.79 ± 0.06e	417.14 ± 58.84b	2.92 ± 0.23bc
MG	1760.00 ± 98.62cd	5.69 ± 0.10cd	572.89 ± 96.16bc	3.69 ± 0.11bc	1605.48 ± 140.64cd	5.42 ± 0.13d	413.68 ± 32.73b	2.80 ± 0.36abc
MH	1978.24 ± 36.05cde	5.72 ± 0.09cd	497.93 ± 56.90abc	3.38 ± 0.22abc	1855.25 ± 23.49de	5.57 ± 0.04de	368.42 ± 2.57ab	2.47 ± 0.29a
MO	2225.82 ± 100.31e	5.76 ± 0.06d	527.30 ± 53.54abc	2.66 ± 0.25a	1839.13 ± 106.18de	5.56 ± 0.08de	476.39 ± 58.12b	2.62 ± 0.21ab

Chao 1 index showed the richness of the soil microbial community and Shannon index showed the diversity of the soil microbial community. Values are means ± SE of three replicates. Means in column followed by the same letter are not significantly different ($p > 0.05$). CK, the control treatment; CF, fallow soil with mineral fertilizer; HAF, fallow soil with humic acid fertilizer; OMF, fallow soil with organic manure; RG, ryegrass combined with mineral fertilizer; RH, ryegrass combined with humic acid fertilizer; RO, ryegrass combined with organic manure; MG, Chinese milk vetch combined with mineral fertilizer; MH, Chinese milk vetch combined with humic acid fertilizer; MO, Chinese milk vetch combined with organic manure.

application treatments, except that urease activity in MH was higher than in HAF. The two green manure species also had different effects on enzyme activities. Urease activity was significantly higher in MH than in RH, while β-glucosidase and catalase activities were 1.41- and 1.38-fold higher in RO than in MO, respectively.

3.3 Soil microbial community during green manure incorporation

3.3.1 Soil microbial community composition

We detected a total of 4,365 bacterial OTUs and 2,162 fungal OTUs from soil and identified to 43 bacterial phyla and 12 fungal phyla after green manure incorporation. The dominant bacterial phyla included *Chloroflexi* (11.30%–44.23%), *Proteobacteria* (11.35%–22.72%), *Actinobacteria* (8.34%–27.95%), *Firmicutes* (1.02%–10.63%), *Acidobacteria* (5.25%–11.89%), *Desulfobacteriota* (0.77–16.39%), *Myxococcota* (4.21%–12.77%), *Patescibacteria* (0.51–4.89%), *Bacteroidota* (0.41%–8.07%), GAL-15 (0.10%–8.63%), and *Nitrospirota* (0.29%–2.28%), which accounted for 97% of the total bacterial community (Figure 5A). Ryegrass incorporation significantly increased the relative abundance of Firmicutes in all three fertilizer combinations, compared with the fallow treatments (Supplementary Figure S3). Both ryegrass and Chinese milk vetch incorporation significantly increased the relative abundance of *Bacteroidota* under mineral fertilizer and *Patescibacteria* under humic acid fertilizer (Supplementary Figure S3). The fungal phylum *Ascomycota* was dominant, representing 55.37%–94.35% of the fungi in all treatments, followed by *Basidiomycota*, *Chytridiomycota*, and *Glomeromycota* (Figure 5B). Compared with the OMF treatment, RO significantly increased the relative abundance of *Ascomycota*, in which the class *Sordariomycetes* was significantly higher in RO. Also, the relative abundance of *Glomeromycota* was significantly higher in MG than in *CF* and RG ($p = 0.039$), while it was lower in MH and RH than in HAF ($p = 0.027$; Supplementary Figure S4).

According to the LEfSe (LDA > 3.5), the bacterial orders *Bacillales* and *Clostridiale* were significantly impacted by ryegrass incorporation, and the order *Rhizobiales* was more affected in Chinese milk vetch treatments (Figure 5C). For the fungal community, the classes *Dothideomycetes* and *Mortierellomycetes* were significantly impacted by ryegrass and Chinese milk vetch, respectively (Figure 5D).

3.3.2 Soil microbial alpha diversity

In terms of the alpha diversity of the soil bacterial community during green manure incorporation, both the Chao1 and the Shannon indices exhibited significantly higher values in the green manure incorporation treatments compared to the fallow treatments, except for the Chao 1 in the RH and MO treatments (Table 1). Compared to the fallow treatments, the Chao1 exhibited increase ranging from 23.7 to 38.8% due to the green manure incorporation, while the Shannon index showed a range of increases from 7.0% to 14.7%. The RO treatment showed the greatest improvement in the Chao1 and Shannon indices among the 10 treatments (Table 1).

