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Emerging frontiers of microbial functions in sustainable agriculture

Edited by
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Emerging frontiers of microbial functions in sustainable agriculture

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hand, Zheng et al. emphasized the importance of tree ages and soil texture in bacterial diversity and composition. *Proteobacteria, Acidobacteria*, and *Actinobacteria* were the dominant bacterial taxa in pomelo tree soil. Soil properties, such as pH and phosphorus availability, play important roles in shifts in bacterial communities. Bacterial genera *Sinomonas* and *Streptacidiphilus* were found to be unique in red soil, while *Actinoallomurus* and *Microbacterium* were found in paddy soil. The microbial co-occurrence network showed that old trees (20 and 30 years) have more complex networks and are more stable than young trees. Li et al. showed that phytase-producing *Pseudomonas* spp. is predominantly found in the alpine grassland of the Qinghai-Tibetan Plateau. These bacteria can promote the growth of *Lolium perenne* L and show multiple plant growth-promoting traits, such as P solubilization, plant production, nitrogen fixation, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, and antimicrobial activity. Nong et al. described *Burkholderia* sp. strain GXS16, a diazotrophic bacteria colonization response in sugarcane roots. Bacterial colonization enhances antioxidants, such as H_2O_2 and malondialdehyde. Differentially expressed genes linked to ethylene pathways were much more highly expressed than those linked to abscisic acid and gibberellin.

Microbes and agricultural practices

Plant rhizosphere and endosphere-associated microbes are strongly involved in nutrition transport and plant growth development (Solanki et al., 2019). Organic manure positively influences microbial functions and improves soil nutrient availability and uptake by plants (Alori et al., 2017; Jiao et al., 2019). Zhao et al. revealed that cattle manure improves oat plant root length and surface. Bacterial genera *Pseudoxanthomonas*, *Pseudomonas*, and *Sphingomonas*, and the fungal phylum Ascomycota, were positively related to oat biomass and nutrient accumulation during cattle manure application. Results revealed that Basidiomycota is more abundant in cattle manure deposition treatment than the control. Moreover, cattle manure disrupts the growth of pathotrophs, such as the fungal genera *Alternaria* and *Fusarium*, and encourages the development of saprotrophic and symbiotrophic fungi.

Soil health restoration through different kinds of microbes is an ecological process (Solanki et al., 2019; Solanki et al., 2021), and soil health monitoring through microbial activity and response plays an essential role in all restoration processes. Bhaduri et al.. discussed the major strategies that can help restore and maintain ecosystem stability. Various bio-indicators, such as microbial biomass, enzymes, genetic markers, metabolites, and microbial communities, could be used to identify soil health in the presence of different pollutant-contaminated soil samples. Next, Choudhary et al. reported that soil properties and fungal communities play a significant role in climate-smart agricultural (CSA) practices. The fungal taxon Ascomycota was found to be abundant in rice-based CSA scenarios. Additionally, higher levels of soil organic carbon and nitrogen were found in CSA scenarios, improving crop yield.

Microbes and plants: Biotic and abiotic stresses

Biotic and abiotic factors are considerable limitations in sustainable agriculture production. In recent years, drought stress has become a major issue for agriculture sectors in developing countries (Yandigeri et al., 2012; Wang et al., 2018). Morales-Quintana et al. showed that the fungal endophytes *Penicillium brevicompactum* and *P. chrysogenum,* isolated from Antarctic vascular plants, provoke drought stress regulation in strawberry plants. These endophytes enhance photosynthetic activity, antioxidants, and proline content and reduce lipid peroxidation, which helps plants regulate drought stress. These symbiotic fungi can also be used as an eco-friendly strategy to cope with drought in other crops. Palmieri et al. revealed that patulin biosynthesis by *Penicillium expansum* strongly correlates with extracellular pH in wounded apples. The pH modulation by *Papiliotrema terrestris* LS28 is vital for reducing the amount of patulin. Jia et al. delved into the microbial diversity associated with healthy and wilted *Paeonia suffruticosa* rhizosphere soil. Fungal genera *Fusarium*, *Cylindrocarpon*, and *Neocosmospora* were directly associated with plant yield reduction and disease incidence. Bacterial and fungal networks were more complex in diseased plants than in healthy ones. The bacterial network significantly impacted the diseased plants that provide a comfortable environment in which the fungal group can grow efficiently. Dastogeer et al. showed that the microbiomes of leaf and grain tissues are altered significantly at the *Magnaporthe oryzae* infection site. The bacterial genus *Rhizobium* increased, whereas the fungal genera *Tylospora, Clohesyomyces*, and *Penicillium* declined in the symptomatic leaf and grain tissues. The microbial network identified several direct interactions between *Magnaporthe oryzae* and other microbes. A higher percentage of soil bacteria was tracked from healthy root samples.

Fruit-associated microbiome

Microbial communities are associated with fruit surfaces and internal tissues and are impacted by the host's age, evolution, and diversity. The fruit carposphere harbors a wide diversity of microbes (Droby and Wisniewski, 2018). In this regard, Zhimo et al. identified 15 bacterial and 35 fungal core taxa that are abundant at different stages of the apple carposphere of three cultivars that grow in the same environmental conditions. This study represents the strong microbial cross-domain associations, uncovers potential microbe-microbe correlations in the apple carposphere and provides essential information regarding microbial recruitment in the fruit carposphere and its influence over time.

Conclusion

The non-judicial use of agrochemicals, including fertilizers, and mismanagement of natural soil and water resources greatly impact soil microbial community function, which may result in barren or unproductive soil in the long term. Moreover, soil degradation through the depletion of soil carbon is a critical factor for judging soil carrying capacity and its future utility. In this regard, it is highly pertinent that plant rhizosphere microbes and their symbiotic associations or endophytes play an optimistic candidature, and that their proper use sustains the utility of the soil in the long term.

Perspectives

- Microbial resources and their significance for maintaining soil ecosystems has been highly recognized in recent decades.

However, microbial performance varies greatly depending on the various biotic and abiotic factors that are directly linked with the agroecological conditions.

- Nutrient cycling, restoration of pollutant-contaminated soil, and protection of soil and plant diversity are only possible by the virtue of symbiotic association among microbes in plants and soil.
- Soil health represents the accumulation of healthy soil biota or biodiversity over a period of time or through the proper management of soil biotic components.
- Bridging multiple technologies to better understand the microbial relationships in plant growth and soil productivity. Here, several molecular approaches are applied to extract the insight information. However, research and obstacles run parallel with each other, and advanced technologies may help unlock the secret information in the future.

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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Limited Impacts of Cover Cropping on Soil N-Cycling Microbial Communities of Long-Term Corn Monocultures

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Cover cropping (CC) is a promising in-field practice to mitigate soil health degradation and nitrogen (N) losses from excessive N fertilization. Soil N-cycling microbial communities are the fundamental drivers of these processes, but how they respond to CC under field conditions is poorly documented for typical agricultural systems. Our objective was to investigate this relationship for a long-term (36 years) corn [*Zea mays* L.] monocultures under three N fertilizer rates (N0, N202, and N269; kg N/ha), where a mixture of cereal rye [*Secale cereale* L.] and hairy vetch [*Vicia villosa* Roth.] was introduced for two consecutive years, using winter fallows as controls (BF). A 3 × 2 split-plot arrangement of N rates and CC treatments in a randomized complete block design with three replications was deployed. Soil chemical and physical properties and potential nitrification (PNR) and denitrification (PDR) rates were measured along with functional genes, including *nifH*, archaeal and bacterial *amoA*, *nirK*, *nirS*, and *nosZ-I*, sequenced in Illumina MiSeq system and quantified in high-throughput quantitative polymerase chain reaction (qPCR). The abundances of *nifH*, archaeal *amoA*, and *nirS* decreased with N fertilization (by 7.9, 4.8, and 38.9 times, respectively), and correlated positively with soil pH. Bacterial *amoA* increased by 2.4 times with CC within N269 and correlated positively with soil nitrate. CC increased the abundance of *nirK* by 1.5 times when fertilized. For both bacterial *amoA* and *nirK*, N202 and N269 did not differ from N0 within BF. Treatments had no significant effects on *nosZ-I*. The reported changes did not translate into differences in functionality as PNR and PDR did not respond to treatments. These results suggested that N fertilization disrupts the soil N-cycling communities of this system primarily through soil acidification and high nutrient availability. Two years of CC may not be enough to change the N-cycling communities that adapted to decades of disruption from N fertilization in corn monoculture. This is valuable primary information to understand the potentials and limitations of CC when introduced into long-term agricultural systems.

Keywords: N cycle genes, *nosZ*, *amoA*, *nifH*, *nirK*, *nirS*, maize (*Zea mays* L.), N fertilization

INTRODUCTION

As one of the most promising conservation practices, cover cropping (CC) bolsters the natural functions of an agricultural system through ecological intensification (Tittonell, 2014). This leads to various benefits like reducing soil erosion, returning organic matters to the soil, and weed suppression (Blanco-Canqui et al., 2015; Blesh et al., 2019; Reiss and Drinkwater, 2020). Many of such benefits contribute toward soil health, the soil's capacity to support ecological services crucial for sustainable agriculture (Lehmann et al., 2020). Thus, CC has been widely proposed as a crucial strategy to improve the soil health of intensely managed systems that are vulnerable to soil degradation and nutrient pollution (IL-EPA et al., 2015). Simplified cropping systems concentrated on commercially important crops like corn [*Zea mays* L.] and soybean [*Glycine max* (L.) Merr.] dominate the crucial agricultural regions like the United States Midwest (Hatfield et al., 2018). As these systems, especially those based on corn, rely on frequent and heavy N fertilization, an estimated four Tg of inorganic N is applied each year in this region alone (USDA-ERS, 2020; Socolar et al., 2021). However, the low N use efficiency of these systems leads to excess soil N, which causes chemical imbalances that not only degrade the soil health but also become major sources of water pollution and greenhouse gas (GHG) emissions (Rengel, 2011; Fagodiya et al., 2017). Here, replacing bare fallows with CC can scavenge the excess N and release them slowly upon decomposition, thereby reducing the soil N in forms vulnerable to loss Indeed, past primary research and research syntheses have demonstrated that CC mixtures that include non-legumes generally reduce NO_3^- leaching (Thapa et al., 2018) and nitrous oxide (N_2O), a potent GHG, and emissions (Basche et al., 2014; Muhammad et al., 2019).

The soil microbes are the fundamental drivers of soil processes that constitute ecological services. Likewise, the soil N-cycling is largely determined by the complex web of activities by the N-cycling microbial communities. Therefore, changes in the soil environment from management practices can perturb the N-cycling communities and their functionality (Behnke et al., 2022; Kim et al., 2022b). Indeed, soil chemical imbalances and N losses in vulnerable agricultural systems are symptoms of excessive N fertilization disturbing the soil microbial N-cycling (Hirsch and Mauchline, 2015; Li et al., 2022). Likewise, various effects of CC, including excess soil N removal, organic matter return, and root exudation may also modify the soil environment and lead to unintended responses from the N-cycling communities. For instance, compared to leaving the terminated CC residues on the surface, incorporating them into the soil can rather increase N_2O emissions by accelerating the N mineralization by the decomposers (Muhammad et al., 2019). Also, Foltz et al. (2021) have shown that although cereal rye CC decreased field N_2O emission from a corn-soybean rotation, it increased the N_2O production potential under assay settings, suggesting that CC might increase emissions without proper nutrient management. Thus, evaluating the effectiveness of CC in mitigating the detriments of excessive N fertilization must include how the soil N-cycling communities respond to changes in the soil environment under CC.

Abundances of the functional genes that code N-cycling enzymes represent the population sizes of their respective N-cycling communities. Indeed, the abundances of N-cycling functional genes tend to highly correlate with their respective inorganic N products (Lourenço et al., 2022). For example, N_2O emission correlates negatively with functional genes that code N_2O reductase but does so positively with those of nitrifiers (You et al., 2022). The commonly used N-cycling functional genes include *nifH* (nitrogenase; N-fixation), ammonia-oxidizing archaea (AOA) and bacterial (AOB) *amoA* (ammonia monooxygenase; nitrification), *nirK* and *nirS* (nitrite reductase; denitrification), and *nosZ-I* (nitrous oxide reductase; denitrification; Hirsch and Mauchline, 2015). As N fertilizers directly interact with the microbes harboring these genes, many studies have reported their sensitivity to N fertilization. For example, an Illinois study on the bioenergy production system of Mollisols reported that N fertilization (112 kg N/ha) increased the abundances of AOB *amoA,* but decreased those of *nirS* and *nifH* (Kim et al., 2022a). Meanwhile, a meta-analysis by Ouyang et al. (2018) compiled the results from 47 primary studies on how N fertilization affects these genes. The authors suggested that N inputs can intensify microbial N-cycling. Furthermore, the responses of these genes were sensitive to cofactors like fertilizer forms (organic vs. inorganic) and cropping systems (monoculture vs. rotation). Like these cofactors, CC might also alter the interactions between these functional genes and N fertilization. While these functional genes provide crucial insights into specific N-cycling communities, they do not equate to overall functionality as population size may not necessarily translate to activity. Thus, they need to be complemented by more direct measures of functionality like potential nitrification (PNR) and denitrification (PDR) rates determined by enzyme assays. Indeed, reports from various systems show that functional genes do correlate with PNR and PDR (Xu et al., 2012; Chen et al., 2013; Li et al., 2022). For instance, a study in acidic soils under soybean-corn-corn rotation reported significant correlations between *amoA* genes and PNR (Xu et al., 2012). However, primary research that coupled functional genes and more direct measures functionality is scarce for N-cycling communities under CC, especially within typical Midwestern cropping systems.

A few past studies have characterized the N-cycling functional genes under CC. For example, Hu et al. (2021), on cotton monoculture on Tennessee Alfisols, compared bacterial functional genes among bare fallow, legume CC, and grass CC between two N rates (0 and 67 kg N/ha), and between tillage and no-till. The authors reported that AOB *amoA* increased in abundance with legume CC and more so with N input, while grass CC only increased AOB *amoA* when unfertilized. They also showed that *nifH* increased sequentially from bare fallow, legume CC, and grass CC. Yet, the N rates of this study are much lower than those typically simplified cropping systems. Another recent study by Castellano-Hinojosa et al. (2022) also reported that CC, especially a legume and non-legume mixture, generally increased the abundance of the N-cycling functional genes. Yet, their system was a citrus orchard, which is also different from the United States Midwestern cropping systems. Thus, currently

available studies on this topic are done in systems dissimilar to the intensely managed and simplified cropping systems typical to the major agricultural regions like the United States Midwest. Therefore, there is a critical lack of primary information on how the N-cycling functional genes respond to introducing CC to such systems.

Three past reports exist on this study's experimental site where Kim et al. (2022b) and Huang et al. (2019) each characterized its soil and the N-cycling functional genes, respectively, before CC; while Kim et al. (2022c) described the soil microbiota with high taxonomic resolution using genus-level bioindicators after introducing CC. Kim et al. (2022b) demonstrated that significant soil acidification, increase in NO_3^-, and nutrient depletion marked the long-term effects of heavy N inputs in corn monocultures over resilient Mollisols. Huang et al. (2019) found that N fertilization decreased the abundance of *nifH* while increasing that of AOB *amoA*. Kim et al. (2022c) observed more diversified N-cycling communities in unfertilized soils, including an increased abundance of an indicator of AOA. They also reported soil acidification as the major modulating factor for the soil microbes of this system. Meanwhile, CC increased the abundances of N-fixers and *nirK* denitrifiers, while decreasing those of N_2O reducers (Kim et al., 2022c). These potential shifts in the N-cycling guilds after introducing CC may have translated into the abundance of the functional genes, and, ultimately, into soil microbial N-cycling.

Thus, we aimed to investigate whether CC can mitigate the disruptions in the soil microbial N-cycling after long-term N fertilization in typical simplified agricultural systems. In accordance with aforementioned past reports on this study's site, we hypothesized that: (i) N fertilization would decrease the number of *nifH* and AOA *amoA* but increase that of AOB *amoA*; (ii) introducing CC will increase the abundances of *nifH* and *nirK*; and that (iii) these changes will translate to changes in PNR and PDR. Our objective was to characterize the soil N-cycling communities after introducing CC to a long-term corn monoculture of 36 years of consistent management with and without N fertilization, using six commonly used functional genes (*nifH*, AOA and AOB *amoA*, *nirK*, *nirS*, and *nosZ-I*). This study also determined the soil properties, and PNR and PDR to test whether these genes are consistent with the changes in the soil environment and functionality by treatments. This study will provide primary information on how CC affects the soil microbial N-cycling in a simplified cropping system under heavy N inputs. This will be valuable for future research and decision-makers to evaluate CC as a strategy to improve soil health and nutrient cycling.

MATERIALS AND METHODS

Experimental Site Description and Management Practices

The field experimental site was established at the Northwestern Illinois Agricultural Research and Demonstration Center (40° 55′ 50″ N, 90° 43′ 38″ W) in 1981 to study the effects of N fertilization rates (Nrates) on corn yields of continuous corn and corn-soybean short rotations (**Figure 1**). The mean annual precipitation and temperature of this site are 914 mm and 10.6°C, respectively, Illinois State Water Survey (2010). The soil series is Muscatune silt loam (fine-silty, mixed, and mesic Aquic Argiudoll), dark-colored and very deep soils of moderate permeability, and low surface runoff potential that developed under prairie vegetation in a layer of 2–3 m thick loess over glacial till (Soil Survey Staff et al., 2020). The topography of this site is nearly flat (Soil Survey Staff et al., 2020). Kim et al. (2022b) described further information on this experimental site and field management up to 2018.

This study started in 2018 and focused on introducing CC to the corn monoculture plots, spanning two CC growing seasons in 2018–2019 and 2019–2020. The experiment was arranged in a split-plot of Nrates (N0, N202, and N269; kg N/ha) and CC (cover crop, CC; bare fallow control, BF) in a randomized complete block design with three replicates. The main plot dimension was 18 m × 6 m, and the subplots were 18 m × 3 m. Fertilizer, weed, and pest management decisions were based on the best management practices as recommended by the Illinois Agronomy Handbook (Fernández and Hoeft, 2009). No P and K fertilizers or lime were applied during this experiment. Corn was harvested in mid-October with a plot combine (Almaco, Nevada, IA, United States). Following corn harvest, on October 3, 2018, and on October 19, 2019, a mixture of cereal rye [*Secale cereale* L.] and hairy vetch [*Vicia villosa* Roth.] was no-till drill-seeded at 84 kg seeds/ha (70% cereal rye, and 30% hairy vetch). Cover crop termination occurred in early May, both years following soil sampling, using glyphosate [*N*-(phosphonomethyl)glycine] (Roundup WeatheMAX®, Bayer AG, Leverkusen, Germany) at the rate of 1.89 kg ai/ha. Spring N fertilizer treatments were applied as incorporated urea ammonium nitrate (UAN, 28%) on May 15, 2019 and May 5, 2020, following CC suppression and before planting corn. Spring tillage using a rotary tiller (Dyna Drive Cultivator, EarthMaster, Alamo Group, Inc., Seguin, TX, United States) was conducted on June 3, 2019 and May 11, 2020. Corn cash crop was planted on June 3, 2019 and May 26, 2020, at 88,000 seeds/ha.

Soil and Cover Crop Biomass Sampling and Properties Measurement

Soil sampling occurred on April 26, 2019 and April 30, 2020. Within each experimental unit, three composited soil subsamples, each up to 500 *g*, were taken from random points at 0–10 cm depth with an Eijelkamp grass plot sampler (Royal Eijkelkamp Company, Giesbeek, Netherlands) for microbial DNA extraction and PNR and PDR rates. These soil samples were transported in coolers filled with ice from the experimental site and then stored at −20°C. Also, three 0–90 cm depth soil core subsamples were taken per experimental unit using a tractor-mounted soil sampler with soil sleeve inserts (Amity Tech, Fargo, ND, United States). These soil cores were divided into depths of 0–30, 30–60, and 60–90 cm, and transported to the lab to determine the water content (%) and bulk density (Bd; Mg/m^3). Only the data from 0 to 30 cm is reported in this study. About 10 *g* of soil per subsample was oven-dried at 105°C

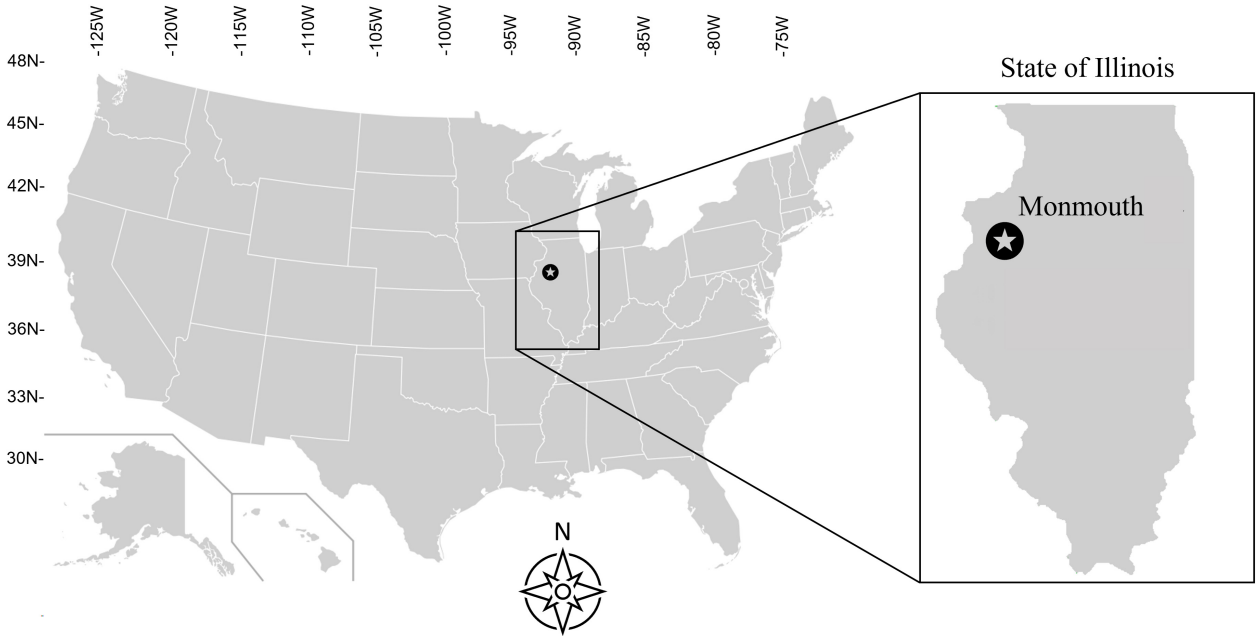

FIGURE 1 | Location and coordinates of the experimental site within the United States and the state of Illinois.

to measure the gravimetric water content and obtain Bd using the core method (Blake and Hartge, 1986). About 4 *g* of each subsample was used to determine water soil aggregate stability (WAS,%) with a west sieving apparatus (Eijkelkamp, Giesbeek, Netherlands). The remaining was sent to a commercial laboratory (Brookside Laboratories, Inc., New Bremen, OH, United States) to determine additional soil properties using standard methods recommended for United States-North Central region[1]. Thus, soil organic matter (SOM,%) content was determined by loss-on-ignition at 360°C (Schulte and Hopkins, 1996). Soil available P was measured with Bray I extraction (Pbray, mg/kg), and the extractable elements, including sulfur, calcium, magnesium, sodium, and potassium (K, mg/kg), with the Mehlich 150 III method (Ziadi and Tran, 2008). The summation of these exchangeable cations was used to estimate the cation exchange capacity (CEC, $cmol_c$/kg; Sumner and Miller, 1996).

The PNR and PDR were measured with the methods adapted from Drury et al. (2008) and Malique et al. (2019), respectively. For PNR, a mixture of 6 *g* of the soil subsample and 40 mL of working solution was activated for assay by shaking them for an hour in a 125 mL jar covered with parafilm with several holes, on a rotary shaker (Corning® LSE™ orbital shaker, Corning, NY, United States) at 180 RPM at room temperature. After assay activation, 5 mL of initial aliquot was taken and it was centrifuged for 10 min at 9,000 RPM (Sorvall® RC 5C Plus, Kendro Laboratory Products, Asheville, NC, United States) and the supernatant was transferred and stored frozen for further analysis. NO_3^- and NO_2^- were measured using SmartChem 200 (Westco Scientific Instruments, Inc., Danbury, CN, United States). For the dry mass of the soil used in this equation, the mass of 1 *g* of soil subsample was measured after drying it for 48 h in the DKN-810 Mechanical Convection Oven (Yamato Scientific America Inc., Santa Clara, CA, United States). For PDR, 10 *g* of soil and 20 mL of working solution were put in 125 mL jars with septa caps. The headspace was vacuumed and, then, refilled with 100 mL of N_2 gas with a syringe; this process was repeated once. Then, 20 mL of acetylene was inserted into the jar to prevent N_2O reduction, and a 15 mL initial gas sample was taken and replenished with 15 mL of N_2 gas. The jar that was shaken by hand to initiate reaction was incubated in a shaker (Eberbach, Ann Arbor, MI, United States). Gas samples were taken three times in 30 min intervals, replenishing the jars with 15 mL N_2 gas each time. The gas samples were analyzed in a gas chromatograph equipped with an electron capture detector and autosampler (Shimadzu GC-2014 and AOC 5000 Plus, Kyoto, Japan). The N_2O concentrations were calculated with the equation used in Malique et al. (2019), but the PDR (ng N/g of dry soil hour) was calculated using the $t = 0_{min}$ N_2O concentration instead of that of $t = 30_{min}$.

Soil DNA Extraction and Real-Time Quantitative Polymerase Chain Reaction Analysis

Soil DNA was extracted from 0.25 *g* of each homogenized soil subsample, using PowerSoil® DNA isolation kits (MoBio Inc., Carlsbad, CA, United States) according to the manufacturer's instruction, and stored at −20°C. A Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) was used to test the quantity and quality of the extracted DNA. The DNA samples were sequenced

[1]https://www.blinc.com/resources/testing-methods (accessed on 20 February 2022).

using primers for the bacterial V4 region 16S rRNA and archaeal 16S rRNA, fungal internal transcribed spacer (ITS) region, nitrogenase *nifH*, bacterial and archaeal ammonia monooxygenase *amoA*, nitrite reductase *nirK* and *nirS*, and nitrous oxide reductase *nosZ-I*. For this, the DNA samples were sent to W.M. Keck Center for Comparative and Functional Genomic lab at the University of Illinois Biotechnology Center (Urbana, IL, United States) for Illumina MiSeq paired-end System (2 × 250 bp; Illumina, Inc., San Diego, CA, United States). The maximum sample DNA concentration was set to 50 ng/μL. The primer sets used for amplification were 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACVSGGGTWTCTAAT) for the bacterial 16S rRNA gene (Fierer et al., 2005); 349F (GTGCASCAGKCGMGAAW) and 806R (GGACTACVSGGGTATCTAAT) for the archaeal 16S rRNA gene (Colman et al., 2015); 3F (GCATCGATGAA GAACGCAGC) and 4R (TCCTCCGCTTATTGATATGC) for the fungal ITS region (Crawford et al., 2012); CS1-*nifH*-PolF-124-for (TGCGAYCCSAARGCBGACTC) and CSs-*nifH*-PolR-118-rev (ATSGCCATCATYTCRCCGGA) for *nifH* (Poly et al., 2001); Cren*amoA*23f (ATGGTCTGGCTW AGACG) and Cren*amoA*616r (GCCATCCATCTGTATG TCCA) for AOA *amoA* (Tourna et al., 2008); *amoA*-1F-10-for (GGGGTTTCTACTGGTGGT) and *amoA*-2R-10-rev (CCCCTCKGSAAAGCCTTCTTC) for AOB *amoA* (Rotthauwe et al., 1997); CS1-*nirK*876-209-for (ATYGGCGGVCAYG GCGA) and CS2-*nirK*1040-197-rev (GCCTCGATCAGRTTRT GGTT) for *nirK* (Henry et al., 2004); CS1-*nirS*Cd3aF-211-for (AACGYSAAGGARACSGG) and CS2-*nirS*R3cd-199-rev (GASTTCGGRTGSGTCTTSAYGAA) for *nirS* (Kandeler et al., 2006); and CS1-nosZ1F-16-for (WCSYTGTTCMTCGACA GCCAG) and CS2-nosZ1R-15-rev (ATGTCGATCARCTGV KCRTTYTC) for *nosZ-I* (Henry et al., 2006).

The resulting pooled amplicons of template DNA from the Illumina MiSeq system from all samples were prepared into PCR mixtures by mixing 1 μL of the pooled amplicon, 25.8 μL of ddH_2O, 10 μL of 5X GoTaq green reaction buffer (Promega Corp., Madison, WI, United States), 4 μL of dNTP at 2.5 mM, 5 μL of $MgCl_2$ at 25 mM, 0.2 μL of GoTaq DNA polymerase (Promega Corp., Madison, WI, United States), and 2 μL of primers for each forward and reverse sequences for each of the nine target sequences described above. This procedure was repeated by replacing the pooled amplicon with water to make controls. The mixtures were amplified in PCR with BioRad T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, United States) with parameters of 95°C for 10 min, followed by 34 cycles of amplification (45 s at 95°C; 45 s at 58°C; 45 s at 72°C), and a final extension at 72°C for 10 min. The amplified mixtures were run in electrophoresis with 1.5% agarose gel containing GreenGlo™ Safe DNA dye (Denville Scientific, Inc. Metuchen, NJ, United States), so that each pure, amplified marker genes are contained in one of the wells. The thick bands in the gel visible under UV light at the respective molecular weights of each target gene were cut out. Then, DNA was extracted from the cut gels using Monarch® Genomic DNA Purification Kit (New England Biolabs, Ipswich, MA, United States), following the manufacturer's instructions. The resulting DNA samples were again checked for quantity and quality using Nanodrop 1000 Spectrophotometer. Then, to make the standard curve, aliquots of each target gene were taken in unique amounts that equalized their estimated gene counts to 1.51×10^{10} copies for each of the nine marker genes. The aliquots were mixed and topped to 500 μL with 1× TE buffer. Then, the aliquots were diluted in 10-fold series and used 10^8 to 10^1 dilutions, so that the range of dilution encompasses the possible DNA copies per sample. Finally, quantitative polymerase chain reaction (qPCR) plates were prepared, including 70 μL of primers for each of the nine marker genes, 10 μL for each dilution of the standard curve for each plate, and 10 μL of aliquots for each sample. The prepared plates were run for high-throughput qPCR in a BioMark HD™ System (Fluidigm Corporation, South San Francisco, CA, United States) at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois Biotechnology Center.

Statistical Analysis and Visualization

Linear mixed models were fitted using the GLIMMIX procedure in SAS software version 9.4 (SAS Institute, Cary, NC, United States) to determine the effects of Nrate, CC treatments (CC), and their interactions on the response variables: soil properties, the $\log_{10}$-transformed gene copy counts, and PNR and PDR (Littell et al., 2006). The Nrate, CC, and their interaction were considered fixed effects, while the blocks, years, and their interactions with the fixed effects were considered random terms in the analysis of variance (ANOVA). For any significant treatment effects on the response variables in ANOVA, their least-square means were separated by treatment levels, with the *lines* option and setting the probability of a type I error at $\alpha = 0.10$. To assess the correlations among the soil properties, PNR, PDR, and the nine marker genes, their Spearman's rank correlation coefficients were calculated using the R function *cor* with option method = "spearman." Relationships with coefficients (Spearman's rho, ρ) above |0.8| were considered "very strong," those between |0.6–0.8| as "strong," and those between |0.4–0.6| as "moderate," using the ranges from Huang et al. (2019) and setting the Type I error rate at $\alpha = 0.05$. Weak relationships between |0.2–0.4| were disregarded even if they were statistically significant, to make the analysis more selective. The Spearman correlation also included soil pH, NH_4^+, and NO_3^- as their correlations with the functional genes and PNR and PDR represent new information not reported by Kim et al. (2022c). The ggplot2 package in R version 4.1.0 was used to create the figures (Wickham, 2016; R Core Team, 2019).

RESULTS

Responses of Soil Properties and Potential Nitrification Rate and Potential Denitrification Rate to N Rate and Cover Cropping Treatments

Table 1 shows the estimated treatment means, the standard errors of the mean (SEM), sample size (*n*), and the results of mean

separation procedures for each level of Nrate, CC, and their interactions for selected soil properties (CEC, SOM, Pbray, K, Bd, and WAS) at 0–30-cm depth. **Table 1** also shows the degrees of freedom (df) and probability values (*p*-value) associated with the ANOVA for each source of variation. The ANOVA and mean separation results for Nrate and CC main effects and their interaction effects on PNR and PDR are also included in **Table 1**. This study detected statistically significant Nrate main effects on CEC ($p = 0.0098$), Pbray ($p = 0.0209$), and K ($p = 0.0005$). The estimated means for CEC decreased sequentially with higher N rates. The mean Pbray and K decreased significantly with N fertilization, by 75 and 31%, respectively. Meanwhile, PNR and PDR did not have any statistically significant treatment effects. No statistically significant CC main effect nor Nrate × CC interaction effect was detected.

Responses of N-Cycling Functional Genes to N Rate and Cover Cropping Treatments

The estimated means, the SEM, and the mean separation by treatment levels of Nrate, CC, and their interactions for each $\log_{10}$-transformed count of the bacterial and archaeal 16S rRNA, fungal ITS, and the six N-cycling genes are summarized in **Table 2**. The sample size (*n*), df, and *p*-values associated with the results of ANOVA for each source of variation are also shown in **Table 2**. **Figures 2–4** each illustrate the treatment means and the SEM of these genes for the Nrate main effect, CC main effect, and their interaction effect, respectively. The number of bacterial 16S rRNA region copies ranged between 1.95×10^7 and 1.12×10^8 copies/μg DNA, with the average count of 5.89×10^7 copies/μg DNA. The number of archaeal 16S rRNA regions ranged between 2.04×10^5 and 1.73×10^6 copies/μg DNA, with the average count of 6.76×10^5 copies/μg DNA. Bacterial and archaeal 16S rRNA did not have any treatment effect as a significant source of variation. The fungal ITS region counts ranged between 1.05×10^7 and 1.70×10^8 copies/μg DNA and averaged at 4.90×10^7 copies/μg DNA. The fungal ITS region counts had a statistically significant ($p = 0.0499$) Nrate main effect, where the mean counts increased with N fertilization.

The nitrogenase-coding *nifH* gene counts were used to infer the abundance of the N-fixing community, and they ranged between 7.41×10^3 and 7.41×10^5 copies/μg DNA and averaged at 6.31×10^4 copies/μg DNA. This gene had a statistically significant ($p = 0.0278$) Nrate main effect, where its means decreased with N fertilization. The AOA and AOB *amoA* gene counts were each used as proxies for the abundances of AOA and AOB communities, respectively. The AOA *amoA* counts ranged between 7.08×10^3 and 6.46×10^5 copies/μg DNA and averaged at 5.62×10^4 copies/μg DNA. The bacterial *amoA* gene counts ranged between 3.98×10^4 and 3.54×10^6 copies/μg DNA, with an average of 5.25×10^5 copies/μg DNA. AOA *amoA* had a statistically significant ($p = 0.0148$) Nrate main effect and decreased with N fertilization. Statistically marginal ($p = 0.1094$) N rate and CC interaction effects were detected for AOB *amoA*. Within BF, the mean abundance was greater with N202 than N269, with N0 being intermediate, while the means did not differ by N rate within CC; its mean count increased with CC when fertilized at N269. The *nirK* and *nirS* genes that code NO_2^- reductase were both used as proxies for the NO_2^--reducing denitrifier community. The *nirK* counts ranged between 2.51×10^6 and 1.15×10^7 copies/μg DNA, with an average of 4.79×10^6 copies/μg DNA. This gene had a statistically significant ($p = 0.0404$) Nrate and CC interaction effect. The mean counts of *nirK* increased with N fertilization within CC, and they were greater with N202 than N269 within BF, with N0 being intermediate. The *nirS* count ranged between 81 and 3.89×10^5 copies/μg DNA and had an average of 1.20×10^4 copies/μg DNA. It had a statistically significant ($p = 0.0363$) Nrate main effect, so that its mean counts decreased sequentially with higher N rates, with N202 being intermediate. Finally, the N_2O reductase coding *nosZ-I* gene, used to represent the N_2O-reducing community, ranged between 6.03×10^5 and 3.31×10^6 copies/μg DNA with an average of 1.48×10^6 copies/μg DNA. This gene did not have any statistically significant treatment effect.

Correlations Among Gene Counts, Soil Properties, and Potential Nitrification Rate and Potential Denitrification Rate

Supplementary Table 1 shows the Spearman's rank correlation matrix with coefficients among bacterial and archaeal 16S rRNA, and fungal ITS regions, the six N-cycling functional genes, selected soil properties (CEC, pH, SOM, Bd, WAS, NH_4^+, NO_3^-, Pbray, and K), PNR, and PDR. This matrix is also visualized as a heatmap in **Figure 5**. We observed five very strong (|>0.8|), twelve strong (|0.6–0.8|), and 24 moderate (|0.4–0.6|) associations. The bacterial 16S rRNA gene counts had a very strong positive association with those of archaea, followed by moderate positive association with WAS and negative ones with Bd and K. Archaeal 16S rRNA associated moderately positively with archaeal *amoA* and WAS, and negatively with Bd. Fungal ITS region counts are associated strongly and positively with AOB *amoA* and *nosZ-I*, moderately positively with *nirK*, and moderately negatively with soil pH. The N-fixing *nifH* a had strong positive association with AOA *amoA*, *nirS*, pH, and Pbray, and moderately with CEC and K. AOA *amoA* had a very strong positive association with *nirS*, and moderate positive correlations with pH and Pbray. AOB *amoA* had strong positive associations with *nirK* and moderately positive associations with *nosZ-I* and NO_3^-. Denitrifying *nirK* gene counts are associated very strongly and positively with *nosZ-I*, while *nirS* is associated positively with pH strongly, and with CEC, Pbray, and K moderately. Besides the above-mentioned relationships, *nosZ-I* did not have further associations. Other than those already mentioned, CEC is very strongly and positively associated with pH and K, strongly with Pbray, and moderately with Bd and PNR. Soil pH is positively and strongly associated with K and moderately with Pbray and PNR, while having a moderate and negative association with NO_3^-. Meanwhile, SOM had a positive moderate association with NH_4^+. Bd

TABLE 1 | Estimated treatment means, standard errors of the mean values (SEM), and sample size (*n*) of selected soil chemical properties, including cation exchange capacity (CEC, $cmol_c/kg$), soil organic matter (SOM,%), bulk density (Bd, Mg/m^3), water aggregate stability (WAS,%), available phosphorus (Pbray, mg/kg), and extractable potassium (K, mg/kg), along with the potential nitrification (PNR, mg N/kg dry soil day), and denitrification (PDR, ng N/g dry soil hr) rates determined under the N fertilization (Nrate) and cover cropping (CC) treatments, and their interactions.

Treatment*	*n*	CEC	SOM	Bd	WAS	Pbray	K	PNR	PDR
Nrate									
0	12	19.8 a	3.91	1.22	80.7	56.7 a	199 a	50.8	0.96
202	12	17.0 b	4.16	1.28	81.4	17.0 b	142 b	28.6	0.87
269	12	15.4 c	4.65	1.18	78.9	11.4 b	133 b	23.1	0.61
SEM		1.25	0.31	0.05	6.12	8.15	15.8	13.2	0.32
CC									
BF	18	17.2	4.04	1.22	79.3	28.4	158	23.9	0.74
CC	18	17.6	4.44	1.23	81.4	28.4	158	44.4	0.89
SEM		1.23	0.27	0.05	5.95	5.54	15.2	10.6	0.30
Nrate × CC									
0BF	6	19.7	3.94	1.20	80.5	58.3	204	47.0	0.76
0CC	6	19.9	3.89	1.23	80.9	55.2	194	54.6	1.17
202BF	6	16.8	4.11	1.27	80.8	15.5	138	9.0	1.04
202CC	6	17.3	4.20	1.28	82.0	18.5	146	48.1	0.70
269BF	6	15.0	4.07	1.18	76.7	11.3	132	15.7	0.43
269CC	6	15.7	5.23	1.18	81.2	11.5	133	30.4	0.80
SEM		1.28	0.43	0.05	6.28	8.40	16.93	15.0	0.40
Sources of variation	**df**	**CEC**	**SOM**	**Bd**	**WAS**	**Pbray**	**K**	**PNR**	**PDR**
Nrate	2	*0.0098*	0.2997	0.2573	0.7338	*0.0209*	*0.0005*	0.3436	0.5711
CC	1	0.1906	0.2790	0.7142	0.4138	1.0000	0.9746	0.2549	0.6738
Nrate × CC	2	0.8046	0.3437	0.5962	0.4923	0.5861	0.5344	0.2844	0.4813

The degrees of freedom (df) and the probability values (p-values) associated with the analysis of variance (ANOVA) results are shown below. For each treatment and within a given column, mean values followed by the same lowercase letter were not statistically different ($\alpha = 0.10$).
**Nrate treatment levels: 0, 202, and 269 kg N/ha. CC treatment levels: bare fallow controls (BF) and hairy vetch and cereal rye cover crop mixture (CC).*

had a very strong negative correlation with WAS. Pbray and K had a strong positive correlation, and K had a positive moderate association with PNR. In addition, there were 16 statistically significant ($p < 0.05$) but weak ($p < |0.4|$) correlations (**Supplementary Table 1**).

DISCUSSION

This study observed that CC had little impact on the soil environment of an intensely managed and simplified cropping system; thus, the soil properties remained stable since Kim et al. (2022b) before CC introduction. Higher N rates decreased CEC, which Kim et al. (2022b) explained, was due to the increasing soil acidity from the nitrifying N fertilizers and increased crop root uptakes (Tang and Rengel, 2003), leading to loss of exchange sites within soil particles (Barak et al., 1997). N fertilization also significantly depleted the soil available P and K *via* increased crop uptake. Before CC, Kim et al. (2022b) reported a statistically significant N rate main effect on Bd, but this study could not detect this. These contrasting results may owe to the thicker layer of topsoil analyzed in this study (30 cm) than in the previous study (15 cm). Similar to Kim et al. (2022b), SOM and WAS did not differ significantly by N rate. Indeed, Necpálová et al. (2014) estimated that statistically detectable changes in SOM of resilient Mollisols like in this study would take several decades. As for CC, only Kim et al. (2022c) found that CC decreased NO_3^- in this experimental site. Similar results have been observed by Acuña and Villamil (2014), also in Illinois, where CC only affected NO_3^- among various soil properties. Before CC, Kim et al. (2022b) demonstrated soil acidification and high nutrient availability as the main results of long-term N fertilization and corn monoculture over fertile United States Midwestern soils. Overall, in addition to the innate resilience of the Mollisols of this region, this study suggests that these soils may have become resistant to conservation practices after long-term exposure to disruptive practices. Thus, desired improvements from deploying CC may take more time and effort. The marginal CC effects on N-cycling communities and their functionality in this study may have stemmed from this resistant soil environment.

Consistent with Huang et al. (2019), this study found a statistically significant N rate's main effect on fungal ITS counts, while bacteria and archaea did not respond to treatments. In both studies, fungal ITS region counts increased with N fertilization. This result agrees with the fungal species richness that also increased with N input in this site (Kim et al., 2022c). The fungal community may have benefited from N fertilization due to the stoichiometric differences between fungi and bacteria. Because fungal biomass has a greater C:N ratio than bacteria, fungi might benefit from N fertilizers increasing the returns of corn residues high in C:N ratio (Strickland and Rousk, 2010).