The Chao1 of the fungal communities in RG and MG treatments were significantly increased by 77.0% and 90.0%, respectively, compared to the CF treatment. Whereas there was no significant difference between the green manure incorporation treatments and

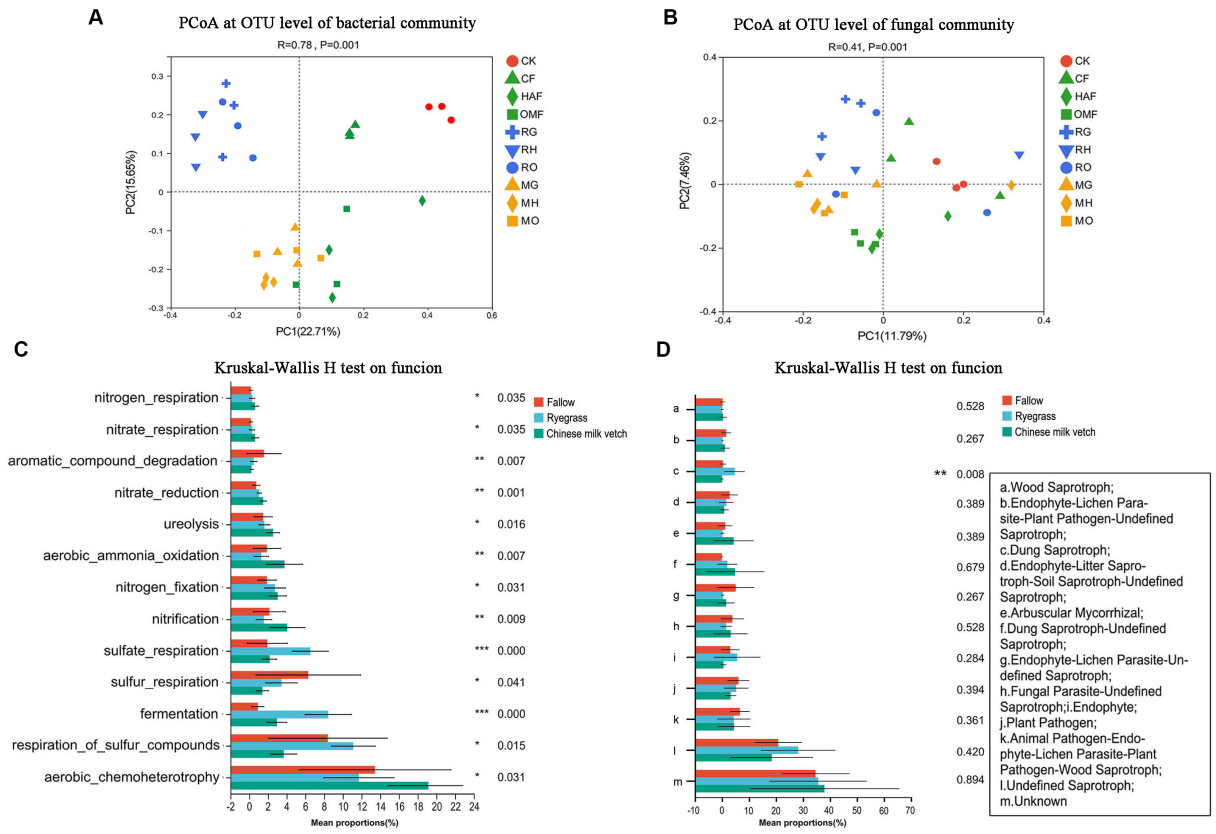

FIGURE 3
PCoA analysis at OTU level of **(A)** soil bacterial community and **(B)** fungal community in green manure growth period. Samples of CK are shown as red circles. The fallow treatments, ryegrass incorporation treatments and Chinese milk vetch incorporation treatments are shown as green, blue and orange symbols, respectively. The combined mineral fertilizer, humic acid fertilizer and organic manure are indicated by triangle, diamond and square, respectively. Regardless of the combined fertilizers, the functional prediction of **(C)** bacterial community by FAPROTAX and **(D)** fungal community by FUNGuild analysis. The fallow, ryegrass and Chinese milk vetch treatments are shown as red, blue and green bars. Values are means ± SE of three replicates. The *, **, and *** was indicates the $P < 0.05$, $P < 0.01$, and $P < 0.001$ (Kruskal-Wallis H-test), respectively.

the fallow treatments when combined with humic acid fertilizer or organic manure. The Shannon index was significantly decreased in MH and MO by 29.6% and 30.1% compared to HAF and OMF, respectively (Table 1). Additionally, there was no significant difference observed in the alpha diversity of the soil fungal community among the three combined fertilizer treatments for each green manure species.

3.3.3 Soil microbial community structure and functional prediction

Based on the abundances of bacterial OTUs, the PCoA indicated that samples were clustered significantly according to the different green manure treatments (ANOSIM: R = 0.524, $p = 0.001$), and the samples from the organic manure treatments were separated from others (ANOSIM: R = 0.502, $p = 0.001$; Supplementary Figure S5A). For the fungal community, there were significant differences among all samples (ANOSIM: R = 0.617, $p = 0.001$). Additionally, green manure incorporation (ANOSIM: R = 0.426, $p = 0.001$) and fertilizers (ANOSIM: R = 0.279, $p = 0.001$) weakly but significantly affected the soil fungal communities (Supplementary Figure S5B).

The FAPROTAX functional prediction showed that the dominant functions such as chemoheterotrophy, nitrogen fixation and fermentation were significantly improved by the green manure application, which were related to C and N cycling. However, the functions related to sulfur cycle, such as respiration of sulfur compounds and sulfur respiration, were decreased in the green manure treatments, except for the relative abundance of sulfate respiration in Chinese milk vetch treatments (Figure 5E). FUNGuild analysis found that the relative abundance of dung saprotroph was significantly higher in ryegrass than Chinese milk vetch and fallow treatments, while the opposite was true in arbuscular mycorrhizal and endophyte litter saprotroph-soil saprotroph (Figure 5F).