TABLE 2 | Estimated treatment means and standard errors of the mean (SEM) of the log_{10}-transformed copies (per μg DNA) of microbial marker genes, including bacterial (Bacteria) and archaeal (Archaea) 16S rRNA, fungal ITS region (Fungi), *nifH*, archaeal (AOA) and bacterial (AOB) *amoA*, *nirK*, *nirS*, and *nosZ-I* determined under N fertilization (Nrate), Cover crop (CC) treatments, and their interactions.

Treatment*	*n*	Bacteria	Archaea	Fungi	*nifH*	AOA	AOB	*nirK*	*nirS*	*nosZ-I*
Nrate										
0	12	7.75	5.88	7.41 b	5.40 a	5.21 a	5.54	6.64 b	4.90 a	6.07
202	12	7.81	5.85	7.84 a	4.62 b	4.66 b	5.95	6.76 a	4.04 ab	6.25
269	12	7.75	5.75	7.82 a	4.38 b	4.39 b	5.66	6.66 b	3.31 b	6.20
SEM		0.09	0.12	0.11	0.17	0.19	0.14	0.06	0.35	0.08
CC										
BF	18	7.76	5.84	7.65	4.74	4.72	5.70	6.66	3.81	6.15
CC	18	7.77	5.81	7.73	4.86	4.78	5.74	6.71	4.36	6.20
SEM		0.08	0.12	0.10	0.11	0.17	0.11	0.06	0.29	0.07
Nrate × CC										
0BF	6	7.72	5.82	7.46	5.37	5.13	5.60 abc	6.69 ab	4.84	6.12
0CC	6	7.78	5.94	7.35	5.44	5.28	5.48 bc	6.58 b	4.95	6.02
202BF	6	7.82	5.89	7.76	4.59	4.57	6.02 a	6.72 a	3.63	6.20
202CC	6	7.79	5.80	7.92	4.65	4.75	5.89 abc	6.79 a	4.45	6.29
269BF	6	7.75	5.82	7.71	4.26	4.47	5.47 c	6.56 b	2.94	6.11
269CC	6	7.75	5.69	7.93	4.50	4.31	5.85 ab	6.75 a	3.67	6.28
SEM		0.11	0.14	0.16	0.19	0.21	0.17	0.07	0.44	0.11
Sources of Variation	**df**	**Bacteria**	**Archaea**	**Fungi**	***nifH***	**AOA**	**AOB**	***nirK***	***nirS***	***nosZ-I***
Nrate	2	0.7685	0.3385	*0.0499*	*0.0278*	*0.0148*	0.2470	*0.0916*	*0.0363*	0.3361
CC	1	0.9054	0.5570	0.5333	0.2652	0.6824	0.7149	0.3611	0.1494	0.5367
Nrate × CC	2	0.8524	0.1309	0.5464	0.7333	0.3086	*0.1094*	*0.0404*	0.5373	0.4183

The sample size (n), degrees of freedom (df), and the probability values (p-values) associated with the analysis of variance (ANOVA) results are shown below. For each treatment and within a given column, mean values followed by the same lowercase letter were not statistically different (α = 0.10).
**Nrate treatment levels: 0, 202, and 269 kg N/ha. CC treatment levels: bare fallow controls (BF) and hairy vetch and cereal rye cover crop mixture (CC).*

Furthermore, fungi generally tolerate soil acidity better than bacteria, which allows them to take advantage of the acidifying soils from prolonged N fertilization (Strickland and Rousk, 2010; Kim et al., 2022c). Indeed, the ITS counts correlated negatively with soil pH. Another study in Illinois Mollisols on the effects of crop rotation and tillage also showed similar results, where only fungal abundance responded to crop rotation, due to soil acidification from corn monoculture (Behnke et al., 2020). Meanwhile, the bacterial and archaeal 16S counts had a strong monotonic relationship and correlated negatively and positively with Bd and WAS, respectively. Indeed, less dense soil and more stable soil aggregates are associated with higher soil carbon (C; Trivedi et al., 2017), wherein the soil's microbial growth depends on (Kamble and Bååth, 2018). These results suggest that bacterial and archaeal abundances might depend more on these soil's physical properties and perhaps soil C, than on N fertilizers and CC.

Responses of the Soil N-Cycling Functional Genes

Detrimental Effect of N Fertilization on N-Fixing Community

As hypothesized, *nifH* decreased in abundance with N fertilization, consistent with the report before CC introduction (Huang et al., 2019). Similarly, a continuous wheat study of 22 years on the effects of N and P fertilization in Saskatchewan Mollisols also found that N fertilization decreased *nifH* and bacterial N-fixation (Li et al., 2020). Also, in a semi-arid grassland study in China, testing 3 years of N application (0, 25, 50, and 100 kg N/ha), the authors found that *nifH* initially increased in abundance at a low N rate but decreased sequentially as the N rate increased (Liao et al., 2021). High N availability from fertilizers mitigates the plant dependence on microbial N-fixers and disincentivizes their recruitment, which may explain why *nifH* decreased with higher N rates in this study (Liao et al., 2021). In addition, soil acidification is known to repress N-fixation (Bakari et al., 2020; Zhang et al., 2021), which also agrees with its strong positive association with soil pH. Therefore, soil acidification from excessive N fertilization could also be a factor. Kim et al. (2022c) reported that the genus *Mesorhizobium*, which includes nodule-forming N-fixers, increased in abundance with CC. Accordingly, this study hypothesized that this would reflect on the *nifH* counts. However, CC was not a significant source of variation for *nifH*. Indeed, *Mesorhizobium* also includes NO_2^- reducers (Okada et al., 2005). Perhaps, Kim et al. (2022c) detected this genus not as an indicator of N-fixers but of denitrifiers. There have been studies reporting either positive (Castellano-Hinojosa et al., 2022) or insignificant (Hu et al., 2021) CC effects on *nifH*. The authors explained that the non-legume CC in the mixture depletes soil N, thus, encouraging N-fixation to meet the legume's N demand (Castellano-Hinojosa et al., 2022). If this also applies to corn monoculture, this study might have not detected a

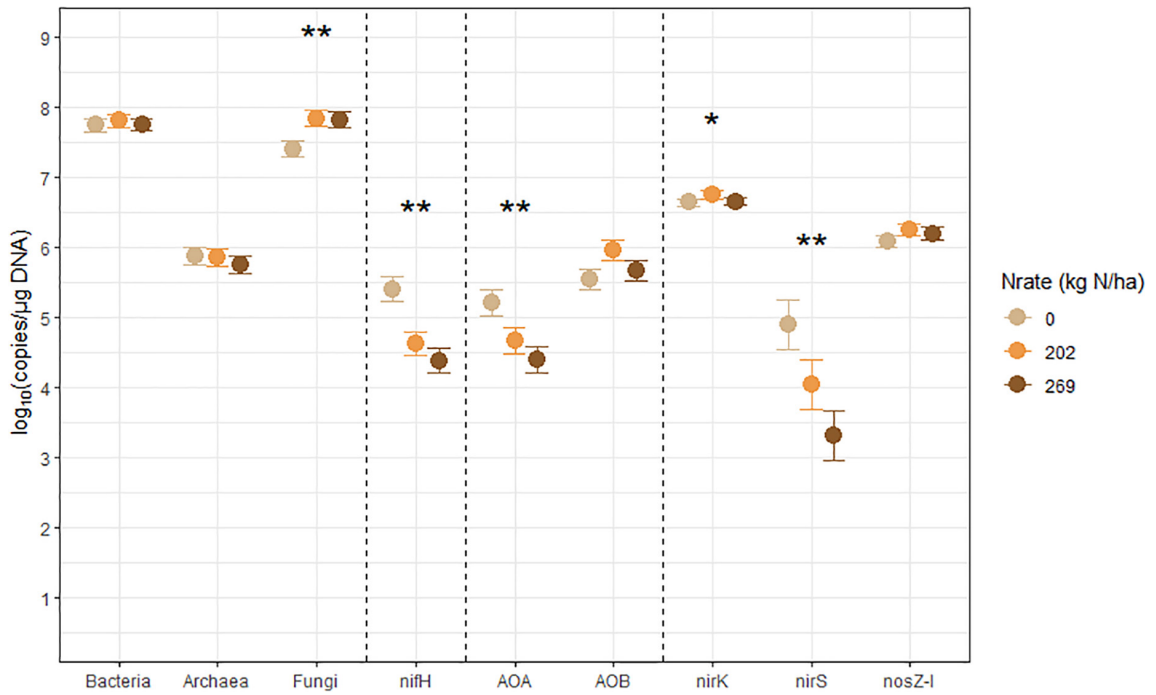

FIGURE 2 | Treatment means of soil microbial marker genes (log_{10} copies/µg DNA), including bacterial (Bacteria) and archaeal (Archaea) 16S rRNA, fungal ITS region (Fungi), *nifH*, archaeal (AOA) and bacterial (AOB) *amoA*, *nirK*, *nirS*, and *nosZ-I* separated by N fertilization rate (Nrate), with their standard errors of the mean as whiskers. Asterisks indicate the level of significance associated with the probability value from analysis of variance of the factors (*$p < 0.1$, **$p < 0.05$). Nrate treatment levels were: 0, 202, and 269 kg N/ha.

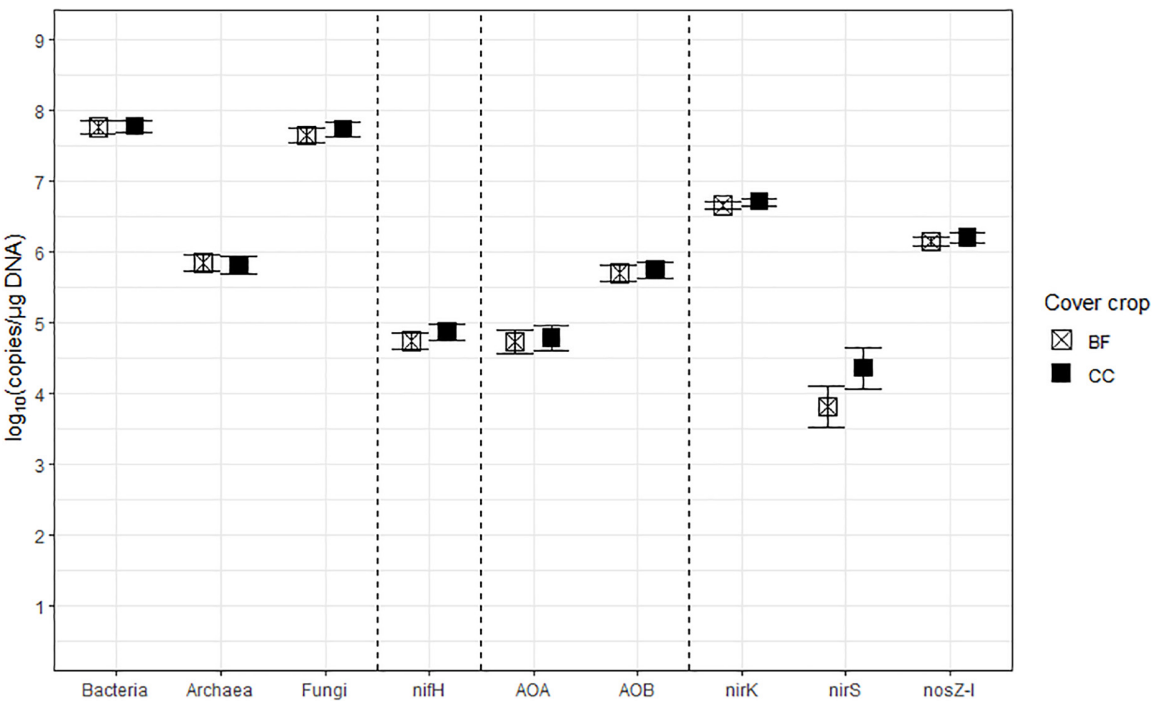

FIGURE 3 | The treatment means of soil microbial marker genes in log_{10} (copies/µg DNA), including bacterial (Bacteria) and archaeal (Archaea) 16S rRNA, fungal ITS region (Fungi), *nifH*, archaeal (AOA) and bacterial (AOB) *amoA*, *nirK*, *nirS*, and *nosZ-I* separated by cover cropping (CC), with their standard errors of the mean as whiskers. The CC treatment levels were bare fallow control (BF) and hairy vetch and cereal rye cover crop mixture (CC).

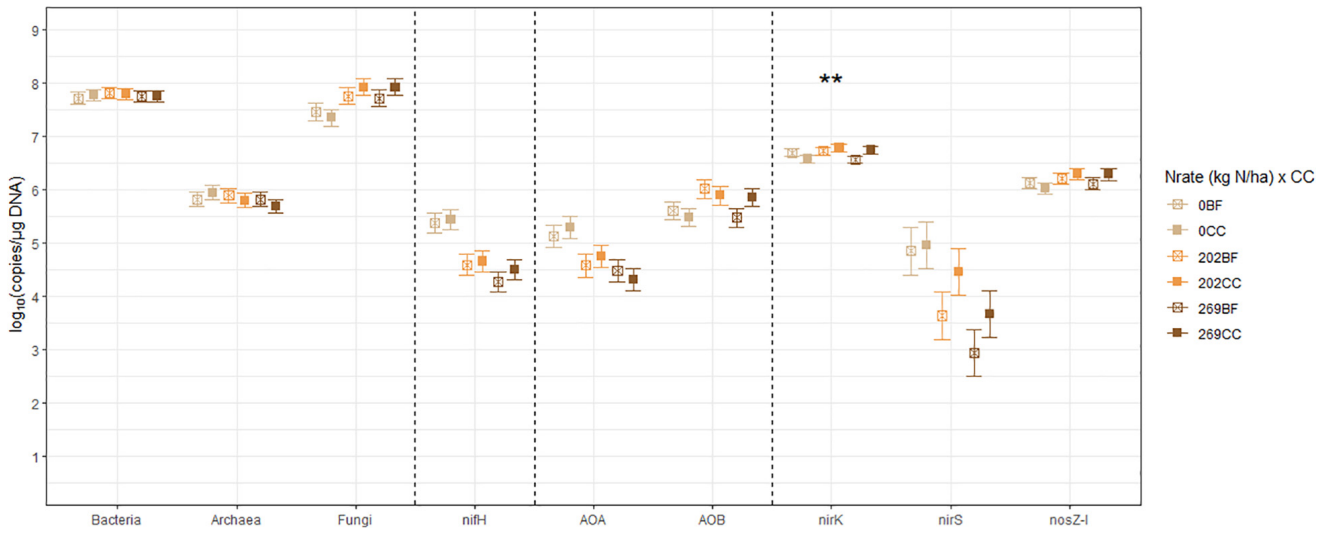

FIGURE 4 | The treatment means of soil microbial marker genes in (log_{10} copies/µg DNA), including bacterial (Bacteria) and archaeal (Archaea) 16S rRNA, fungal ITS region (Fungi), *nifH*, archaeal (AOA) and bacterial (AOB) *amoA*, *nirK*, *nirS*, and *nosZ-I* separated by the interactions between N fertilization rate (Nrate) and cover cropping (CC), with their standard errors of the mean as whiskers. The asterisks indicate the probability value of the treatment effect from analysis of variance (**$p < 0.05$). Nrate treatment levels were: 0, 202, and 269 kg N/ha. CC treatment levels were bare fallow control (BF) and hairy vetch and cereal rye cover crop mixture (CC).

significant CC effect on *nifH* because heavy fertilization overrode the N depletion by non-legume CC.

Ammonia-Oxidizing Archaea and Ammonia-Oxidizing Bacteria Responded Differently to Treatments (*amoA*)

In this study, a higher N rate sequentially decreased the abundance of AOA *amoA*. This agreed with Kim et al. (2022c), who also found that N fertilization decreased the abundance of *Nitrososphaera*, a genus, including neutrophilic AOA. Furthermore, AOA *amoA* correlated positively with soil pH. These results implied that AOA responded negatively to soil acidification, to which they may have contributed as nitrifiers themselves (Bolan and Hedley, 2003). Sun et al. (2019) studied the relationships between various soil properties and nitrifier communities in long-term fertilized, slightly acidic to neutral Chinese Vertisols (Wei et al., 2018). Similar to this study, the authors reported that AOA correlated the most with soil pH and decreased with soil acidification. AOA may adapt and form communities of distinct compositions at different soil pH (Hirsch and Mauchline, 2015). Thus, responses of these distinct AOA communities to changes in the soil conditions may vary by the initial soil pH they adapted to. For example, AOA is better adapted to acidic soils than AOB (Zhang et al., 2012) and can respond positively to N inputs in this condition (Gubry-Rangin et al., 2010). Yet, another AOA community that is initially adapted to more neutral soils may respond negatively to N inputs, like on Sun et al. (2019). Therefore, this study further suggested that soil acidity may have a detrimental impact on the AOA community that adapted to the slightly acidic to neutral soils typical to the US Midwest region. Meanwhile, CC did not have a significant impact on AOA *amoA*. This also agrees with Kim et al. (2022c), who reported no AOA, as indicators, is associated with CC. Castellano-Hinojosa et al. (2022) observed that legume and non-legume CC mixture increased AOA *amoA* in the citrus orchard, but this may not be the case for corn monoculture. A greenhouse study on Argentinian Mollisols by Allegrini et al. (2021) compared the nitrifier community responses to different CC termination methods. They found that AOA *amoA* increased with mechanical CC termination compared to chemical (glyphosate) termination and control without CC, which they explained that glyphosate can hamper the growth of AOA (Allegrini et al., 2021). Perhaps, chemical CC termination masked CC effects on AOA in this study as well, which might be worthwhile investigating in a field study.

According to the findings of Huang et al. (2019), this study hypothesized that AOB *amoA* will increase in abundance with higher N rates. Instead, the mean separations between fertilized and unfertilized control within CC were not statistically significant for AOB *amoA* in this study. Moreover, under bare fallow, AOB *amoA* mean counts did not differ statistically and significantly between fertilized plots and the unfertilized control. Overall, these results suggested that the AOB of this system was not as sensitive as AOA to N fertilization and its subsequent soil acidification. Indeed, past reports showed that AOB responds more sensitively to nutrient availability than soil pH. The meta-analysis by Ouyang et al. (2018) showed that AOB *amoA*, consistently, had positive effect sizes with N fertilization regardless of soil pH, while AOA *amoA* had an insignificant effect size below pH 6. Sun et al. (2019) also reported that soil acidification rather increased AOB abundance, although at the cost of their diversity. A microcosm study by Hink et al. (2018) showed that while AOA preferred a low rate of N inputs from organic sources, AOB increased in abundance with high rate of

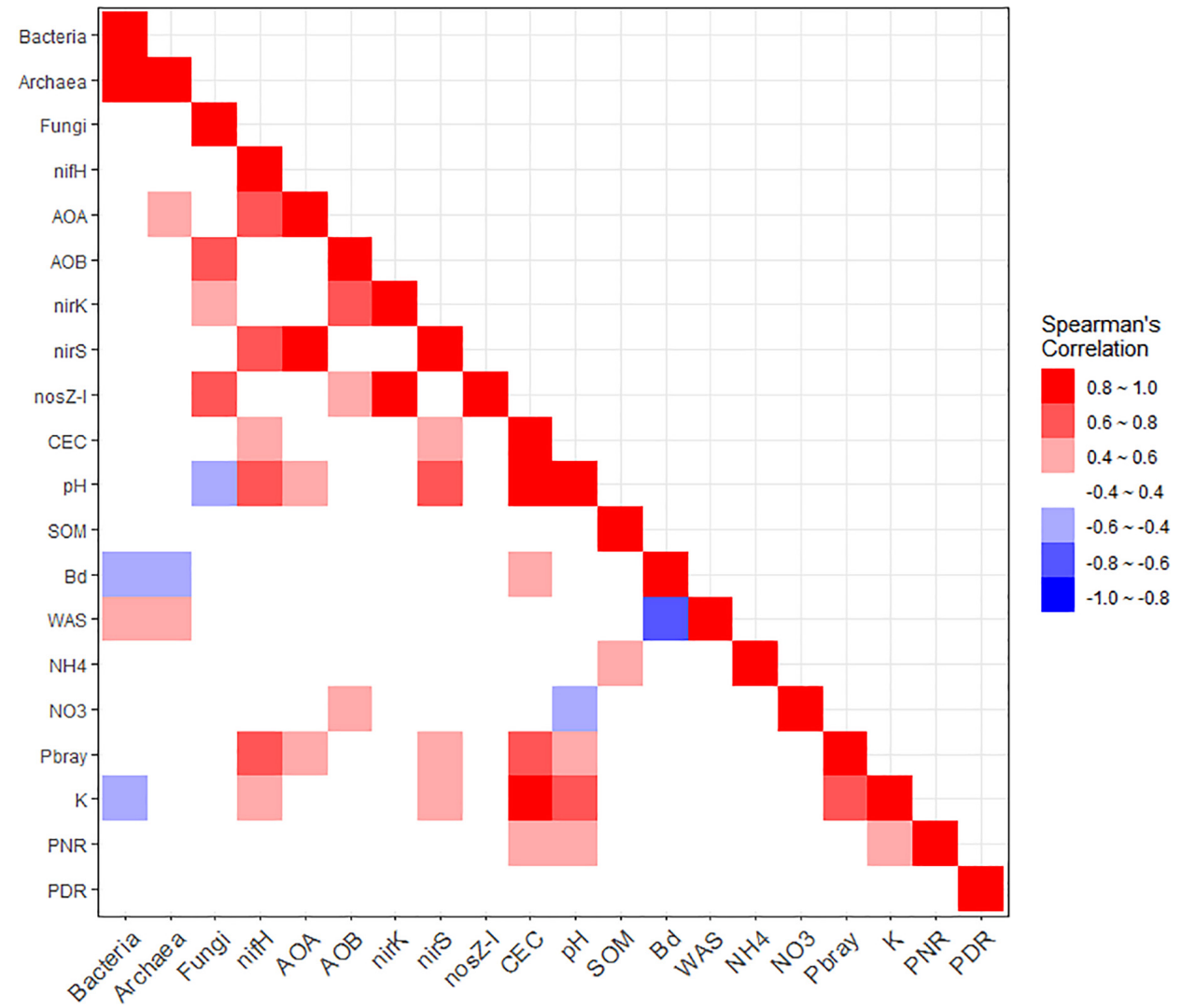

FIGURE 5 | Heatmap depicting the matrix of Spearman's rank correlation coefficients among the soil microbial marker genes, soil properties, and potential nitrification (PNR) and denitrification (PDR) rates. Marker genes included bacterial (Bacteria) and archaeal (Archaea) 16S rRNA, fungal ITS region (Fungi), *nifH*, archaeal (AOA) and bacterial (AOB) *amoA*, *nirK*, *nirS*, and *nosZ-I*. Soil properties included cation exchange capacity (CEC, $cmol_c/kg$), pH, soil organic matter (SOM,%), bulk density (Bd, Mg/m^3), water aggregate stability (WAS,%), ammonium (NH_4, mg/kg), nitrate (NO_3, mg/kg), available phosphorus (Pbray, mg/kg), and extractable potassium (K, mg/kg). The red and blue hues each indicate positive and negative associations, respectively. Higher color saturation indicates greater absolute values of Spearman's rank correlation coefficients.

inorganic N inputs, showing that AOB may better exploit the inorganic N from fertilizers than AOA. Sun et al. (2019) suggested that the AOB community may occupy broader niches than AOA because they have more ecophysiological diversity. Perhaps, this ecological versatility allows AOB to adapt to acidifying soils and maintain their abundance. Also, Sun et al. (2019) showed that AOB significantly correlated with soil C. Thus, labile C from CC as root exudates and residues could explain why AOB *amoA* was more abundant with CC compared to bare fallow when fertilized at the highest N rate (Wang et al., 2021).