3.3.4 The relationship between the microbial community and soil properties and enzyme activities.

The results of the db-RDA at the genus level showed that soil bacterial communities in green manure application were significantly correlated with the SOM and TN when combined with humic acid fertilizer and organic manure. The soil bacterial communities of RG, RH, and MH had a stronger positive correlation with TP, while soil from RO and MO correlated more positively with pH and AN (Figure 6A; Supplementary Table S3). While, the soil fungal communities were significantly correlated with the pH, SOM, AN, TN and OP (Supplementary Table S4). The soil fungal communities of OMF, RO, and MO showed a strong correlation with SOM, while

TABLE 2 Effects of different green manure and fertilizers combinations on soil properties including pH, soil organic matter (SOM), total nitrogen content (TN), total phosphorus content (TP), Olsen-phosphorus (OP), and alkali-hydrolyzale nitrogen (AN).

	pH	SOM (g kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	AN (mg kg^{-1})	OP (mg kg^{-1})
CK	5.04 ± 0.03b	7.55 ± 0.44a	0.46 ± 0.01a	1.04 ± 0.09a	52.87 ± 3.38a	4.27 ± 0.04a
CF	4.83 ± 0.05a	7.60 ± 0.62a	0.50 ± 0.01a	1.09 ± 0.07ab	54.50 ± 1.79ab	4.41 ± 0.21ab
HAF	5.16 ± 0.03b	18.85 ± 1.02c	0.63 ± 0.02c	1.13 ± 0.08abc	62.93 ± 1.46abc	4.37 ± 0.20b
OMF	5.05 ± 0.04 b	10.46 ± 0.72b	0.68 ± 0.01de	1.18 ± 0.03abc	69.37 ± 2.78c	4.48 ± 0.27ab
RG	5.43 ± 0.07c	8.13 ± 0.17a	0.57 ± 0.03b	1.02 ± 0.01a	87.43 ± 3.03d	4.36 ± 0.15ab
RH	5.63 ± 0.04d	20.44 ± 0.18c	0.65 ± 0.01cd	1.12 ± 0.02abc	60.73 ± 6.20abc	4.91 ± 0.05b
RO	5.65 ± 0.06d	10.86 ± 0.48b	0.70 ± 0.01e	1.13 ± 0.01abc	81.73 ± 7.29d	4.41 ± 0.18ab
MG	5.03 ± 0.07b	7.81 ± 0.95a	0.57 ± 0.02b	1.11 ± 0.12ab	65.37 ± 2.68bc	4.84 ± 0.20b
MH	5.42 ± 0.06c	20.65 ± 0.91c	0.67 ± 0.02cde	1.27 ± 0.05c	64.80 ± 5.52abc	4.80 ± 0.19ab
MO	5.17 ± 0.07 b	10.87 ± 0.17 b	0.64 ± 0.01 cd	1.24 ± 0.06bc	65.57 ± 3.39bc	4.71 ± 0.31ab

Values are means ± SE of three replicates. Means in column followed by the same letter are not significantly different ($P > 0.05$). CK, the control treatment; CF, fallow soil with mineral fertilizer; HAF, fallow soil with humic acid fertilizer; OMF, fallow soil with organic manure; RG, ryegrass combined with mineral fertilizer; RH, ryegrass combined with humic acid fertilizer; RO, ryegrass combined with organic manure; MG, Chinese milk vetch combined with mineral fertilizer; MH, Chinese milk vetch combined with humic acid fertilizer; MO, Chinese milk vetch combined with organic manure.

samples from MG and MH were positively correlated with pH, AN, and TN (Figure 6B).

We also found that different soil enzyme activities had strong and unique correlations with both bacterial and fungal communities, varying among the different green manure treatments by using RDA and CCA (Figures 6C,D). In the fallow treatments, the soil bacterial and fungal communities were significantly positively correlated with catalase activity. Meanwhile, in the ryegrass and Chinese milk vetch treatments, the soil bacterial and fungal communities were significantly positively correlated with β-glucosidase activity and urease activity, respectively (Supplementary Tables S5, S6).

4 Discussion

Previous studies have reported that the incorporation of green manure with fertilizers affected soil physical and biological properties in long-term rice-green manure rotation systems (Chen et al., 2017). Our findings suggested that the utilization of green manure crops had a substantial influence on the soil microbial community throughout the growth and incorporation stages, especially the bacterial community. Additionally, we observed that these effects varied depending on the species of green manure crop applied and combined fertilizers.

4.1 Effects of green manure on soil microbial diversity and structure

Consistent with previous studies (He et al., 2020; Gao et al., 2021), the application of green manure resulted in a significant increase in bacterial diversity. However, our study also showed a contrasting trend in the diversity of the fungal community (see Table 1). The rhizosphere is a crucial area for nutrient exchange between plants and soil during green manure growth, which affects the microbial communities in this region (Bell et al., 2015). Previous studies have demonstrated that the diversity of the soil microbial community in the rhizosphere is influenced by both plant and soil properties (Lundberg et al., 2012; Mendes et al., 2014). In this study, the changes of bacterial and fungal diversities observed in rhizosphere soil may be attributed to the symbiotic and competitive interactions between green manure plants and microorganisms (Kuzyakov and Xu, 2013). For instance, green manure plant sediments serve as an effective and plant-specific source of organic matter for microbial growth in low-organic matter soil (Gmach et al., 2020; Saleem et al., 2020). However, green manure plants also obtain available nutrients from the soil to grow, which may form a competitive relationship with certain microbial groups in the rhizosphere soil. Furthermore, the effects of plant growth on rhizosphere microbial diversity were associated with soil nutrient conditions (Tedersoo et al., 2016). In our study, the bacterial diversity in the green manure rhizosphere significantly improved when combined with mineral fertilizer, while the effects of green manure on the diversity of rhizosphere microbes appeared to be less obvious when organic fertilizers were included, compared with the fallow soils.