Despite a significant decrease in AOA *amoA* with a higher N rate, PNR did not differ by treatments in this study. While reports on PNR from similar cropping systems are scarce, a study from Northeastern Chinese Alfisols under soybean-corn-corn rotation found that PNR decreased with higher N rates (Xu et al., 2012). Another study from Kentucky Alfisols with corn monoculture and grass CC without bare fallow control found that PNR increase with N fertilization (Liu et al., 2017). Thus, currently, there is no consensus on how N fertilization affects PNR. The N rate effect on PNR may have not been statistically significant because stable AOB population size may have masked the decreasing contributions from AOA. Indeed, AOB *amoA* was 9.33 times more abundant than AOA *amoA* on average. Also, while AOA *amoA* abundance differed up to 15.13 times by treatment levels, AOB *amoA* only did so by 3.55 times. Likewise, the topsoil NO_3^- level had a statistically significant

positive correlation with AOB *amoA*, suggesting that AOB could have mainly driven the nitrification in this system. Meanwhile, there currently is no report on how PNR responds to CC in systems comparable to this study. While this study found no significant effects, further research will have to accumulate on this relationship.

Distinct Responses Between *nirK*- and *nirS*-Harboring Nitrite-Reducing Groups

In this study, *nirK* and *nirS* responded differently to the treatments. While N fertilization consistently decreased *nirS*, it slightly increased *nirK* within CC and had no difference from unfertilized control within bare fallow. These responses of *nirK* and *nirS* were analogous to AOB and AOA *amoA*, respectively, but the *nirK* differences by N rates within CC were statistically significant. Indeed, past reports agree with this study that N fertilization effects on *nirK* tend to range from insignificant to positive, while they are negative for *nirS* (Yin et al., 2014; Yang et al., 2017). For example, Yang et al. (2017) on a wheat-corn rotation system over alkaline soils of northern China reported that a higher N rate increased the abundances of *nirK* but decreased that of *nirS*. Yin et al. (2014) on soybean-corn-corn rotation observed that inorganic fertilizers decreased the abundances of *nirS* but had no impact on *nirK*. These genes may be responding differently to each other because the denitrifier guilds that each harbor adapt differently to soil acidification, similar to the *amoA*-nitrifiers discussed above. Indeed, this study observed that soil pH correlated strongly and positively with *nirS*, but not with *nirK*, which agreed with the past reports (Čuhel et al., 2010; Yang et al., 2017; Bowen et al., 2018). For example, Čuhel et al. (2010), on the effects of soil pH changes in pasture systems over Czech Mollisols, showed that *nirS* increased in abundance from acidic to alkaline soils, while *nirK* did not differ by soil acidity. Therefore, soil acidification from N fertilization in this study's system may negatively impact the *nirS*-harboring denitrifiers. Also, Yang et al. (2017) showed that the abundance of *nirS* correlated significantly with the soil pH, while *nirK* did so with soil inorganic N levels. Thus, the *nirK*-harboring denitrifiers might be better adapted to N fertilizers, although this was only detected within CC in this study. Furthermore, fungi hold significant shares of the *nirK* community. For example, Xu et al. (2019) found that the fungal *nirK*-harboring community may contribute up to 50% of the N_2O production during corn crops over black soils of Northeast China. Indeed, the present study observed statistically significant monotonic relationships among fungal ITS and *nirK*. As discussed earlier, fungi have better tolerance for soil acidity, which could have factored into *nirK* having less sensitivity to soil pH (Bowen et al., 2018).

This study hypothesized that CC would increase *nirK* but have no effect on *nirS*, based on the bioindicators of Kim et al. (2022c) that included *nirK*-harboring NO_2^--reducers (*Mesorhizobium* and *Luteimonas*), but not *nirS* (Falk et al., 2010; Yin et al., 2014; Zhong et al., 2020). This hypothesis somewhat held true as *nirS* did not respond to CC, while *nirK* increased in abundance with CC under the context of N fertilization. However, past studies reported that CC tends to benefit both genes. The microcosm study by Wang et al. (2021) showed that ryegrass CC increased the abundances of *nirK* and *nirS* regardless of N rate (0 to 200 kg N/ha). They attributed these results to the labile C from CC root exudates and residues that generally promote the soil microbial community, including the denitrifiers. Similarly, Castellano-Hinojosa et al. (2022), on citrus orchards, found that CC increased the abundance of both genes. Still, their systems are very different from that of this study. Thus, the primary information on how *nirK* and *nirS* respond to CC within the typical corn-based cropping systems is critically lacking. In this study, *nirS* did have higher mean counts with CC than bare fallow control, but the CC main effect was still not statistically significant. Overall, this study found that *nirK* of this system may increase with CC when heavily fertilized, which also agreed with the bioindicators found by Kim et al. (2022c). However, whether *nirS* also responds positively to CC in this system will need further investigation.

Similar to nitrification, PDR did not have a statistically significant treatment effect, yet this result contrasts with past reports on PDR that increased with N fertilization and CC (Foltz et al., 2021; Li et al., 2022). Similar to *amoA* communities, *nirK* was much more abundant (nearly 400 times) than *nirS*, which decreased significantly with N fertilization. Also, *nirK* was more resistant to change than *nirS*, only differing up to 1.55 times in abundance by treatment levels compared to *nirS* that differed more than a hundred times. Maeda et al. (2017) reported that *nirK*-denitrifiers were more responsible for N_2O emission in a compost pile despite their smaller number than *nirS*. Meanwhile, Liang et al. (2021) showed that denitrification was associated more with the *nirS* community because it was more responsive to climate factors and soil pH. Therefore, the lack of statistically significant responses from PDR could be reflecting both the dominant *nirK* contribution to PDR and sensitive changes in *nirS* communities to the treatments. Yet, this is beyond the insight that PDR can provide overall; moreover, nitrifier denitrification complicates this even more. Therefore, further investigation should determine how much each nitrifier and denitrifiers contributes to PDR, by using inhibitors of specific groups, for example.

N_2O-Reducing Denitrifiers Unresponsive to N Fertilization and Cover Cropping (*nosZ-I*)

Consistent with past reports from this study's site (Huang et al., 2019), the treatments had no statistically significant effect on *nosZ-I*. Conversely, Kim et al. (2022c) identified *Gemmatirosa* as a *nosZ*-harboring bioindicator that decreased with CC. Indeed, the denitrifiers in this genus belong to the *nosZ-II* clade (Xu et al., 2020), implying that *nosZ-I* and *nosZ-II* may respond differently to treatments in this system. This could be because these denitrifier groups each perform different soil functions and occupies distinct niches (Shan et al., 2021). Bowen et al. (2018) reported that the *nosZ-II* clade might be more sensitive to soil properties like pH than *nosZ-I*. Also, Wang et al. (2021) showed that the presence of ryegrass CC and high N rate increased the abundance of the *nosZ-I* clade, while *nosZ-II* did not respond to the treatments. Possibly, *nosZ-II* may have responded to N

fertilizers and CC in this study, but this gene was not analyzed in this study. Therefore, future research should include *nosZ-II* for a more complete picture of how the N_2O-reducers respond to CC in this system. Meanwhile, although high-rate N fertilization might not change the abundance of *nosZ*, soil acidification can interfere with the functionality of its product, N_2O reductase, potentially exacerbating N_2O emission (Qu et al., 2014). Thus, further investigation on the transcripts or enzyme activity of these genes might be necessary to determine how N_2O reduction itself responds to CC in this system.

CONCLUSION

This study is the first to determine how the soil N-cycling microbial community responds to cover crops in highly fertile, yet intensely manage cropping systems that are common to critical agricultural regions like the United States Midwest. Overall, the results on functional genes, the measured PNR and denitrification rates, and soil properties were consistent with each other without unexplainable discrepancies. Excessive N fertilization disrupts the N-fixer, AOA, and *nirS*-denitrifier communities, likely due to their sensitivity to soil acidification and a disincentivized plant-symbiosis for the N-fixers. Conversely, AOB and denitrifiers harboring *nirK* and *nosZ-I* responded subtly to N fertilization, likely because they are less sensitive to soil acidity or prefer high soil nutrient availability. Therefore, adaptation to soil acidification and nutrient availability from excessive N fertilization might be the discerning factor for the N-cycling communities in this system. Moreover, as in the cases of AOA *amoA* and *nirK*, some functional genes mirrored the previously reported genus-level bioindicators of the same site that are also known to harbor the respective genes. Thus, this study further supported the high-taxonomic resolution bioindicators as useful predictors of the soil microbial functions, although further investigation and reproduction of these results should follow. Despite these changes in the N-cycling communities, the enzyme assays did not respond to treatments, showing that overall functionality could be more resilient to changes. Contrary to the initial hypothesis, CC had a limited impact on the soil properties, N-cycling communities, and their functionality. This suggested that the soil environment and its N-cycling communities became resistant to changes after decades-long adaptation to consistent disruptions from heavy N fertilization and corn monocultures. Therefore, short-term CC may not be enough to improve heavily disrupted N-cycling communities. Further research should expand on this study with long-term CC, different cropping systems, and more functional genes for a more complete understanding of cover cropping as a sustainable practice to mitigate soil degradation and nutrient loss.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MV and NK: conceptualization and formal analysis. NK, CR, MZ, MV, and SR-Z: methodology. MV, SR-Z, and CR: resources. MV, MZ, CR, and NK: data curation. NK: visualization and writing—original draft preparation. MV and MZ: writing—review and editing. MV: supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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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.2022.926592/full#supplementary-material

Supplementary Table 1 | Spearman rank correlation coefficients for bacterial and archaeal 16S rRNA, fungal ITS, *nifH*, AOA and AOB *amoA*, *nirK*, *nirS*, *nosZ-I*, estimated cation exchange capacity (CEC), pH, soil organic matter (SOM), bulk density (Bd), water aggregate stability (WAS), ammonium (NH_4), nitrate (NO_3), available phosphorus (Pbray), potassium (K), and potential nitrification (PNR) and denitrification (PDR) rates. Statistically significant coefficients ($p < 0.05$) are in bold. Cells are colored based on their coefficients, with red being positive and blue being negative, and darker the color the greater the value.

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Transfer of Nitrogen and Phosphorus From Cattle Manure to Soil and Oats Under Simulative Cattle Manure Deposition

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Simulated cattle manure deposition was used to estimate nutrient transfer to soil and oats and to investigate changes in microbial community composition and functional groups in oat rhizospheres. Nutrient absorption and return efficiency were calculated as a series of standard calculation formulas, and total nutrient transfer efficiency was nutrient absorption efficiency plus nutrient return efficiency. In total, 74.83% of nitrogen (N) and 59.30% of phosphorus (P) in cattle manure were transferred to soil and oats, with 11.79% of N and 7.89% of P in cattle manure absorbed by oats, and the remainder sequestered in the soil for 80 days after sowing. Cattle manure increased oat root length, surface, and volume under 0.2 mm diameter, and improved relative abundance of the microbiome known to be beneficial. In response to cattle manure, several bacteria known to be beneficial, such as Proteobacteria, Bacteroidota, and Firmicutes at phyla the level and *Pseudoxanthomonas*, *Pseudomonas,* and *Sphingomonas* at the genus level, were positively related to oat biomass and nutrient accumulation. For fungal communities, the relative abundance of Ascomycota is the predominant phylum, which varied in a larger range in the control treatment (81.0–63.3%) than the cattle manure deposition treatment (37.0–42.9%) as plant growing days extend. The relevant abundance of Basidiomycota known as decomposer was higher in cattle manure deposition treatment compared to that in control treatment at 15 days after sowing. More importantly, cattle manure deposition inhibited trophic mode within pathotroph like *Alternaria* and *Fusarium* fungal genus and promoted saprotroph and symbiotroph.

Keywords: cattle manure deposition, nutrient return, root length, bacteria, fungi

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INTRODUCTION

In native grassland ecosystems, soil N and P are absorbed to support plant growth, and 30–50% of above-ground plant biomass is consumed by grazing animals (e.g., cattle), with portions of N and P returned to the soil in excreted wastes (Yoshitake et al., 2014). Appropriate application of animal manure and its subsequent decomposition provide highly plant-available nutrients that

stimulate plant production (Van der Waal et al., 2011). In cattle feces, the N concentration ranges from 18.0 to 26.2 g kg^{-1} (dry matter basis; Sordi et al., 2014), with 80% of total consumed plant N subsequently deposited on soil in cattle feces (Cardenas et al., 2016). Although the amount of N and P from plant to cattle is easily evaluated, the amount of N and P from cattle manure to soils is not as well evaluated. Appropriate application of cattle manure to grasslands can maintain soil quality (Pätzhold et al., 2013), whereas excessive applications cause substantial reactive N and P, with volatilization and environmental contamination (Kumar et al., 2017).

Large amounts of organic matter from cattle dung provide energy sources and beneficial rhizospheric conditions for the growth of the microbiome (Chaparro et al., 2012; Poeplau and Don, 2015). In this situation, soil microbiome degrades macromolecular organic matters, promoting microbial nutrient recycling and energy flowing in terrestrial ecosystems (Rosinger et al., 2019). These changes in grassland promoted the catalyzers of nutrient cycling such as carbon sequestration (Smith et al., 2015), ammonia oxidation (Prosser and Nicol, 2012), and phosphorus transformation (Parniske, 2008).

Rhizosphere microbes are closely associated with soil nutrition and plant growth (Kumar et al., 2016). Microbial communities are species-specific in the rhizosphere environment, as plant roots acquire specific microorganisms from soil (Hein et al., 2008). These species-specific microorganisms have high functional diversity (Escalas et al., 2019) and drive soil functions at specific time points (Bastida et al., 2016). Plant rhizosphere microbial communities are influenced by numerous factors, mainly plant development (Schlemper et al., 2017) and soil nutrient status (Meena et al., 2017). As plants develop, they release specific metabolites to select microorganisms, presumably to support specific functions (Chaparro et al., 2014; Zwetsloot et al., 2020) and perhaps in association with changes in nutrient requirements. For example, legumes release flavones to enhance rhizobia-legume symbiosis as plants usually require more N later in development (Zhang et al., 2009). However, it was previously reported that microbial communities are modified by fertilization, with decreased dependence of the microbiome on root exudates (Ai et al., 2015; Zhao et al., 2019, 2020). Cow manure provides the main source of energy for microbes as it contains a lot of labile carbon that can stimulate microbial aggregation, activity, and diversity (Delgado-Baquerizo et al., 2016). Subsequently, the microbiome decomposed the organic matter in cow manure and increased nutrient availability (Jacoby et al., 2017).

Applying cattle manure and incorporating it into the soil has been widely investigated in agriculture ecosystem (Sha et al., 2012). Rhizosphere microbes, e.g., autotrophic archaea oxidize ammonium ($NH4^+$) to nitrate ($NO3^-$) by nitrification (Leininger et al., 2006; Chen et al., 2015), whereas the rhizosphere core microbiome, *Acidobacteria* and *Sphingobacteriales*, are involved in N cycling (Hester et al., 2018). Arbuscular mycorrhizal fungi (AMF) can access P even in root P-depletion zones (Walder and Heijden, 2015). Moreover, plant beneficial rhizospheric microorganisms or plant growth-promoting microorganisms, such as *Azospirillum, Enterobacter, Pseudomonas, Klebsiella, Serratia,* and *Pantoea* species, were associated with plant growth in a variety of grass species (Lange et al., 2015). The surface application of cattle manure affects functional microbes, e.g., autotrophic and heterotrophic nitrifiers (Wu et al., 2020). Applying cattle manure to grassland soils can cause rapid changes to the availability of N and P (Geisseler and Scow, 2014) which stimulate microbes, especially those related to ammonia oxidation (Mooshammer et al., 2014) and phosphorus transfer (Richardson et al., 2009). However, fecal N could be lost because of ammonia volatilization and leaching during the growing season (Maris et al., 2021). Ammonia volatilization is the main loss pathway for N from cattle dung deposited on grassland influenced by multiple factors such as temperature, rainfall, and wind speed (Fischer et al., 2015). Generally, dry weather conditions will increase the volatilization of N (Meisinger and Jokela, 2000). Besides, rainfall increases t N leaching and subsequent denitrification of nitrate (NO_3^-) (Barger et al., 2013). Characterizing dynamics of microbial community composition, especially specific functional microbial groups, in response to the application of cattle manure, may provide new knowledge to optimize plant growth at various stages.

The root system connects the plant and soil and enables the absorption of nutrients and water in grassland ecosystems (Zhou et al., 2018). Roots are covered by highly complex rhizosphere microorganisms (Wilson, 2014). Rhizosphere, defined as the volume around living roots, is one of the most dynamic zones for nutrient influx (Sasse et al., 2017). As roots develop, vigorous root branching, high specific root length, and increased root length occupy a larger volume of soil, greatly increasing the rhizosphere and microbial activity (Sasse et al., 2017). The root system is relatively plastic and can change in response to soil fertility gradients that vary in time and space (Correa et al., 2019). Root functional traits, including root diameter (RD), root length (RL), root surface area (SA), root volume (RV), specific root length (SRL), and root tissue density (RTD), are affected by fertilizer applications in agriculture ecosystems (Kramer-Walter et al., 2016; Wen et al., 2019). However, responses of root traits to the application of cattle manure in grassland ecosystems are poorly understood (Carmona et al., 2013). Understanding the response of root morphology to the application of manure should improve understanding of nutrient transfer and predictions of plant production.

Oat (*Avena sativa* L.) is a very important forage in animal husbandry in China as it has high yield and nutritive value. From 2015 to 2017, the imported oat grass increased from 0.15 million tons to 0.30 million tons. Besides, from 2012 to 2015, the planting area increased from 21,800 hm^2 to 334,200 hm^2, with the output of oat increasing from 1.8416 million tons to 2.7436 million tons (Guo T. et al., 2019). However, demand for oat is high in China, and dairy farming alone requires a minimum of about 1.75 million tons of quality oat at present (Guo T. et al., 2019). In China, oat grass is fed to cattle and the resulting manure is returned to the grassland which was a new mode of planting and breeding circular agriculture in recent years.

In the present study, by using oats (*A. sativa* "Baiyan No.2") growing in pots as a model of cattle manure application to oat grassland, the objectives of this study were to: (1)

estimate the transfer of N and P from manure to soils; (2) investigate microbial community dynamics, especially specific functional microbial groups in response to cattle manure; and (3) determine root dynamical morphology changes in response to available nutrients.

MATERIALS AND METHODS

Plant Growth Conditions and Experimental Design

A pot experiment was performed from July to September 2019 at an automatic greenhouse at the Agricultural Ecology Station of Northeast Institute of Geography and Agroecology Chinese Academy of Sciences, in Changchun City, Jilin province (43°59′54′ N, 125°23′57′E). This site has a temperate continental monsoon climate. The mean annual temperature is 6.4°C, and the mean annual precipitation is 614 mm. This soil is classified as typical thin black soil. The soil is a clay loam (Typic Hapludoll, USDA Soil Taxonomy) with an average of 36.0% clay, 24.5% silt, and 39.5% sand. In this area, the maize crop was planted under conventional tillage management since 2012. Soil (0–25 cm depth) was collected from a farmland near the greenhouse. After passage through a 2-mm sieve, the soil was well mixed and packed into 24 pots (20 cm diameter and 25 cm high) with 6 kg soil per pot (1.21 g cm^{-3} bulk density). Each pot was sown with 2.0 g of oat seeds (50 seeds pot^{-1}) on July 1. After sowing, 12 pots were covered with 0.5 kg fresh cattle manure from a cattle fattening farm, as simulated cattle manure deposition (CMD) treatment, whereas cattle manure was not applied to 12 pots and they were the controls (CON). The basic properties of soil and fresh cattle manure are shown (**Supplementary Table 1**). Plants were grown under a photoperiod of 16 h light (from 6 am to 10 pm), followed by 8 h dark, at a light intensity of 200 ± 20 μmol m^{-2} s^{-1} (LED-T8) and 50–75% RH, with an average temperature of 25/18°C (day/night) (Wen et al., 2019). An artificial watering method (three times per week) was used, with equal volumes of water for all pots.