Alterations in microbial diversity subsequent to the incorporation of green manure exhibited a congruent trend with the growth period, while the negative effect of green manure on fungal richness was mitigated (Table 1). Numerous of studies have suggested that the type and chemical composition of organic materials input to the soil affect the diversity of soil microorganisms (Khan et al., 2019; Liu et al., 2020). The microbial taxa that contribute to organic matter decomposition may benefit from the green manure residues (Wang et al., 2021). Fungal communities have been recognized as pivotal decomposers of organic materials, particularly with regard to cellulose and lignin in soil (Cox et al., 2001). Thus, the incorporation of green manure is expected to stimulate more fungal taxa in the degradation process. Sun et al. (2015) found that pig manure had a more positive effect on bacterial community diversity than other organic materials in a long-term green manure-rice system. The consistent findings were observed in our short-term experimental study, indicating that the incorporation of green manure with organic manure is advantageous for enhancing bacterial community diversity. Also, the highest diversity of bacterial

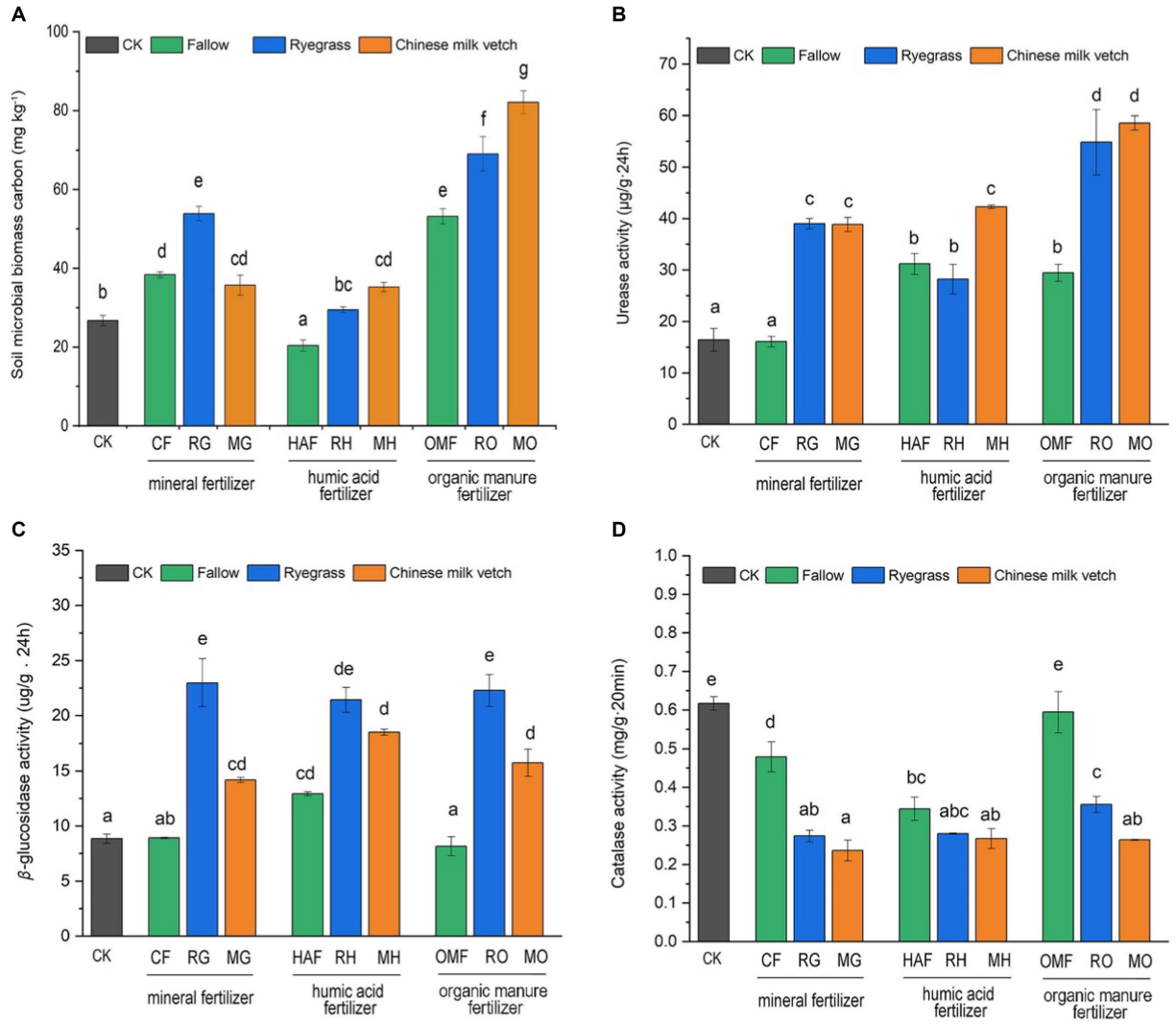

FIGURE 4
Effects of application of green manure combined with different fertilizers on **(A)** the soil microbial carbon, **(B)** urease activity, **(C)** β-glucosidase activity and **(D)** catalase activity during the green manure incorporation period. The CK is shown as the gray bar, and the fallow treatments, ryegrass incorporation treatments and Chinese milk vetch incorporation treatments are shown as green, blue and orange bars, respectively. Different lowercase letters indicate significant differences ($p < 0.05$).

community in RO could be linked to the more carbon and nitrogen provided by this combination and resulted in a wider range of ecological niches allowing for greater microbial growth in immature red soil. Consequently, the combination of green manure and organic manure may contribute to the augmentation of potential genetic diversity and the ability to resist environmental stress in immature red soil.