Sample Collection and Measurement

At the 0 days (July 1, sowing date), six soil samples were collected before packing pots as the initial soil and were sifted through a 2-mm sieve and homogeneously mixed. Approximately 6 g of each initial soil sample was put into a 5-ml PE tube and placed in liquid N for microbial sequencing, whereas the remainder was air-dried at room temperature for 1 week and used for chemical analyses. During growth, three random pots in the control treatment and cattle manure deposition treatment were collected for shoot, root, and bulk soil samples on the following days: 15 days after sowing (July 16, trefoil stage), 47 days after sowing (August 17, elongation stage), 66 days after sowing (September 5, pustulation stage), and 80 days after sowing (September 19; maturity stage). The rhizosphere soil (soil firmly attached to roots) of each pot was only collected 15 days after sowing and 66 days after sowing. Briefly, at each sampling, all shoots of each pot were separated from below-ground parts, dried at 65°C for 48 h in a forced-air drying oven, and weighed to determine the total above-ground biomass (AB). Subsequently, all shoot samples were shaken on a 1-mm sieve and stored at 4°C for subsequent chemical analyses. Then, the complete root system of each pot was carefully extracted by removing most soil around the root system. The remaining fine roots in the soil were collected using forceps to minimize soil losses. Then, complete roots and fine roots were washed in a mesh bag (1 μm; 10 cm × 10 cm × 25.5 cm) and stored at 4°C prior to analysis. Finally, the bulk soil of each control treatment pot was well mixed before sampling, whereas for cattle manure deposition treatment pots, surface cattle manure was first removed and then the bulk soil was well mixed and sampled. All soil samples were passed through a 2-mm sieve and air-dried before chemical analysis. For rhizosphere soil sampling at trefoil and pustulation stages, rhizosphere soil from each pot was collected before sampling root by gently shaking the whole plant root system to remove loosely attached soil, and then the soil adhering to the root system was placed into two replicated 5-ml PE tubes. Rhizosphere soil samples were immediately transferred into liquid N and stored at −80°C prior to microbial sequencing.

For root morphology analysis, all roots for each control treatment or cattle manure deposition treatment pot were spread out in water on a glass tray to reduce root overlap and scanned using a flatbed scanner (EPSON Perfection V700 Photo, Seiko Epson Corp., Japan) at a resolution of 600 dpi, as described (Bouma et al., 2001). Subsequently, all roots of each control treatment or cattle manure deposition treatment pot were oven-dried at 65°C for 48 h and weighed as below-ground biomass (BB). Images were saved in an uncompressed TIFF format. RL, SA, and V at various RD classes (<0.2, 0.2–0.5, 0.5–1, 1–2, and >2.0 mm) were measured by WinRHIZO image analysis software (Epson 1680, WinRHIZO Pro2003b, Regent Instruments Inc., Quebec, Canada). Other traits, including the SRL (the ratio of the root length to its BB) and the RTD (the ratio of BB to its fresh volume) were calculated.

Soil organic carbon (SOC) was analyzed by the $K_2Cr_2O_7$–H_2SO_4 oxidation–reduction colorimetric method (Yeomans and Bremner, 1988). Total N (TN) and phosphorus (TP) of soil were measured by Kjeldahl digestion and colorimetric analysis, respectively (Institute of Soil Science, Chinese Academy of Sciences [ISSCAS], 1978). Soil available nitrogen (AN) and phosphorus (AP) were measured by alkaline diffusion and $NaHCO_3$ extraction (0.5 mol L^{-1}, pH 8.5), respectively (Bao, 2000; Liang et al., 2014). For the plant, above-ground N content (ANC) was measured by N Kjeldahl, and above-ground P content (APC) was measured colorimetrically at 880 nm after reaction with molybdenum blue, as well as below-ground N and P contents (BNC and BPC). Two samples from each pot were analyzed and averaged.

Nutrient absorption and return efficiency were calculated as follows: Above-ground N or P accumulation (ANA and APA, g pot^{-1}) = AB × ANC or AB × APC, respectively. The same calculation was used for below-ground N and P accumulation content (BNA and BPA, g pot^{-1}). N absorption efficiency (NAE,%) = [(ANA + BNA in cattle manure deposition treatment pot) - (ANA + BNA in control treatment pot)]/amount of nutrient in cattle manure × 100%. The same calculation

was used for P absorption efficiency (PAE,%). Furthermore, N return efficiency (NRE,%) from feces to soil = [(TN in each cattle manure deposition treatment pot) – (TN in each control treatment pot)]/amount of N in cattle manure × 100%, with the same calculation used for P return efficiency (PRE,%). Total N and P transfer efficiency (TNE and TPE) from feces to soil and oat = NAE + NRE and PAE + PRE, respectively.

DNA Extraction and Illumina High-Throughput Sequencing

To extract soil microbial DNA from 0.5 g rhizosphere soil samples, a FastDNA^{TM} SPIN Kit was used according to the manufacturer's protocol (MP Biomedicals, Santa Ana, CA, United States). The DNA was diluted to 1 ng μl^{-1} and stored at −20°C pending analysis. Bacterial 16S and fungal ITS rRNA gene sequences were measured to characterize microbial populations. For bacterial diversity analysis, the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were used to amplify the flexible V4 region of the 16S rRNA gene. For fungal diversity analysis, the primers ITS1-1F-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS1-1F-R (5′-GCTGCGTTCTTCATCGATGC-3′) were used to amplify the ITS1-1F region.

The reaction mix (30 μl) contained 1.5 μl of each primer, 10 μl of template DNA (1 ng μl^{-1}), 15 μl of Phusion High-Fidelity PCR Master Mix (BioLabs, Inc., Waltham, MA, United States), and 2 μl of water. The thermal program included 25 cycles of 98°C for 30 s, 50°C for 30 s, and 72°C for 1 min. PCR amplicon quality was evaluated by gel electrophoresis and the amplicons were purified using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Amplicons from each sample were amplified in the second round of PCR, purified with a Qiagen Gel Extraction Kit, and quantified using a Qubit dsDNA Assay Kit.

Equimolar amounts of purified amplicons were pooled and subjected to high-throughput sequencing on a HiSeq platform (Illumina, San Diego, CA, United States) at Beijing Biomarker Corporation. Raw paired-end reads were quality-filtered with Trimmomatic software (version 0.33; Bolger et al., 2014), and FLASH software (version 1.2.11) was used to joint for paired reads, and potential chimeras were removed using UCHIME (version 8.1) (Edgar et al., 2011) as implemented in MOTHUR (Schloss et al., 2009). QIIME software (version 1.90) was used to analyze sequences (Caporaso et al., 2010). Finally, UPARSE pipeline was used to classify the operational taxonomic units (OTUs) with 97% similarity UCLUST software Version 1.2.22 (Edgar, 2013). SILVA (Quast et al., 2013) was used for taxonomic annotations of bacterial and fungal OTUs and a minimum similarity cutoff of 90% was used for conservative OTU assignments. The data evaluation of sample sequencing for bacteria (**Supplementary Table 2**) and fungi (**Supplementary Table 3**) and multi-samples' rarefaction curves for bacteria (**Supplementary Figure 1**) and fungi (**Supplementary Figure 2**) demonstrated that our sequencing data represented most of their compositions. Ecological and metabolic functions were predicted for bacterial and fungal OTUs using Functional Annotation of Prokaryotic Taxa (FAPROTAX; Louca et al., 2016) and fungi functional guild (FunGuild), respectively (Nguyen et al., 2016). The sequencing reads of the bacterial 16S rRNA gene and fungal ITS rDNA gene were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under the accession numbers PRJNA830643 and PRJNA831303, respectively.

Statistical Analyses

Independent Student's *t*-tests were done to assess the effects of cattle manure deposition on all parameters separately for each plant growth stage. Two-way analyses of variance (ANOVA) were used to analyze the effects of treatment (cattle manure deposition or control) and plant growth stage (plant trefoil stage and plant pustulation stage) on plant and soil parameters, and on nutrient absorption and return efficiency. Non-metric multidimensional scaling (NMDS) of the bacterial and fungal communities based on OTU composition was done using the Bray–Curtis similarity metric to identify differences between cattle manure deposition treatment and control treatment microbial communities at each sampling time. Differences in bacterial and fungal communities between treatments were tested by the analysis of similarities (ANOSIM and ADONIS). Relative abundance at phylum and genus levels and each specific bacterial function and fungal guild in a given sample was calculated as a percentage value by dividing the raw number of sequences associated with the specific taxa by the total number of sequences in the sample. Positive or negative relationships between environmental factors (nutrient absorption and return efficiency, root traits, and soil properties) and microbial communities (bacterial and fungal at phyla and genus level, respectively) were examined using canonical correspondence analysis (CCA). For all parameters (plant and soil parameters, root traits, and rhizosphere soil microbial sequencing), two samples from each pot were analyzed and averaged and three replicates (pots) were used for data analysis. All parameters were analyzed with SPSS version 16.0 (SPSS Inc. Chicago, IL, United States), with $P < 0.05$ considered significant. All figures were generated by Origin 2016 Pro.

RESULTS

Fecal N and P Transfer

There was an interaction ($P < 0.001$) between cattle manure deposition treatment and plant growth stages on above- and below-ground biomass. Briefly, compared to control treatment, above- and below-ground biomass of oat in cattle manure deposition treatment at 80 days after sowing increased 1.9 and 1.5 times, respectively, whereas above- and below-ground biomass N and P contents increased 19–57%. Consequently, both above and below-ground N and P accumulation were 2.1 to 2.9 higher in response to cattle manure deposition treatment compared with the control treatment (**Table 1**). N and P absorption efficiency increased ($P < 0.001$) with the mature stage extension. Finally, 11.79% of total N and 7.89% of total P in cattle manure were absorbed by oats during the entire growth period, of which 95% of absorbed N and P was deposited to the above-ground part of oats.

TABLE 1 | The dynamics of N and P transfer from cattle manure to soil and oats along plant growth stage under cattle manure deposition.

Items	Treatment								*P* - value			
	15 days after sowing		47 days after sowing		66 days after sowing		80 days after sowing		SEM	GS	CMD	GS*CMD
	CON	CMD	CON	CMD	CON	CMD	CON	CMD				
Biomass (g/pot)												
Aboveground biomass	2.33	2.97*	9.93	16.37*	16.70	28.60*	17.73	32.77*	2.167	$P < 0.001$	$P < 0.001$	$P < 0.001$
Belowground biomass	0.45ns	0.43	0.73	1.61*	0.97	1.73*	1.26	1.89*	0.114	$P < 0.001$	$P < 0.001$	$P < 0.001$
C return												
Soil organic carbon (g.kg)	12.47	14.82*	11.57	15.68*	11.13	15.91*	10.87	16.23*	0.319	0.873	$P < 0.001$	$P < 0.001$
N absorption and return												
Aboveground N content (g.kg)	26.32	35.83*	19.83	20.77ns	13.21	15.57*	11.76	14.01*	1.127	$P < 0.001$	$P < 0.001$	$P < 0.001$
Belowground N content (g.kg)	13.36	16.16*	11.34	12.63ns	7.99	12.55*	8.01	12.45*	0.401	$P < 0.001$	$P < 0.001$	0.002
Aboveground N accumulation (g pot^{-1})	0.061	0.106*	0.197	0.342*	0.221	0.445*	0.208	0.459*	0.029	$P < 0.001$	$P < 0.001$	0.001
Belowground N accumulation (mg pot^{-1})	5.97	6.89ns	8.33	20.37*	7.72	21.66*	10.08	23.59*	1.482	$P < 0.001$	$P < 0.001$	$P < 0.001$
Soil total N content (g.kg)	0.894	0.907ns	0.887	1.002ns	0.874	1.082*	0.862	1.097*	0.023	0.304	$P < 0.001$	0.095
Soil available N content (mg.kg)	100.6	244.4*	109.9	256.3*	174.9	297.6*	78.2	247.6*	14.684	0.058	$P < 0.001$	0.875
P absorption and return												
Aboveground P content (g kg)	5.58	7.22*	3.77	5.24*	3.00	4.30*	2.20	3.46*	0.239	$P < 0.001$	$P < 0.001$	0.912
Belowground P content (g kg)	3.34	5.40*	2.30	2.57ns	1.83	2.40*	1.63	2.25*	0.170	$P < 0.001$	$P < 0.001$	$P < 0.001$
Aboveground P accumulation (g pot^{-1})	0.013	0.021*	0.037	0.086*	0.050	0.123*	0.039	0.113*	0.008	$P < 0.001$	$P < 0.001$	0.001
Belowground P accumulation (mg pot^{-1})	1.49	2.30ns	1.69	4.14*	1.77	4.15*	2.05	4.27*	0.250	$P < 0.001$	$P < 0.001$	0.015
Soil total P content (g kg)	0.351	0.362ns	0.348	0.386*	0.343	0.405*	0.341	0.425*	0.005	$P < 0.001$	$P < 0.001$	$P < 0.001$
Soil available P content (g kg)	13.47	16.51*	12.18	17.75*	13.72	19.08*	9.60	16.63*	0.446	$P < 0.001$	$P < 0.001$	$P < 0.001$
Nutrient absorption efficiency (%)												
N absorption efficiency		2.05c		7.00b		10.58a		11.79a	1.229		0.001	
P absorption efficiency		0.94c		5.14b		7.60ab		7.89a	0.906		0.001	
Nutrient return efficiency (%)												
N return efficiency		3.67c		30.95b		55.88a		63.05a	5.316		$P < 0.001$	
P return efficiency		6.20d		23.15c		37.45b		51.41a	3.619		$P < 0.001$	
Total nutrient transfer efficiency (%)												
Total N transfer efficiency		5.72c		37.95b		66.46a		74.83a	8.439		$P < 0.001$	
Total P transfer efficiency		7.14d		28.29c		45.05b		59.30a	5.905		$P < 0.001$	

GS, growth stage. 15 days after sowing corresponds to the plant trefoil stage; 47 days after sowing corresponds to the plant elongation stage; 66 days after sowing corresponds to the plant pustulation stage; 80 days after sowing corresponds to the plant maturity stage. CON, control; CMD, cattle manure deposition. Data represents mean ± SEM (n = 3).The data was based on a pot experiment in one year. All parameters were analyzed with SPSS version 16.0 (SPSS Inc. Chicago, IL, United States). For biomass, C return, N and P absorption and return, significant differences are indicated (represents $P < 0.05$). For N and P absorption and return efficiency, within a growth stage, means without a common superscript differed ($P < 0.05$).*

The SOC had a decreasing tendency in control treatment but an increasing tendency in cattle manure deposition treatment as oat development, with similar variation tendency in TN and TP (**Table 1**). Thus, cattle manure deposition treatment increased 49% SOC ($P < 0.001$), 27% TN ($P < 0.001$), and 25% TP ($P < 0.001$) of soil when compared to control treatment. Moreover, both N and P return efficiency in cattle manure deposition treatment increased ($P < 0.001$) as oats developed, with 63.05% of fecal N and 51.41% of fecal P returned to the soil throughout the whole growth period.

Responses of Root Traits to Cattle Manure Deposition

The root length of oats at <0.2 mm diameter increased ($P < 0.01$) in cattle manure deposition treatment compared with the control treatment for all plant growth stages, except trefoil stage (**Figure 1**). The root length, surface area, and volume of oat root at 0.2–0.5 mm diameter were higher ($P < 0.01$) in cattle manure deposition treatment than control treatment at 80 days after sowing. For oat roots at 0.5–1.0 mm diameter, the root length and surface area in cattle manure deposition treatment were lower ($P < 0.05$) at 47 days after sowing and then higher ($P < 0.05$) in cattle manure deposition treatment compared with the control treatment. As diameter exceeded 1 mm, root length and surface area at 47 days after sowing were not significantly affected by cattle manure deposition treatment, but increased ($P < 0.05$) afterward. The specific root length was decreased ($P < 0.05$) by cattle manure deposition treatment at 47 days after sowing and increased ($P < 0.05$) afterward, with the opposite for root tissue density (**Figure 2**).

Response of Microbial Community Composition to Cattle Manure Deposition

Non-metric multidimensional scaling ordination based on Bray–Curtis distances indicated that cattle manure deposition significantly affected the bacterial (**Figure 3A**) and fungal (**Figure 3B**) community composition.

Bacterial and fungal community structures at the phylum level under cattle manure deposition treatment and plant growth stage are shown in **Figure 4A**. Relative abundances of Proteobacteria, Firmicutes, and Bacteroidota in cattle manure deposition treatment at 15 and 66 days after sowing were higher ($P < 0.01$) than those in the control treatment, with a decreased tendency in cattle manure deposition treatment from 15 to 66 days after sowing. Moreover, cattle manure deposition treatment decreased ($P < 0.001$) the relative abundance of Actinobacteriota and Acidobacteriota at 15 and 66 days after sowing when compared to control treatment (**Figures 4B a–h**). Among fungal communities, Ascomycota was the predominant phylum, accounting for 37.0 and 81.0% relative abundance in cattle manure deposition treatment and control treatment, respectively at 15 days after sowing, and 42.9 and 63.3% relative abundance at 66 days after sowing. The relative abundance of Rozellomycota increased ($P < 0.05$) in cattle manure deposition treatment compared with control treatment at 15 and 66 days after sowing, whereas the relative abundance of Basidiomycota, Chytridiomycota, and Glomeromycota were higher ($P < 0.05$) in cattle manure deposition treatment at 15 days after sowing compared with the control treatment (**Figures 4B i–p**).

Bacterial and fungal community structures at genus level under cattle manure deposition treatment and plant growth stage are shown in **Figure 5A**. In the eight most frequently observed bacterial communities, relative abundances of *Pseudoxanthomonas* and *Pseudomonas* were higher ($P < 0.01$) in cattle manure deposition treatment versus control treatment at 15 and 66 days after sowing (**Figures 5B a–h**). For the fungal community, the relative abundance of fungi was observed, with either a decrease ($P < 0.05$) (*Alternaria*, *Fusarium*, *Gibberella,* and *Bipolaris*) or equal (*Cladosporium* and *Trichocladium*) in cattle manure deposition treatment compared with control treatment at 15 and 66 days after sowing (**Figures 5B i–p**).

Responses of Microbial Functional Groups to Cattle Manure Deposition

Chemoheterotrophy and aerobic chemoheterotrophy bacteria were decreased at 15 days after sowing in cattle manure deposition treatment versus control treatment (**Figures 6A,B**). Denitrification bacteria increased ($P < 0.001$) in the cattle manure deposition treatment at 15 days after sowing compared with control treatment, whereas nitrification bacteria decreased ($P < 0.01$) in cattle manure deposition treatment compared with control treatment at 15 and 66 days after sowing (**Figures 6C,D**). Among fungal functional groups, the relative abundance of Pathotroph and Pathotroph–Saprotroph were higher ($P < 0.01$) in the control treatment than cattle manure deposition treatment at 15 and 66 days after sowing (**Figures 6E,F**), whereas the relative abundance of saprotroph and symbiotroph were higher ($P < 0.01$) in cattle manure deposition treatment versus control treatment 15 days and 66 days after sowing (**Figures 6G,H**).

Relationships Between Microbial Community Composition and Nutrient Absorption, Root Traits, and Nutrient Return

Based on canonical correspondence analysis (CCA), bacterial and fungal community composition (phyla level) responded differently to changes in nutrient absorption, root traits, and nutrient return. The aboveground biomass, above- and below-ground N and P accumulation were the most crucial roles, with longer projection vectors than above- and below-ground N and P content in both bacterial and fungal community composition (**Figure 7A**). The above-mentioned parameters positively shaped Proteobacteria, Bacteroidota, and Myxococcota bacteria. The aboveground biomass and above- and below-ground N and P accumulation had strong positive impacts on Rozellomycota, Chytridiomycota, and Basidiomycota, whereas they had a strong negative impact on the Ascomycota in the fungal community (**Figure 8A**). Root biomass, root length, surface area, volume, and specific root length positively affected, and root tissue density negatively influenced Myxococcota bacteria (**Figure 7B**), whereas root biomass, root length, surface area, volume, and

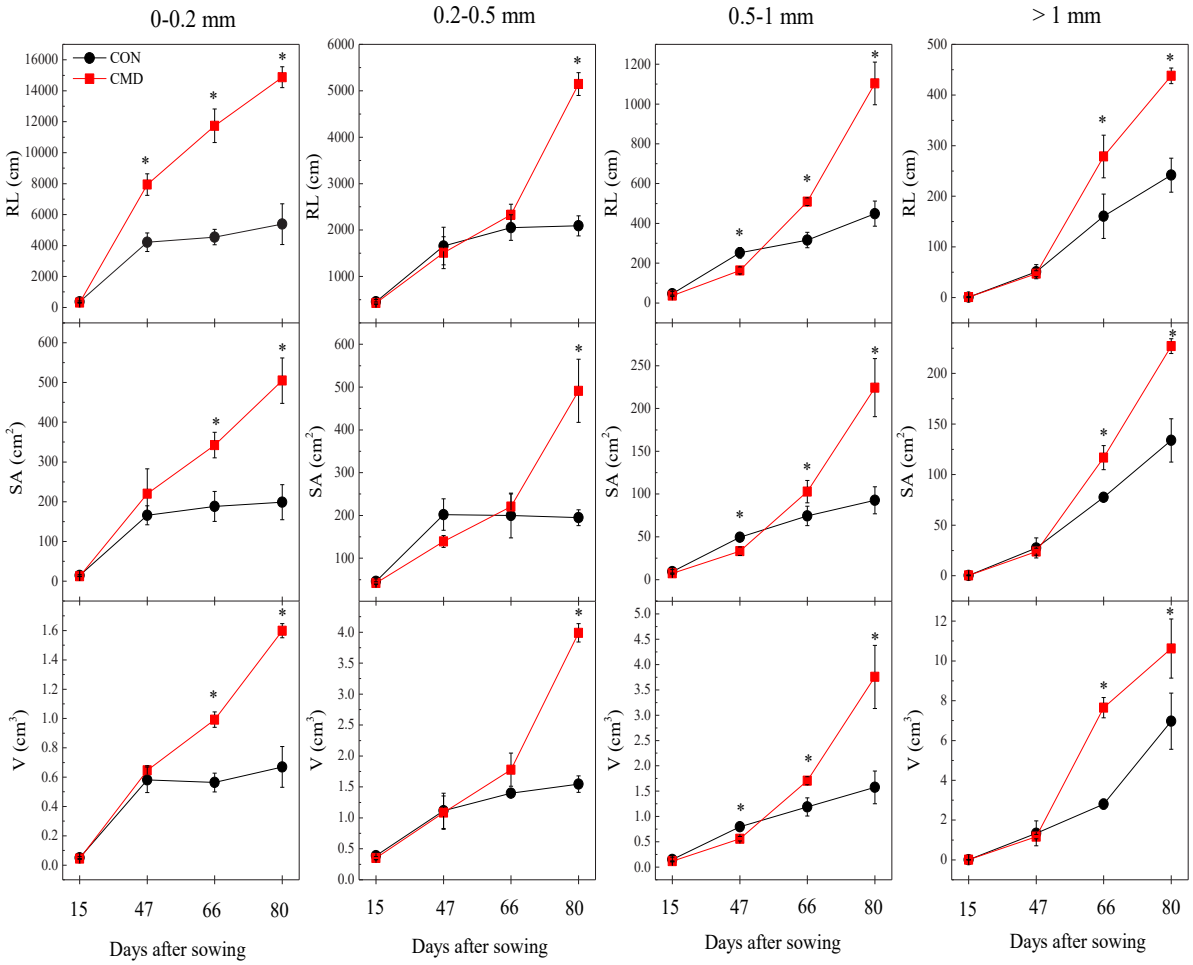

FIGURE 1 | Root length (RL), surface area (SA), and volume (V) at <0.2, 0.2–0.5, 0.5–1, and >1 mm root diameter of oat in CON and CMD treatments, at various growth stages. 15 days after sowing corresponds to the plant trefoil stage; 47 days after sowing corresponds to the plant elongation stage; 66 days after sowing corresponds to the plant pustulation stage; 80 days after sowing corresponds to the plant maturity stage. CON, control; CMD, cattle manure deposition. Data represents mean ± SE (n = 3). The data was based on a pot experiment in one year. All parameters were analyzed with SPSS version 16.0 (SPSS Inc. Chicago, IL, United States). Differences in each root trait whining different diameter classes between CON and CMD treatments at various growth stages are indicated (*represents $P < 0.05$).

specific root length positively affected and root tissue density negatively influenced the Rozellomycota, Chytridiomycota, and Basidiomycota fungi (**Figure 8B**). Soil properties (SOC, TN, TP, AN, and AP) were intensely positively related to Myxococcota, Proteobacteria, and Bacteroidota bacteria and intensely negatively related to Actinobacteriota, Acidobacteriota, Chloroflexi, and Verrucomicrobiota bacteria (**Figure 7C**). In the fungal community, these parameters positively shaped the Rozellomycota, Glomeromycota, Chytridiomycota, and Basidiomycota, and negatively affected Ascomycota (**Figure 8C**).

Regarding microbial community composition at the genus level, aboveground biomass, above- and below-ground N and P accumulation had strong positive impacts on *Pseudoxanthomonas* bacteria, whereas above- and below-ground N and P content positively structured *Sphingomonas*, *Luteimonas,* and *Pseudomonas* bacteria (**Supplementary Figure 3A**). For fungal community, aboveground biomass, above- and below-ground N and P accumulation was positively related to *Corticium* and *Cladosporium* and negatively related to *Alternaria* and *Bipolaris* (**Supplementary Figure 4A**). Root biomass, root surface area, volume, and specific root length had positive effects, whereas root tissue density had negative effects on relative abundance of *Pseudoxanthomonas* bacteria (**Supplementary Figure 3B**). These root traits, with an exception of root tissue density, positively affected *Cladosporium* fungi but negatively influenced *Alternaria* and *Bipolaris* fungi (**Supplementary Figure 4B**). SOC, TN, TP, AN, and AP changed *Pseudoxanthomonas*, *Sphingomonas*, *Pseudomonas,* and *Luteimonas* bacteria and they had a strong negative impact on the Arthrobacter, *RB41*, *MND1*, and *Ellin6067* bacteria (**Supplementary Figure 3C**). In terms of fungal community, these soil properties were positively related to *Corticium* but

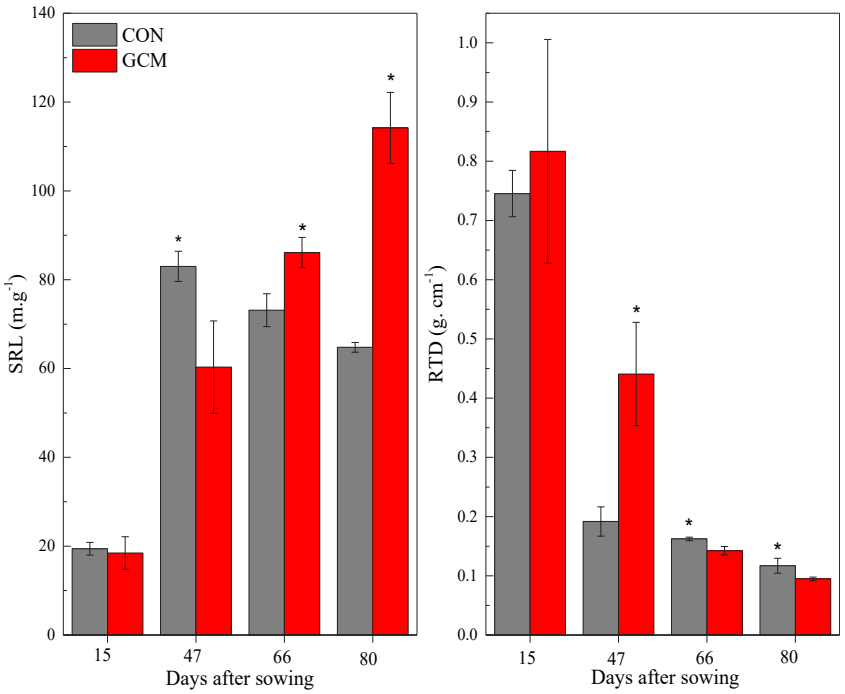

FIGURE 2 | Specific root length (SRL) and root tissue density (RTD) of oat in CON and CMD treatments at various stages. 15 days after sowing corresponds to the plant trefoil stage; 47 days after sowing corresponds to the plant elongation stage; 66 days after sowing corresponds to the plant pustulation stage; 80 days after sowing corresponds to the plant maturity stage. CON, control; CMD, cattle manure deposition. Data represents mean ± SE (n = 3). The data was based on a pot experiment in one year. All parameters were analyzed with SPSS version 16.0 (SPSS Inc. Chicago, IL, United States). Differences between CON and CMD treatments in each root trait at various growth stages are indicated (* represents $P < 0.05$).

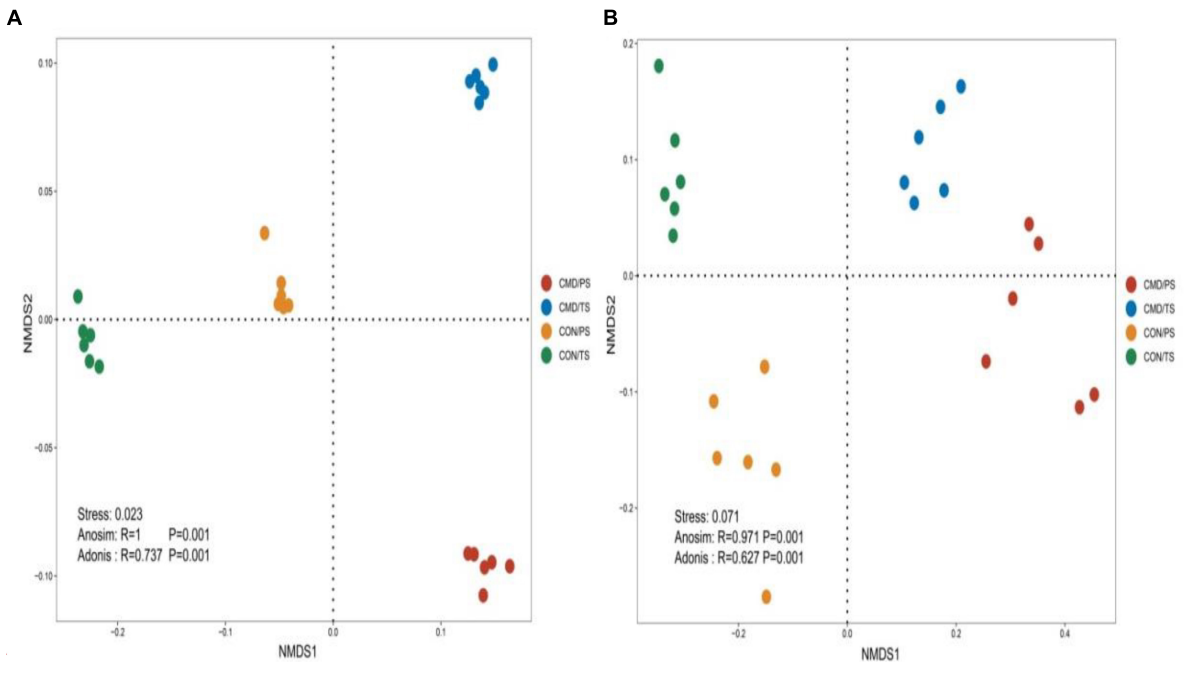

FIGURE 3 | Non-metric multi-dimensional scaling (NMDS) of the bacterial **(A)** and fungal **(B)** community structures in CON and CMD treatments at various growth stages. TS, plant trefoil stage (15 days after sowing); PS, plant pustulation stage (66 days after sowing). CON, control; CMD, cattle manure deposition.

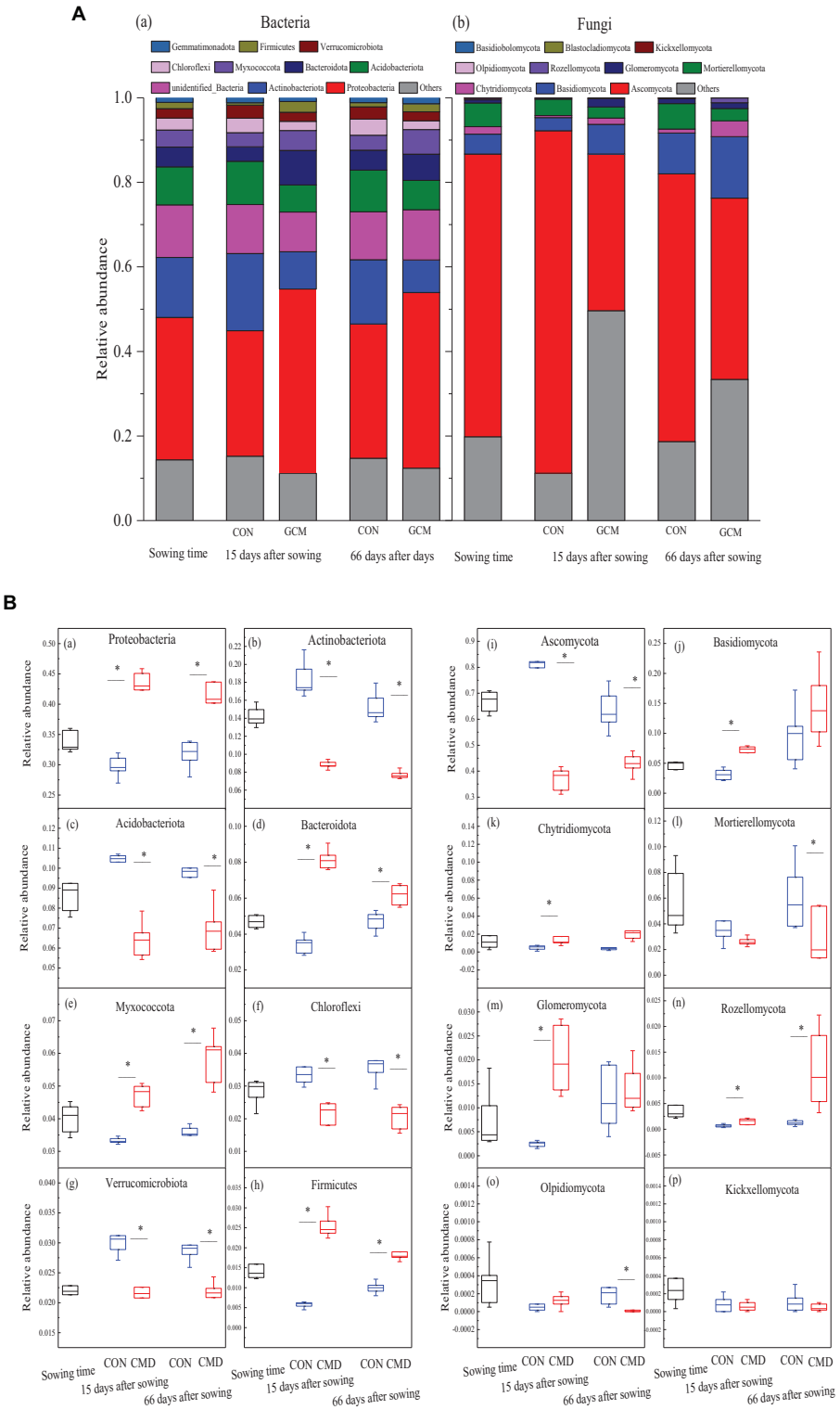

FIGURE 4 | Bacterial and fungal community composition at phyla level under CON and CMD at various growth stages. **(A)** Taxonomic distribution of bacteria **(a)** and fungi **(b)** in different rhizosphere soil. **(B)** Relative abundance of the eight most dominant bacteria **(a–h)** and fungi **(i–p)** in different rhizosphere soil. 0 days corresponds to sowing date; 15 days after sowing corresponds to plant trefoil stage; 66 days after sowing corresponds pustulation stage. CON, control; CMD, cattle manure deposition. Data represents mean ± SE (n = 3). The data was based on a pot experiment in one year. All parameters were analyzed with SPSS version 16.0 (SPSS Inc. Chicago, IL, United States). Asterisk, on bar represent differences ($P < 0.05$) between CON and CMD treatments for bacteria at each growth stage.

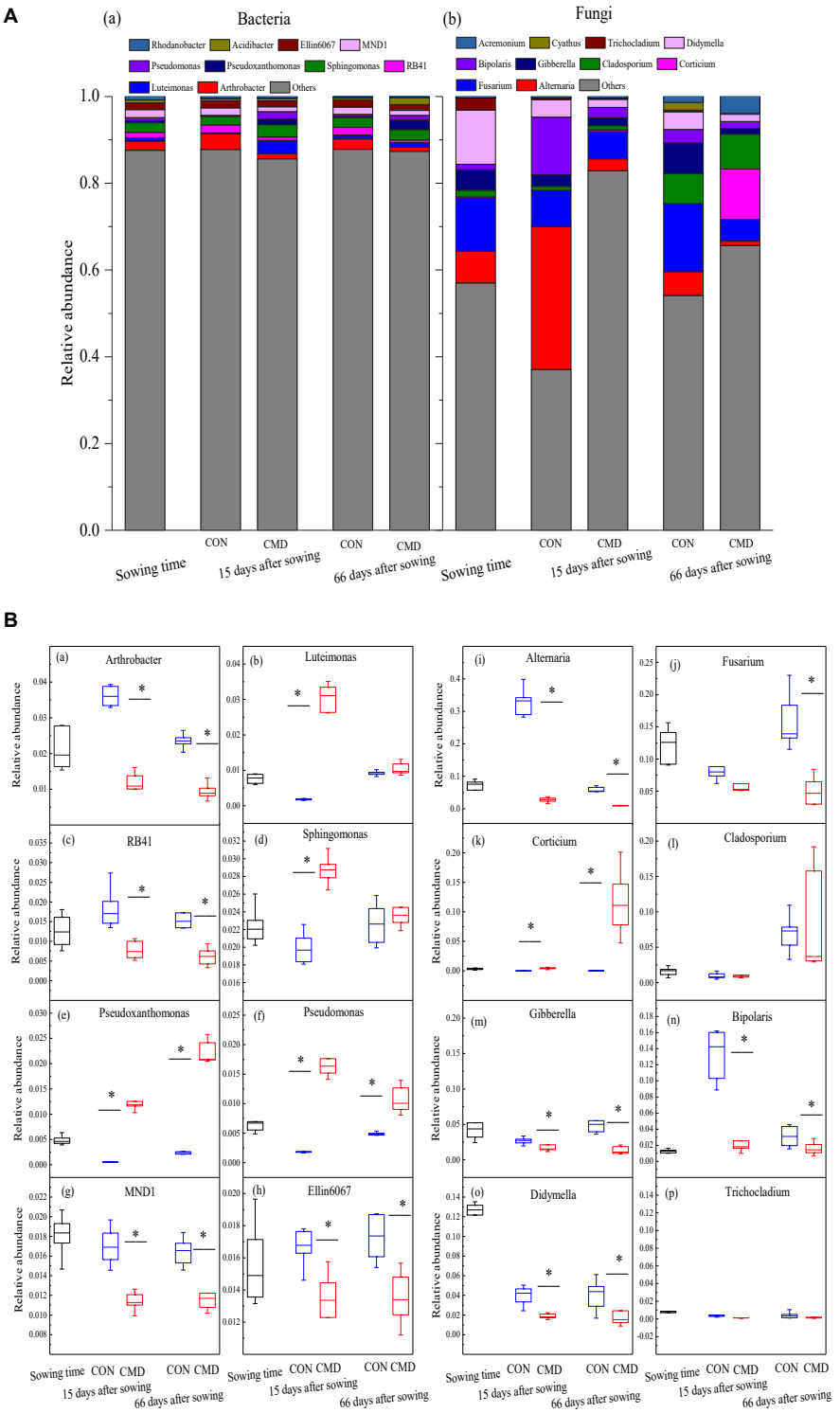

FIGURE 5 | Bacterial and fungal community composition at genus level under CON and CMD at different growth stages. **(A)** Taxonomic distribution of bacteria **(a)** and fungi **(b)** in different rhizosphere soil. **(B)** Relative abundance of the eight most dominant bacteria **(a–h)** and fungi **(i–p)** in different rhizosphere soil. 0 days corresponds to sowing date; 15 days after sowing corresponds to plant trefoil stage; 66 days after sowing corresponds pustulation stage. CON, control; CMD, cattle manure deposition. Data represents mean ± SE (n = 3). The data was based on a pot experiment in one year. All parameters were analyzed with SPSS version 16.0 (SPSS Inc. Chicago, IL, United States). Asterisk, on bar represent differences ($P < 0.05$) between CON and CMD treatments for each microbe at each growth stage.

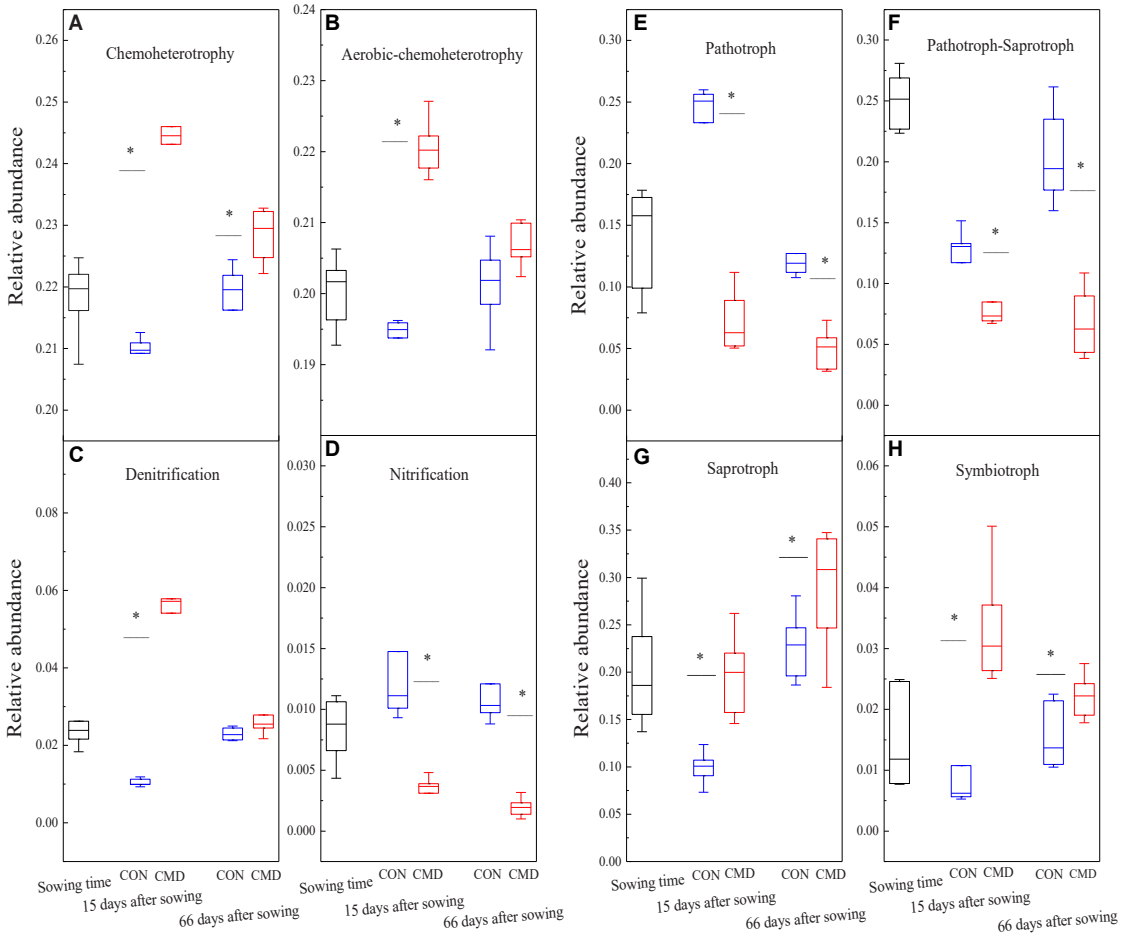

FIGURE 6 | Bacterial **(A–D)** and fungal **(E–H)** functional groups based on FAPROTAX database and FUNguild database, respectively under CON and CMD treatments at different growth stages. CON, control; CMD, cattle manure deposition. 0 days corresponds to sowing date; 15 days after sowing corresponds to plant trefoil stage; 66 days after sowing corresponds pustulation stage. CON, control; CMD, cattle manure deposition. Data represents mean ± SE (n = 3). The data was based on a pot experiment in one year. All parameters were analyzed with SPSS version 16.0 (SPSS Inc. Chicago, IL, United States). Asterisk, on bar represent differences ($P < 0.05$) between CON and CMD treatments for each microbial functional group at each growth stage.

negatively related to *Bipolaris*, *Alternaria*, *Fusarium*, *Didymella*, *Trichocladium,* and *Gibberella* (**Supplementary Figure 4C**).