In addition, our findings indicated that the growth of green manure plants had a more significant impact on the bacterial community structure than fertilizer types in the rhizosphere soil. This was especially prominent in the case of ryegrass (as seen in Figure 3A), which had a strong root system. This may be attributed to the complex microenvironmental conditions within the rhizosphere, which confer a resistance to interference (Lovell et al., 2001). While the effects of green manure incorporation on the soil microbial community structures were related to both the green manure species and fertilizer types (see in Supplementary Figure S5).

4.2 Effects of green manure on soil microbial community composition

Different green manure species share common functional microbes, while can also make specific choices for microbes (Hartmann et al., 2009). In our study, both ryegrass and Chinese milk vetch growth significantly promoted the relative abundance of *Acidobacteria* compared to the fallow treatments. As typical oligotrophic taxa, the members of *Acidobacteria* were found to be advantageous to rhizosphere metabolism, and associated with carbon and nitrogen cycling (Fierer et al., 2007; Jiménez et al., 2012). The phylum *Firmicutes* and its class member *Clostridia* were markedly affected by ryegrass during both growth and incorporation periods (Figures 2C, 5C). *Firmicutes* has been reported to be beneficial for the carbon cycle due to its capacity to decompose plant-based polysaccharides (Pang et al., 2017), and the *Clostridia* can promote plant development and produce cellulase, which played a key role in degrading organic matter (Ueno et al., 2016; Abdallah et al., 2019). Also, the relative abundance of

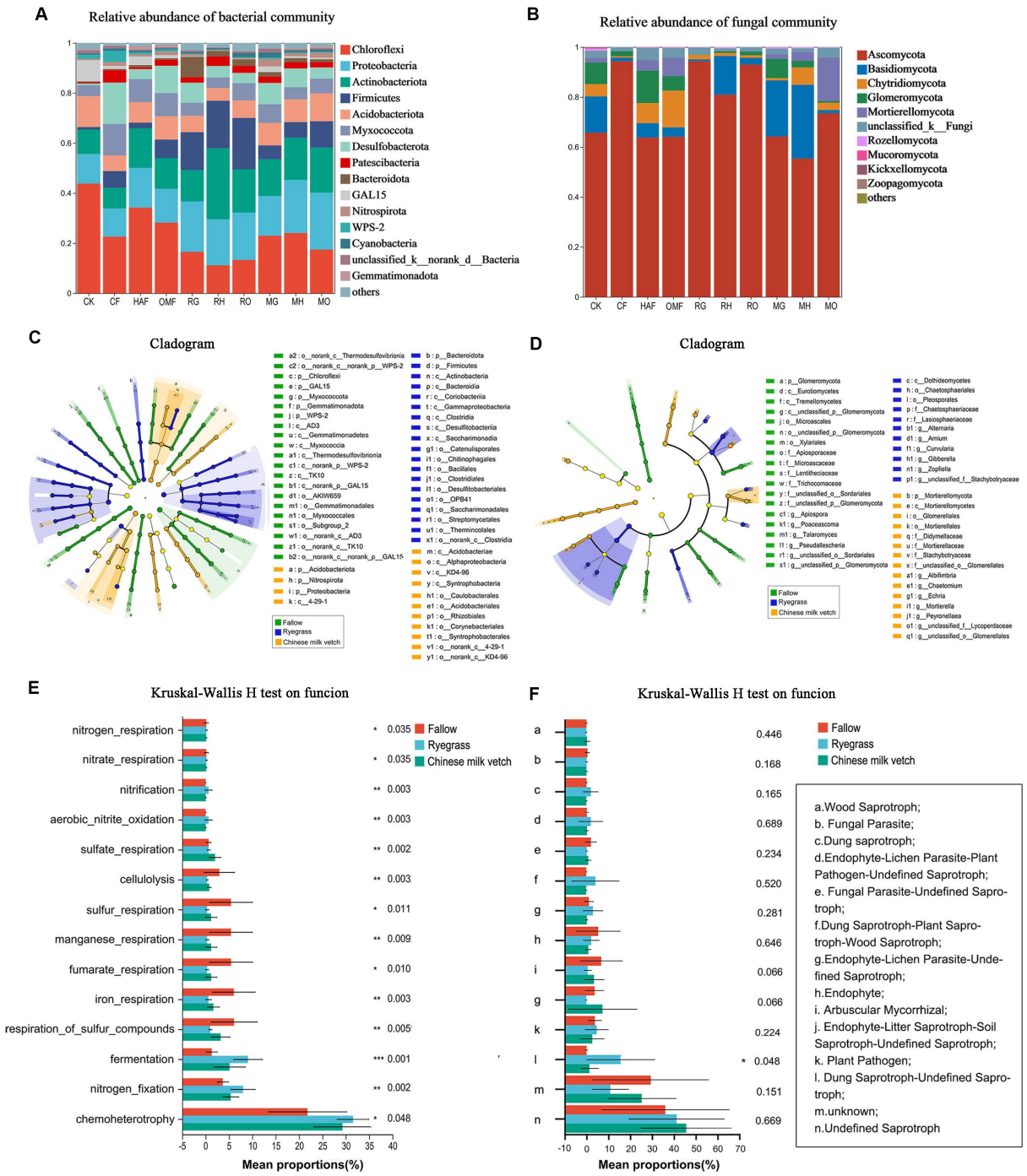

FIGURE 5
During the green manure incorporation period, the relative abundance of **(A)** bacterial community composition and **(B)** fungal community composition at phylum level; the LEfSe of **(C)** bacterial community and **(D)** fungal community regardless of the combined fertilizers; the functional prediction of **(E)** bacterial community by FAPROTAX and **(F)** fungal community by FUNGuild analysis regardless of the combined fertilizers. The *, **, and *** indicate the $P < 0.05$, $P < 0.01$, and $P < 0.001$ (Kruskal-Wallis H test), respectively.

Fibrobacteriota, which have been reported to hydrolyse cellulose under anaerobic conditions, was significantly enhanced during the ryegrass incorporation when combined with organic manure (Supplementary Figure S3). In addition, fungal class *Sordariomycetes* and *Dothideomycetes* were significantly promoted by ryegrass application (Figures 2D, 5D), which are known to secrete a variety of cellulase, hemicellulose and lignocellulosic enzymes (Wang et al., 2021). Therefore, the promotion of these bacterial and fungal groups associated with organic material decomposition and carbon cycle demonstrates that the growth and incorporation of ryegrass are beneficial for improving carbon metabolism in the immature red soil. In the case of Chinese milk vetch, being a legume, it significantly increased the relative abundance of bacterial taxa associated with nitrogen cycling, such as phylum *Cyanobacteria* in rhizosphere soil and order *Rhizobiales* during both