DISCUSSION

Fecal N and P Transfer

In grassland systems, cattle dung patches (0.1–1.75 kg) are an important nutrient source for soil and grass (Carmona et al., 2013; Rochette et al., 2014). In the present study, the transfer of fecal nutrients into soil and absorption by grassland plants were estimated using cattle manure deposition in a pot experiment. In previous studies, plants under cattle manure were temporarily inhibited due to the "smothering effect" of manure (Aarons et al., 2009), with growth stimulated by only 10-30 cm from the cattle dung edge (Yoshitake et al., 2014). However, in the present study, the application of cattle manure did not suppress oat growth, even at the trefoil stage or earlier. Cattle manure improved oat growth at all stages, especially in the late growth stage, in terms of increased biomass, nutrient absorption, nutrient accumulation, and nutrient absorption efficiency. In total, 11.79% of fecal N and 7.89% of fecal P were absorbed by oats.

Application of cattle manure on the soil surface returns fecal nutrients to soil depending on: (i) leaching the original available nutrients and (ii) rhizosphere microbial decomposition to release available nutrients (Jacoby et al., 2017; Jiao et al., 2019). When compared to control treatment, the SOC, TN, TP, AN, and AP of soil in cattle manure deposition treatment at 80 days after sowing were 49, 27, 25, 216, and 73% higher. In total, after the application of cattle manure, 74.83% of fecal N and 59.30% of fecal P were returned to the soil during the whole growing period. A portion of the N and P that returned to the soil were sequestered by oats, with a total of 63.05% of fecal N and 51.41% of fecal P left in the soil. Yoshitake et al. (2014) reported that 70% N of cattle manure entered the soil on a 30 × 100 m temperate area with a typical grazing intensity of ∼1.1 cattle ha^{-1} from late May to late

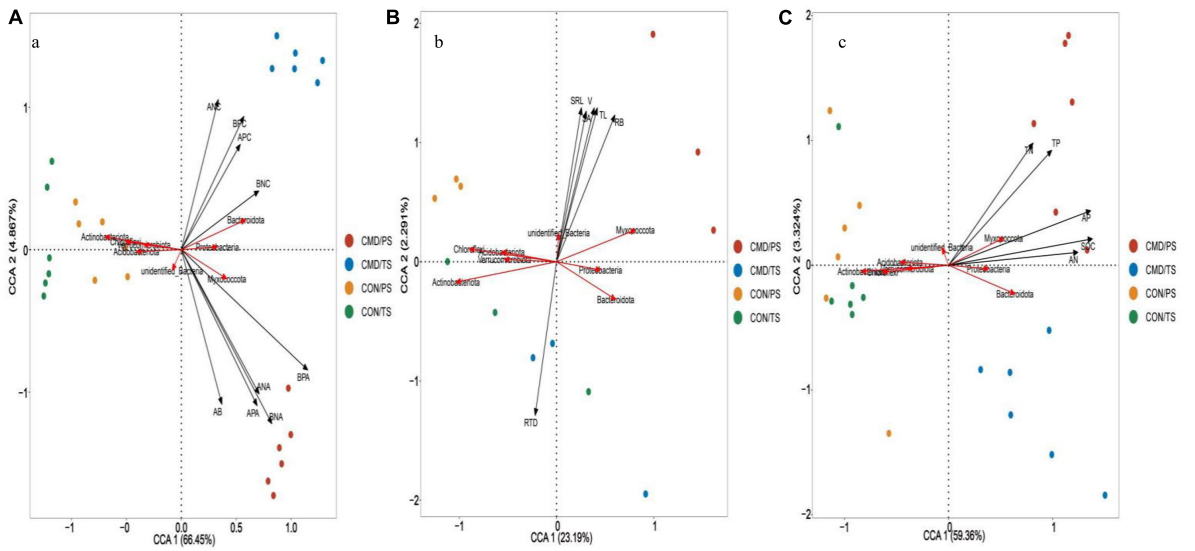

FIGURE 7 | Canonical correspondence analysis (CCA) showing bacterial community composition at phyla level as affected by biomass and nutrient absorption and return **(A)**, and root traits **(B)** soil chemical property **(C)**. Microbial community composition located in the forward direction of the arrow of the environmental factor implies positive relationship between the microbial community composition and the environmental factor, and vice versa. TS, plant trefoil stage (15 days after sowing); PS, plant pustulation stage (66 days after sowing); AB, aboveground biomass; RB, below-ground biomass; ANC, above-ground N content; BNC, below-ground N content; APC, above-ground P content; BPC, below-ground P content; ANA, above-ground N accumulation; BNA, below-ground N accumulation; APA, above-ground P accumulation; BPA, below-ground P accumulation. CON, control; CMD, cattle manure deposition. RL, Root length; SA, root surface area; V, root volume, SRL, specific length; RTD, root tissue density; SOC, soil organic carbon; TN, total nitrogen; AN, available nitrogen; TP, total phosphorus; AP, available phosphorus. Data represents mean ± SE (n = 3). The data was based on a pot experiment in one year. All parameters were analyzed with SPSS version 16.0 (SPSS Inc. Chicago, IL, United States).

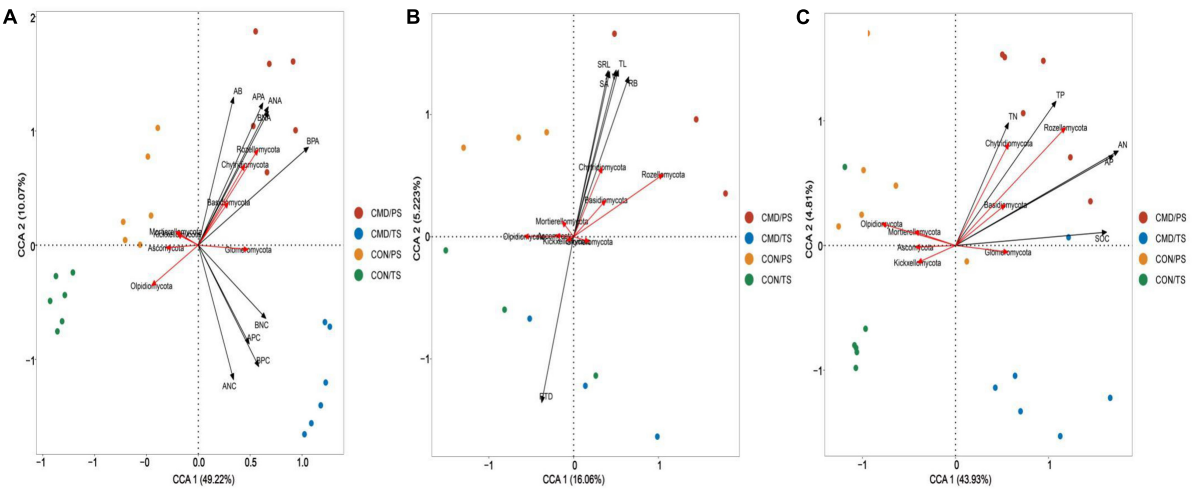

FIGURE 8 | Canonical correspondence analysis (CCA) showing fungal community composition at phyla level as affected by biomass and nutrient absorption and return **(A)**, and root traits **(B)** soil chemical property **(C)**. Microbial community composition located in the forward direction of the arrow of the environmental factor implies positive relationship between the microbial community composition and the environmental factor, and vice versa. CON, control; CMD, cattle manure deposition.

October. However, the decomposition of cattle manure occurred slowly, requiring 350–850 days (Hirata et al., 2009). The weight of cattle manure decreased sharply to about 30% of the initial weight during the first 30 days under cattle manure deposition because of the proper temperature, high water content, and the activity of microbiome (Yoshitake et al., 2014). Another study showed that N of cattle manure in macro-mesh-size litterbags disappeared by 20% during 60 days and 17% during another 60 days (Rashid et al., 2017). In this study, the N transfer efficiency was increased and then decreased during 80 days after cattle manure deposition.

N transfer efficiency was low at 3.67% during 15 days after cattle deposition and then increased to 27.28 and 24.93% between 15 days and 47 days after cattle deposition and between 47 days and 66 days after cattle deposition, respectively. However, the N transfer efficiency was only 8.37% between 66 days and 80 days after cattle deposition. P transfer efficiency had a similar tendency with N transfer efficiency.

In addition, losses of fecal N include ammonia volatilization and fecal AN leaching from fresh dung during the growing season (Maris et al., 2021). Consequently, not all fecal N is transferred into the soil during a single growing season. A better understanding of nutrient return by cattle manure deposition during the growing season will improve nutrient management decisions.

Root Morphology

Root morphology was associated with nutrient absorption and plant biomass. Root growth is directly affected by the chemical properties of soil (Bandyopadhyay et al., 2010). More specifically, one of the most important nutrient-acquisition strategies of plants is thinner roots and larger specific root length (Wen et al., 2019). In the present study, root length of oats <0.2 mm in diameter was more susceptible than other root functional traits, and both root length and specific root length increased after elongation in cattle manure deposition treatment oats compared with the control treatment.

Roots have a close interaction with microorganisms in the rhizosphere. The presence of roots increased microbial abundance, activity, and variance (enrichment of select members or loss of detectable low-abundance taxa) by root exudates and strengthened the attachment of external nutrients to root systems (Landesman et al., 2019). As roots grow, the new tissue surface expands spaces for bacterial community, and the quick root tip elongation assembles more bacterial colonization on the root surface (Bulgarelli et al., 2012). For instance, phylum Proteobacteria are well-known rhizosphere colonizers and mainly positively respond to plant roots (Lundberg et al., 2012), consistent with present results that *Proteobacteria* was strongly positive to root biomass, root length, surface area, volume, and specific root length. Higher abundance of specific bacteria enriched in rhizosphere accelerated mineralization of organic matter and generated available nutrients around the root system, promoting root development (Valencia et al., 2018).

Microbial Community Composition and Functional Groups

Bacterial Community Composition and Functional Groups

Plant growth stages filter and shape rhizosphere microbiomes community by microbial substrate preferences in root exudates (Shi et al., 2011). In addition, Proteobacteria, Actinobacteriota, and Firmicutes prefer easily decomposable substrates, such as sucrose, and consequently were reduced as the plant matured (Zwetsloot et al., 2020), as sucrose concentrations are higher in the early stages of plant growth and allocated primarily to the area behind the root tips (Schlemper et al., 2017). However, we only observed a relative abundance of Actinobacteriota in control treatment soil that distinctly decreased after the trefoil and postulation periods, whereas in Proteobacteria and Firmicutes it slowly increased.

Except for the effects of plant development stages, nutrient availability, especially soil N concentration, is the main driving force shaping the root microbiome community (Qu et al., 2020). Relative abundances of Proteobacteria and Firmicutes of rhizosphere soil increased as soluble organic N concentration increased (Gastal and Lemaire (2002). In this study, cattle manure significantly changed the relative abundance of dominant bacteria and remodeled bacterial variation tendency as plant development proceeded. In particular, manure greatly promoted relative abundance of Myxococcota, slightly promoted relative abundance of Proteobacteria, Firmicutes, and Bacteroidota, and intensely suppressed relative abundance of Actinobacteriota, Acidobacteriota, and Chloroflexi. Differences between cattle manure deposition treatment and control treatment in bacterial species may have been due to differences in adaptation and tolerances to nutrients (Coleman-Derr et al., 2016). In addition, more available nutrients at the late growing stage under cattle manure deposition treatment conditions may have suppressed bacterial growth. Supplementing N likely increased the amount of available nutrients for microbial growth if concentrations were below the threshold (Wei et al., 2013). However, soil N had the greatest impact on the microbial community and additional N suppressed both bacterial and fungal growth (Zhong et al. (2015), implying a potential N threshold for soil microbes in farmland ecosystems. The CCA in this study also supported this assumption, as soil AN and AP had positive impacts on phylum Myxococcota, Proteobacteria, Firmicutes, and Bacteroidota, whereas soil AN and AP had negative impacts on phyla Bacteroidota, Actinobacteriota, Acidobacteriota, and Chloroflexi, with similar results at the genus level.

The main finding of the present study was that cattle manure deposition treatment increased relative abundance of bacteria known to be beneficial. Rhizosphere beneficial bacteria taxa are important links between plants and soil, not only reflecting soil nutrient status but also affecting plant growth (De Araujo et al., 2019). Proteobacteri, Bacteroidetes, and Firmicutes were present in maize, barley, cotton, and wheat rhizosphere (Peiffer et al., 2013; Guo Z. et al., 2019). Similarly, they were also the dominant bacterial phyla in oat rhizosphere, indicating they might be the core microbiome in most plant rhizosphere. Furthermore, cattle manure increased the relative abundance of Proteobacteria, Firmicutes, and Bacteroidota in oat rhizosphere when compared to control treatment at 15 and 66 days after sowing. Proteobacteria and Firmicutes are common in fecal matter and promoted the mineralization of complex organic compounds, reducing the C skeleton required for amino acid formation (Dai et al., 2018). Bacteroidota are important contributors to N and P turnover in the soil (Yousuf et al., 2012). At the genus level, *Pseudoxanthomonas* and *Pseudomonas* (bacterial Pseudomonadales order) were significantly enriched in oat rhizosphere in the present study following the application of manure. Furthermore, *Pseudoxanthomonas* and *Pseudomonas* genera directly solubilized phosphate and had a synergistic

effect on promoting plant growth in soil (Bautista-Cruz et al., 2019; Li et al., 2021), protecting against pathogens (De Corato et al., 2020).

Here, we extended previous findings on fertilization-induced bacterial taxonomic changes to the functional level. It was reported that autotrophic nitrification increased after manure or inorganic N application in loamy (Tian et al., 2015) and neutral soils (Shen et al., 2012). However, nitrification was decreased by N inputs in black soil (Zhao et al., 2020). In that regard, ammonia-oxidizing bacteria (AOB) was suppressed as ionization of ammonia to ammonium was promoted by black soil acidification under fertilization (Zhang et al., 2012). A similar result was observed in the present study, indicating that functional bacteria related to nitrification were decreased by cattle manure, with its high concentrations of TN and AN. Cattle manure significantly increased the relative abundance of chemoheterotrophic and aerobic chemoheterotrophic communities, as they were positively correlated with N-acetyl-glucosaminidase activity, described as N acquisition enzyme (Wahdan et al., 2021).

Fungal Community Composition and Functional Groups

Cattle manure deposition treatment and plant growth stages altered the fungal community. Generally, Ascomycota is a dominant phylum in oat rhizosphere soil and decomposes organic matter in livestock manure (Yao et al., 2017). Similarly, in the present study, Ascomycota was dominant in the oat rhizosphere, but after the deposition of cattle manure, its relative abundance was much lower at 15 days and 66 days after sowing. Inexplicably, Ascomycota were considered key decomposers in agricultural soils and correlated with relatively high N content and availability (Paungfoo-Lonhienne et al., 2015). Perhaps the reduction of relative abundance of Ascomycota in the present study was due to available nutrients exceeding the tolerance limit of Ascomycota. Furthermore, Basidiomycota, another dominant decomposer in plant rhizosphere soil (Wang et al., 2018), decomposed organic matter and released N and P (Osono and Takeda, 2013). In the present study, the addition of manure increased Basidiomycota abundance at 15 days and 66 days after sowing.

More importantly, cattle manure deposition treatment reduced the community of fungi potentially pathogenic to oat. A previous study showed that cattle manure has a strong suppressive effect on soil pathogenic fungal growth and may regulate antagonistic microbial groups (Sun et al., 2016). In the present study, cattle manure deposition treatment decreased microbial groups related to pathotroph but increased beneficial groups of saprotroph and symbiotroph. It is well documented that high nutrient availability e.g., manure, inhibits the growth of pathogenic microbes (Mandavgane and Kulkarni, 2020) due to systemic acquired resistance (De Corato, 2020; Silva et al., 2022). In the present study, almost all fungal genera of the top eight dominant fungi were higher in the control treatment compared to cattle manure deposition treatment. Several of these fungi, e.g., genus *Alternaria* and *Fusarium* are soil-borne fungal pathogens with specific microbiomes in the rhizosphere (Sun et al., 2016). Plant diseases seriously hinder plant production and limit nutrients derived from plants (Raaijmakers et al., 2009).

CONCLUSION

Deposition of cattle manure strongly promoted soil nutrient content and oat biomass. Ultimately 63.05% of fecal N and 51.41% of fecal P of cattle manure returned to the soil, whereas 11.79% of fecal N and 7.89% of fecal P were absorbed by oats throughout the entire growth period (80 days after sowing). The higher oat biomass following the application of manure was closely related to changes in root morphology and microbial community composition in oat rhizosphere soil. Cattle manure increased root length, surface, and volume of oats at all diameter classes, especially root length <0.2 mm diameter. In addition, cattle manure increased the beneficial microbiome, e.g., Proteobacteria, Bacteroidota, and Firmicutes bacteria, and suppressed soil fungal pathogens.

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: NCBI BioProject – PRJNA831303 and PRJNA830643.

AUTHOR CONTRIBUTIONS

RZ designed the experiments, provided the financial support, and helped perform the analysis with constructive discussions. CZ performed the experiments, analyzed the data, and wrote the manuscript. JH, QL, and YF helped revise the language of the manuscript. DL and ZL provided laboratory apparatus. All authors contributed to the article and approved the submitted version.

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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.2022.916610/full#supplementary-material

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Sugarcane Root Transcriptome Analysis Revealed the Role of Plant Hormones in the Colonization of an Endophytic Diazotroph

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Some sugarcane germplasms can absorb higher amounts of nitrogen *via* atmospheric nitrogen fixation through the bacterial diazotrophs. Most endophytic diazotrophs usually penetrate through the root, colonize inside the plant, and fix the nitrogen. To assess the plant's bacterial association during root colonization, strain GXS16 was tagged with a plasmid-bear green fluorescent protein (GFP) gene. The results demonstrated that the strain can colonize roots all the way to the maturation zone. The strain GXS16 showed maximum nitrogenase enzyme activity at pH 8 and 30°C, and nitrogenase activity is less affected by different carbon sources. Further, strain GXS16 colonization response was investigated through plant hormones analysis and RNAseq. The results showed that the bacterial colonization gradually increased with time, and the H_2O_2 and malondialdehyde (MDA) content significantly increased at 1 day after inoculation. There were no substantial changes noticed in proline content, and the ethylene content was detected initially, but it decreased with time. The abscisic acid (ABA) content showed significant increases of 91.9, 43.9, and 18.7%, but conversely, the gibberellin (GA_3) content decreased by 12.9, 28.5, and 45.2% at 1, 3, and 5 days after inoculation, respectively. The GXS16 inoculation significantly increased the activities of catalase (CAT), superoxide dismutase (SOD), polyphenol oxidase (PPO), ascorbate peroxidase (APX), and glutathione reductase (GR) at different timepoint. In contrast, the peroxisome (POD) activity had no changes detected during the treatment. In the case of RNAseq analysis, 2437, 6678, and 4568 differentially expressed genes (DEGs) were identified from 1, 3, and 5 days inoculated root samples, and 601 DEGs were shared in all samples. The number or the expression diversity of DEGs related to ethylene was much higher than that of ABA or GA, which indicated the critical role of ethylene in regulating the sugarcane roots response to GXS16 inoculation.

Keywords: sugarcane root, physiological function, gene expression, endophytic diazotroph, inoculation

INTRODUCTION

Sugarcane (*Saccharum officinarum*) is the primary sugar crop in China, but unbalanced amounts of nitrogen fertilizer have been used in its production that widely enhance the ecological toxicity. Typically, 450–750 kg of nitrogen are applied per hectare, more than twice the world average. It is also one of the reasons for the high costs of production (Carvalho et al., 2011; Yang et al., 2014). Some sugarcane germplasms can obtain high levels of nitrogen *via* nitrogen fixation by associated nitrogen fixing bacteria (NFB) (Reinhold-Hurek and Hurek, 2011). Unlike symbiotic nitrogen fixation, the associated NFB in sugarcane are loosely combined with the plant. After the interaction, they have no observable phenomenon and do not form characteristic symbiotic structures, such as root nodules. However, they are highly efficient at fixing nitrogen. According to studies, biological nitrogen fixation, which is fixed by related NFB, can provide 30–60% of the nitrogen for sugarcane growth (Urquiaga et al., 2012). Inoculation with the endophytic NFB may enable sugarcane to benefit from biological nitrogen fixation, thus, reducing planting costs by limiting the application of nitrogen. Currently, in the study of sugarcane nitrogen fixation, the primary methods for inoculating sugarcane with NFB involve soaking sugarcane seed stems with a bacterial solution, pouring the bacterial solution on stubble cane, and co-culturing the sugarcane tissue culture seedlings with NFB during the rooting stage. When these methods are used for inoculation, the NFB primarily enters the plants from the lateral root origin, root tip, root hair, detached root cap, and other tissues. The bacteria promote plant growth by fixing nitrogen, secreting auxin and ferritin, and dissolving phosphorus and potassium, thus, increasing sugarcane yields (Boller and He, 2009; Wei et al., 2014a; Malviya et al., 2019).