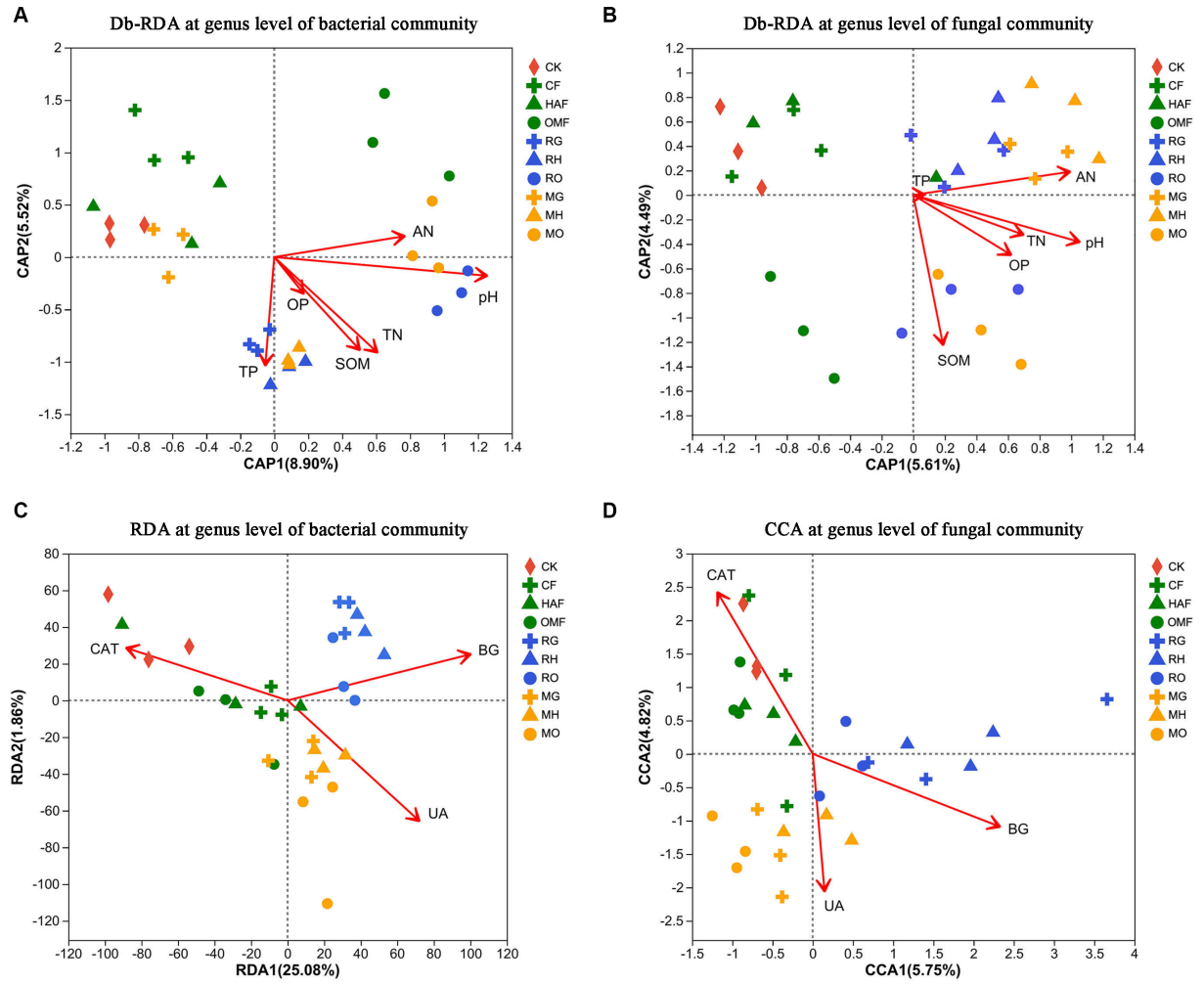

FIGURE 6
Db-RDA at genus level for the relationship **(A)** between soil bacterial communities and soil properties and **(B)** between the soil fungal communities and soil properties after green manure incorporation. The soil properties include pH, soil organic matter (SOM), total nitrogen content (TN), total phosphorus content (TP), Olsen-phosphorus (OP) and alkali-hydrolyzale nitrogen (AN). **(C)** RDA at genus level for the relationship between soil bacterial communities and soil enzyme activities (CAT, catalase; UA, urease; BG, β-glucosidase) after green manure incorporation; **(D)** CCA at genus level for correlation between the soil fungal communities and soil enzyme activities (CAT, catalase; UA, urease; BG, β-glucosidase) after green manure incorporation. Samples of CK are shown as red circles. The fallow treatments, ryegrass incorporation treatments and Chinese milk vetch incorporation treatments are shown as green, blue, and orange symbols, respectively. The combined mineral fertilizer, humic acid fertilizer and organic manure are indicated by triangle, diamond, and square, respectively.

growth and incorporation periods (Supplementary Figure S1; Figures 2, 5). *Cyanobacteria* are widely recognized for their capacity to nitrogen-fixing and their potential for agricultural applications (Singh et al., 2014). The increased abundance of *Rhizobiales* in the rhizosphere soil of Chinese milk vetch is understandable, as it is known to form a symbiotic relationship with legume roots, and fixing nitrogen in nodules (Ferguson et al., 2018). However, the improvement observed during the incorporation of Chinese milk vetch is unexpected since the symbiosis does not occur beyond the roots. This may be attributed to the legacy effects (Jing and Cong, 2022), whereby *Rhizobiales* proliferates in the soil throughout the course of plant growth and eventually evolves into the beneficial group enhanced by Chinese milk vetch. Furthermore, the fungal phylum *Glomeromycota*, whose members are commonly known as arbuscular mycorrhizal fungi (Smith and Read, 2010), was apparently enhanced by Chinese milk vetch in combination with mineral fertilizer (Supplementary Figure S4). This enhancement implies potential advantages for organic matter decomposition and absorption of nutrients by subsequent crops, such as phosphorous and nitrogen (Hodge and Fitter, 2010; Cheng et al., 2012).

4.3 Effects of green manure on soil enzyme activities and microbial functions

Changes in the composition of the microbial community can be partially confirmed by the results of the enzyme activities and microbial functional prediction (Figures 3–5). The activity of soil enzymes, which are produced by various soil microorganisms, plays a central role in soil nutrient cycling and can sensitively reflect the decomposition of organic matter in soil (Sinsabaugh et al., 2008). We found that urease activity was more highly promoted under Chinese milk vetch incorporation, while β-glucosidase activity was higher under ryegrass incorporation. This result was consistent with findings made by Khan et al. (2019), who found that hydrolase activity in the carbon cycle was higher after non-legume

(barley) incorporation, while the activity of soil enzymes involved in the nitrogen cycle was higher after legume (hairy vetch) incorporation. In line with the changes observed in soil enzyme activities, the structures of the soil microbial community and the activities of soil enzymes also exhibited a specific correlation associated with green manure (Figures 6C,D). The incorporation of ryegrass, which has a higher C: N ratio and cellulose content, significantly affected the bacterial and fungal communities correlated with β-glucosidase activity, which is key to organic matter cycling, especially carbon degradation (Bońska and Lasota, 2017). The microbial communities affected by Chinese milk vetch containing more nitrogen content had a strong correlation with the activity of urease, which is considered to be the main enzyme in the nitrogen cycle (Sinsabaugh et al., 2008).

Additionally, the increased relative abundances of microbial functions linked to organic carbon turnover, including chemoheterotrophy, fermentation, and dung saprotroph, confirmed the stimulation of organic carbon metabolism by the utilization of ryegrass. Meanwhile, the proportion of microbial functions involved in nitrogen metabolism was greater in the rhizosphere soil of Chinese milk vetch, suggesting that the rhizosphere activity of Chinese milk vetch promotes nitrogen cycling in immature red soil. The root secretions provide diverse compounds such as amino acids, organic acids and other secondary metabolites that stimulate the growth of various microorganisms under nutrient-limited immature soil and vary with the plant species (El Zahar et al., 2014). In addition, it is notable that the abundance of nitrogen cycle-related functions, such as nitrogen fixation, aerobic nitrite oxidation and nitrification, were also significantly promoted by ryegrass incorporation (Figure 5E), consisting with a previous study in which Italian ryegrass residues promote the growth of microbes involved in the N cycle (He et al., 2020). This may be due to the specific compounds from ryegrass residues that can alter the soil properties and promote the growth of species associated with nitrogen metabolism in the immature soil. In a study by Yan et al. (2018), it was observed that litter from different plant species, with their distinct components, selectively stimulated specific bacteria with diverse biological functions, such as the production of specific extracellular enzymes and promoting community succession. According to our results, we conjectured that the green manure species affected specific soil microbial taxa that could produce different extracellular enzymes and functions, consequently affecting the organic matter degradation and nutrient cycling in immature red soil. Nonetheless, additional investigations are required to gain a deeper understanding.

4.4 Effects of green manure on soil physical properties and microbial growth

The application of green manures combined fertilizers led to changes in soil conditions and thus influenced the bioavailability of nutrients beneficial to microbial growth. Consistent with previous studies suggesting that green manure can improve soil physical and chemical properties (Ma et al., 2021), our results indicated that the application of green manure significantly increased soil pH and nitrogen levels, particularly nitrogen availability, in immature red soil (as shown in Table 2). The improvement in soil pH may be attributed to the alkaline nature of green manure, which neutralizes the protons in acidic soil (Rukshana et al., 2013). Surprisingly, the levels of available nitrogen (AN) in the RG and RO treatments were greatly enhanced, surpassing those in the MG and MO treatments. Previous studies have suggested that non-leguminous cover crops enhance nutrient availability by reducing nutrient leaching (Doltra et al., 2019). However, the ability of leguminous plants to fix nitrogen results in higher nitrogen content in their tissues and thus releases more available nitrogen during degradation (Mathesius, 2022). Although the higher AN content in the ryegrass application aligns with our results in bacterial functional prediction (Figure 5E), further investigation into the specific mechanisms underlying this observation is warranted.

Previously, the input of organic materials has been shown to stimulate the growth of soil microorganisms, so as to improve the soil microbial biomass (Ge et al., 2010). In this study, the incorporation of green manure significantly improved SMBC, with the MO treatment giving the highest increase (Figure 4A). Compared to ryegrass, Chinese milk vetch has a lower fiber content and higher protein content, which is more easily immobilized by soil microorganisms (Watthier et al., 2020). And the organic manure contained more available nutrients for microorganisms, thus the MO combination has an advantage in promoting the growth of microorganisms in immature red soil. It is also noteworthy that the addition of humic acid significantly reduced the SMBC, although humic acid significantly increased the SOC (Figure 4A; Table 2). This finding is consistent with a previous study that observed humic acid application reduced SMBC when combined with biochar (Holatko et al., 2020). It could possibly be attributed to the inhibition of microbial assimilation due to the fact that the organic carbon present in humic acid is stable and difficult to decompose.

4.5 Relationship between the soil microbial community and soil properties regulated by green manure application

Studies have suggested that the soil microbial community was mainly determined by soil properties such as pH, soil nutrients, and organic matter content (Rinklebe and Langer, 2006; Yu et al., 2019). In this study, we found that the improvements of pH, SOM, AN and TN were the key environmental factors affecting both soil bacterial and fungal communities when green manure application combined with organic fertilizers (Figures 6A,B; Supplementary Tables S3, S4). As demonstrated in a previous study, the observed increase in soil total nitrogen (TN) and soil organic matter (SOM) can, in part, be attributed to the rise in the number of potential functional genes following green manure application. This increase is primarily driven by bacteria taxa that are involved in the decomposition process (Zheng et al., 2018b). Our results confirmed the positive correlation between key factors including pH, TN, AN and SOM and the bacterial taxa associated with carbon and nitrogen cycling (e.g., *Firmicutes*, *Fibrobacterota*, and *Bacteroidota*), which were significantly increased following green manure application (Supplementary Figures S3, S6). Also, the significant increase in soil pH under green manure application may related to the notable increase in soil sulfate respiration function, indicating green manuring may be a potential practice in mitigating acidification of immature red soil (Gao et al., 2021). Additionally, the relationships between the soil properties and microbial communities varied with the types of combined fertilizers. Soil pH and soil organic matter positively affected bacterial and fungal community variations, respectively, when combined with organic manure. While, soil total P and alkali-hydrolyzale N were the important variables affecting the soil bacterial community and fungal community, respectively, when

combined with humic acid (Figures 6A,B). The mechanism underlying these effects remains unclear, but it is likely to be associated with the physical structure, composition, and bioactive substances of the two different organic fertilizers.

5 Conclusion

In the present study, we investigated the effects of various green manure-fertilizer combinations on soil microbial community characteristics in low-fertility immature red soil. The application of green manure had a positive effect on soil pH, nitrogen availability, soil microbial growth, activity, bacterial diversity and specific microbial groups involved in soil carbon and nitrogen cycles, such as *Firmicutes*, *Acidobacteria*, and *Glomeromycota*. The significant correlation between soil microbial community and soil properties highlights the crucial role of green manure in stimulating soil nutrient cycling by altering the microbial community, leading to the improvement of soil efficient fertility and ecosystem functioning. Furthermore, the impact of green manure on soil microbial communities and functions exhibited plant species specificity, and were also influenced by the types of combined fertilizers, pointing out the need for targeted management strategies that account for both the type of green manure crops and the specific fertilizer requirements of the soil. In conclusion, our study emphasizes the potential of green manure as a sustainable agricultural practice for improving soil biological health and underscores the importance of considering the role of soil microbes in regulating ecosystem function. However, further research is necessary to determine how these alterations of soil microbial community contribute to the subsequent crops.

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

JX: Formal Analysis, Funding acquisition, Methodology, Visualization, Writing – original draft. LS: Data curation, Funding acquisition, Investigation, Writing – review & editing. XZ: Conceptualization, Validation, Visualization, Writing – review & editing. KC: Investigation, Supervision, Writing – review & editing. JW: Conceptualization, 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.2023.1255056/full#supplementary-material

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