Research has shown that plants gradually form an immune system, thereby defensive them from infection by pathogens (Boller and He, 2009; Takken and Tameling, 2009). However, the interaction pattern between endophytic bacteria and the host differs from pathogenic microorganisms. The immune response of the host will not be over-reactive during the process of colonization, thus, avoiding their mistaken eradication by the host (Vandenkoornhuyse et al., 2015). Reactive oxygen species (ROS) in plant cells (Han et al., 2015; Zhou et al., 2015), antioxidant protection systems (Alqueres et al., 2013), and plant hormones (Vargas et al., 2014; Liu et al., 2017) play essential roles during these types of interactions. RNA-seq is a deep-sequencing method that provides the transcriptome profile of model and non-model plants (Haider et al., 2017). RNA-seq technique helps examine gene functions and expression at the transcriptional level and elucidates the molecular mechanisms of specific biological processes (Stark et al., 2019; Simoneau et al., 2021). Transcriptomics technology mainly was utilized to investigate the different functions such as the interaction between microbes and plants in response to water deficiencies, root exudates, salt stress, biotic and abiotic stresses (Chauhan et al., 2019; Khan et al., 2020; Malviya et al., 2020; Moradi et al., 2021). However, using RNA-seq technology, there is minimal evidence on the physiological and molecular interaction mechanism between diazotrophs and sugarcane.

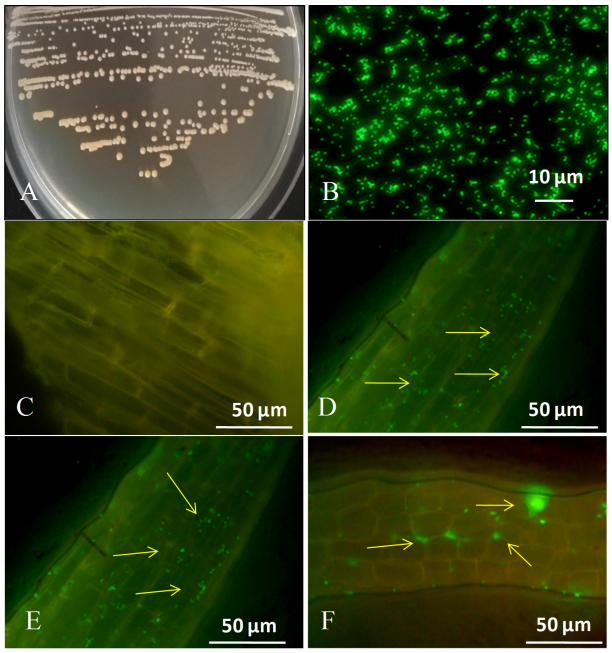

FIGURE 1 | (A) Colony appearance of strain GXS16, **(B)** Microscopy of GFP-tagged strain GXS16 cells, **(C)** Non-inoculated plant root (Control), **(D)** GFP tagged strain GXS16 colonized plant root at 1 day after inoculation, **(E)** GFP tagged strain colonized root at 3 days after inoculation, **(F)** GFP tagged strain colonized root at 5 days after inoculation. The presence of bacteria as a single, bunch, or patch is indicated by arrowheads.

Current studies on endophytic NFB in sugarcane are primarily focused on the following two aspects: first, the isolation of NFB. Sugarcane is inoculated with NFB to confirm their endogenous and growth-promoting effects and measure the efficiency of nitrogen fixation (Wei et al., 2014b; Malviya et al., 2019). Secondly, the regulatory mechanism of the expression of nitrogen-fixing genes is studied using multi-omics technology, and the effects of inoculation with endophytic NFB on the expression of genes or resistance to adversity stress of sugarcane are analyzed (Lery et al., 2010; Vargas et al., 2014; Aguiar et al., 2018; Wang et al., 2019). The root system of sugarcane plays an essential role in nitrogen fixation by endophytic bacteria. However, the physiological and molecular responses of sugarcane roots to the inoculation of endophytic NFB have not been systematically studied. In this study, sugarcane tissue culture seedlings were inoculated with the *Burkholderia* endophytic diazotrop strain GXS16 (Nong et al., 2021), which has a stronger association with nitrogen fixation capacity at the rooting stage, to detect the physiological and biochemical effects and changes in gene expression of the sugarcane roots at various time interval. The focus was on analyzing the impact of diazotroph inoculation on plant hormone-related gene expression. It will explain the recognition and response mechanism of sugarcane roots to GXS16 inoculation and provide a theoretical basis to understand better how NFB promotes sugarcane growth.

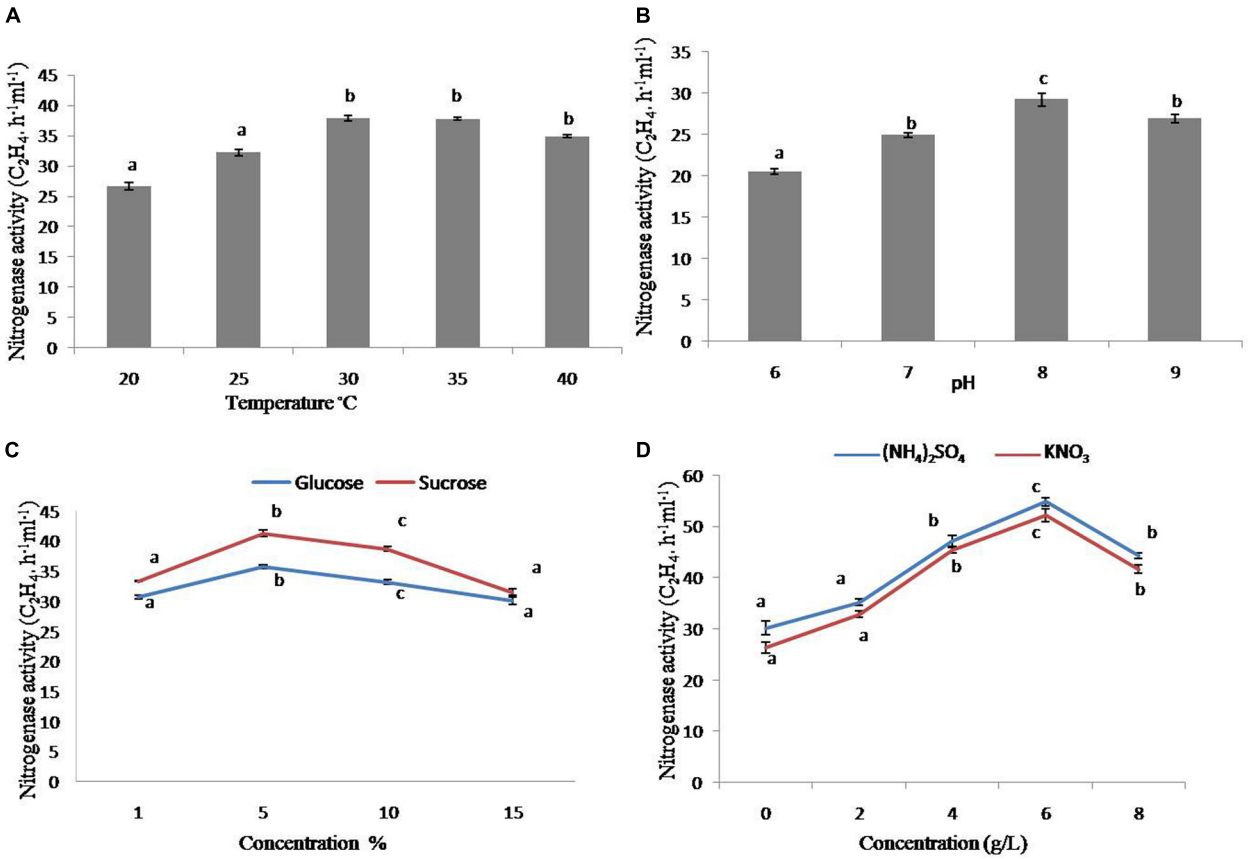

FIGURE 2 | Nitrogenase activity of strain GXS16. **(A)** Temperature, **(B)** pH, **(C)** Glucose and sucrose, **(D)** $(NH_4)_2SO_4$ and KNO_3. Values are mean, and vertical bars indicate the standard deviation of three replications. Means followed by the same letter within a column are not significantly different ($p < 0.05$) according to Duncan's multiple range test (DMRT).

MATERIALS AND METHODS

Experimental Materials

Burkholderia sp. strain GXS16 was previously isolated from the sugarcane variety Guitang 31. Our previous study reported that strain GXS16 has nitrogenase activity of 2.42 μmol C_2H_4 h^{-1} ml^{-1}, strong ACC deaminase activity, and the ability to secrete auxin and degrade inorganic phosphorus, among others (Nong et al., 2021). The height of the sugarcane plant inoculated with GXS16 increases by more than 15% compared with the control, and its dry weight increases by more than 20%. The efficiency is significantly higher than that of model strain *Gluconacetobacter diazotrophicus* PAL5 (Nong et al., 2021). The plasmid standard of the 16S rRNA gene of GXS16 was prepared and stored. The Sugarcane Research Institute of the Guangxi Academy of Agricultural Sciences (Nanning, China), provided the tissue culture seedlings of sugarcane variety RB86-7515.

TABLE 1 | The copy number of GXS16 strain in sugarcane roots.

Treatment	Average *Ct* value	*X*-value	Copies (0.05 g^{-1})
Control-1	32.09	1.46	2.86×10^2
Inoculation-1	26.95	2.96	9.23×10^3
Control-3	31.26	1.69	5.01×10^2
Inoculation-3	17.21	5.82	6.68×10^6
Control-5	32.13	1.44	2.75×10^2
Inoculation-5	15.31	6.38	2.41×10^7

Green Fluorescent Protein Technique for Tagging of Strain

Plasmid transformation has been done as described by Malviya et al. (2019). *Burkholderia* strain was chosen as a recipient for genetic tagging with GFP-pPROBE-pTetr-AP because it was ampicillin-resistant (80 μg mL^{-1}). Kanamycin sensitivity was observed in this strain (50 μg mL^{-1}). Biparental mating with donor *Escherichia coli* (*E. coli*) strain TG1 resulted in the introduction of plasmid pPROBE-pTetr-AP (2.6 kb) carrying the GFP and kanamycin genes produced under the control of a Tetr promoter. The recipient and donor strains were combined in a 1:2 ratio. An aliquot (100 μL) of this combination was spread on Luria Bertani (LB) agar and incubated at 30°C for 24 h. After incubation, the bacteria cells were collected and plated onto selective media containing ampicillin (80 mg mL^{-1})

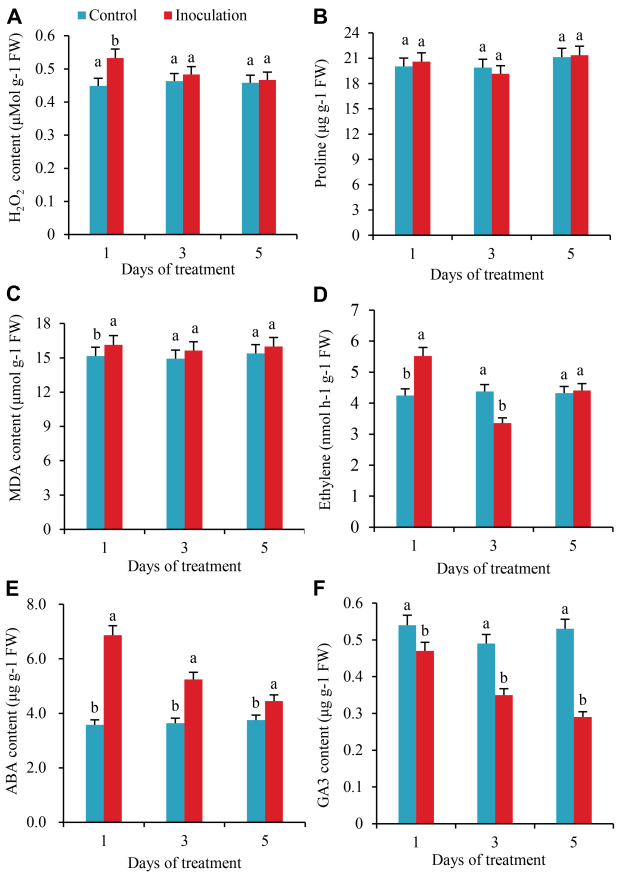

FIGURE 3 | Effect of inoculation with GXS16 on **(A)** H_2O_2, **(B)** Proline, **(C)** MDA, **(D)** Ethylene, **(E)** ABA, and **(F)** GA_3. Values are mean, and vertical bars indicate the standard deviation of three replications. Means followed by the same letter within a column are not significantly different ($p < 0.05$) according to Duncan's multiple range test (DMRT). H_2O_2, hydrogen peroxide; MDA, malondialdehyde; ABA, abscisic acid; GA_3, gibberellic acid.

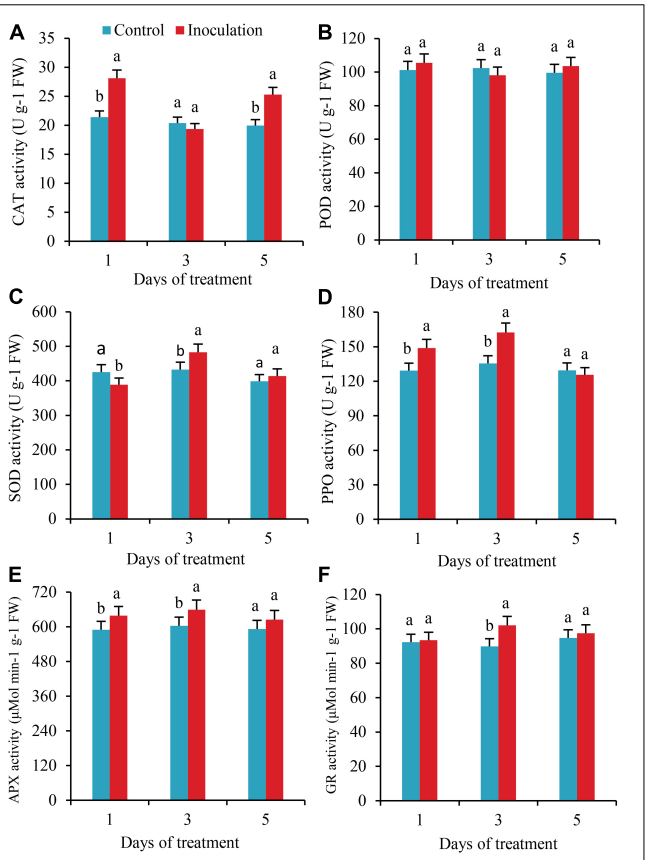

FIGURE 4 | Effect of Inoculation with GXS16 on **(A)** CAT, **(B)** POD, **(C)** SOD, **(D)** PPO, **(E)** AXP, and **(F)** GR activity in sugarcane roots. Values are mean, and vertical bars indicate the standard deviation of three replications. Means followed by the same letter within a column are not significantly different ($p < 0.05$) according to Duncan's multiple range test (DMRT). CAT, catalase; POD, peroxidase; PPO, polyphenol oxidase; SOD, superoxide dismutase; PPO, polyphenol oxidase; AXP, ascorbate peroxidase; GR, glutathione reductase.

and kanamycin (50 mg mL^{-1}). A fluorescence microscope was used to select ex-conjugants that fluoresced green under the UV irradiation (Leica Microsystems, Wetzlar, Germany).

Colonization Analysis Under Gnotobiotic Assays

Active cells of GFP-tagged GXS16 strain was injected into 50 mL of LB medium and incubated at 30°C until late log phase. Centrifugation at 4500 xg for 15 min yielded the tagged bacterial cells. The bacterial cells were then rinsed with phosphate-buffered saline (PBS) at pH 6.5 and the cell density was set to 10^5 CFU/mL. Micropropagated sugarcane tissue culture plants (variety RB86-7515) were cultivated at 30°C in 50 mL tubes containing 10 mL of agar-free MS media with tagged bacteria. In the control plants, PBS was used in place of the bacterial suspension. After bacterial inoculation, sugarcane tissue culture plantlets (inoculated and uninoculated) were taken out on the first, third, and fifth days and cleaned with autoclaved distilled water. The root tissues were sliced into small pieces and put on a slide with 10% (v/v) glycerol before being examined using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany).

Nitrogenase Activity Assay

The nitrogenase activity of an isolated strain was determined using gas chromatography (Shimadzu GC-17A) ethylene and the acetylene reduction technique (Baldani et al., 1986). The nitrogenase activity was calculated using the method described by Yao et al. (2004). The experiments were carried out in triplicate.

Effects of Temperature and pH on Nitrogenase Activity

In a 50 mL flask with a tight-fitting rubber stopper, the strain GXS16 was inoculated into 10 mL sterile culture media (Xing et al., 2016) and incubated at 200 revolution per minute (rpm) for 36 h on rotary shaker. Five mL of acetylene gas was introduced into the flask and incubated at the same rpm for the following 36 h at different temperatures (20, 25, 30, 35,

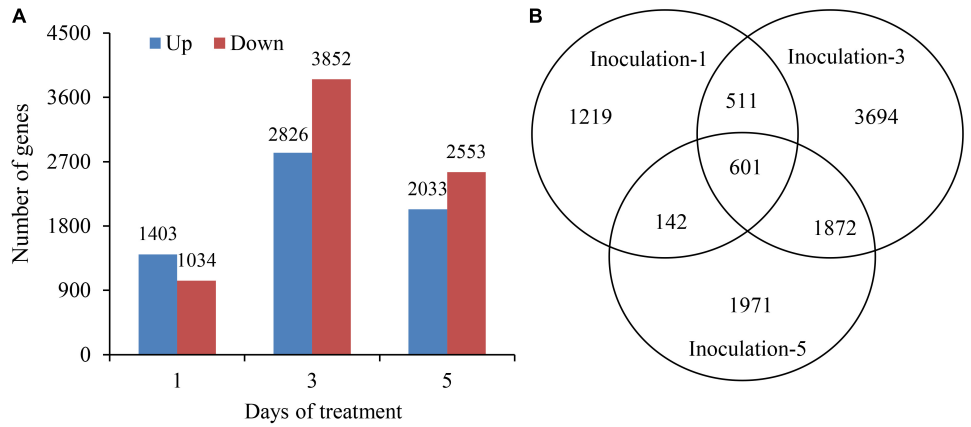

FIGURE 5 | Summary of differentially expressed genes at 1st, 3rd, and 5th days after inoculation. **(A)** Up and down regulated differentially expressed genes. **(B)** Venn diagram showing the differentially expressed gene at all three sampling conditions.

and 40°C, respectively) nitrogenase activity was measured. To determine the effect of pH on nitrogenase activity, culture medium pH was adjusted in the ranges of 6, 7, 8, and 9, using filter-sterilized solutions of sodium hypo-chloride (NaOCl) and hydrochloric acid (HCL), and strain GXS16 was inoculated and followed the 200 rpm on 28°C, and inoculated the acetylene gas, and nitrogenase activity was detected. Both tests were carried out in triplicate.

Effects of Carbon and Nitrogen Sources on Nitrogenase Activity

Sucrose and glucose were added as carbon sources to the sterile culture medium at various concentrations (1, 5, 10, and 15%). The culture GXS16-inoculated broth flasks were incubated at 28°C at 200 RPM, and nitrogenase activity was measured as described before. In addition, at doses of 0, 2, 4, 6, and 8 g/L, Ammonium sulfate [$(NH_4)_2SO_4$] and potassium nitrate (KNO_3) were utilized as nitrogen sources in the growth medium. The culture was incubated at 28°C and 200 rpm for 36 h before being used for the nitrogenase activity test. Both tests were carried out in triplicate.

Inoculation of Sugarcane Tissue Culture Seedlings With GXS16

Rooting tufted sugarcane tissue culture seedlings were divided into individual plants and placed in a culture flask (1/10 MS liquid media, without vitamins and plant hormones), as Lin et al. (2012) described. The seedlings underwent gnotobiotic culture at 30°C, 14 h light/10 h dark, and 60 μmol photons m^{-2} S^{-1} light intensity. After 7 days, the plants with consistent growth were transferred to plastic pots (21 cm in diameter and 19 cm high) with a mixture of thoroughly sterilized sand and perlite [v/v = 1:1], placed in a light incubator for adaptation, and watered with 200 mL 1/10 MS nutrient solution each time as required. After the new roots grew long enough, the GXS16 in the logarithmic phase were collected by centrifugation (30°C, 100 rpm for 5 min), washed twice with 1/10 MS culture solution, and diluted to 2×10^8 CFU mL^{-1} bacterial suspension. A suspension of 200 mL of bacteria was used to water the plant roots. An equal amount of sterile water was used as the control. The young roots of the plants were collected at 1, 3, and 5 days after inoculation. They were packaged in units, quick-frozen in liquid nitrogen, and stored at −80°C.

Measurement of Physiological Indices

The contents of proline (Pro), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) in the roots were calculated as described by Health and Packer (1968); Bates et al. (1973), and Jaleel et al. (2007), respectively. The activities of catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) were measured using commercial kits from Suzhou Comin Biotechnology Co., Ltd. (Suzhou, China) following the manufacturer's instructions.

The contents of abscisic acid (ABA) and gibberellic acid (GA_3) were measured as described by Iriti et al. (2009). The content of ethylene was measured using gas chromatography (GC) (Shimadzu, Tokyo, Japan). A total of 0.5 g of fresh root sample was placed in a 10 mL wide-mouth bottle with a rubber plug that contained 1 mL of pH 6.8 phosphate buffer to react for 24 h at 30°C in the dark. A volume of 1 mL of gas was extracted from the top of the wide-mouth bottle with a sterile injector. The content of ethylene was measured using Gas Chromatography. Measurement conditions: hydrogen flame ion detector, a temperature of 70°C, a temperature of 150°C in sample introduction chamber, detector temperature of 250°C, air pressure of 49 kPa, H_2 pressure of 70–80 kPa, and N_2 pressure of 110 kPa.

Detection of the Strain Copy Number

The Biospin Omni Plant Genomic DNA Extraction Kit (Bioer Technology, Hangzhou, China) was used to extract the root DNA, and the copy number of GXS16 in the roots was determined

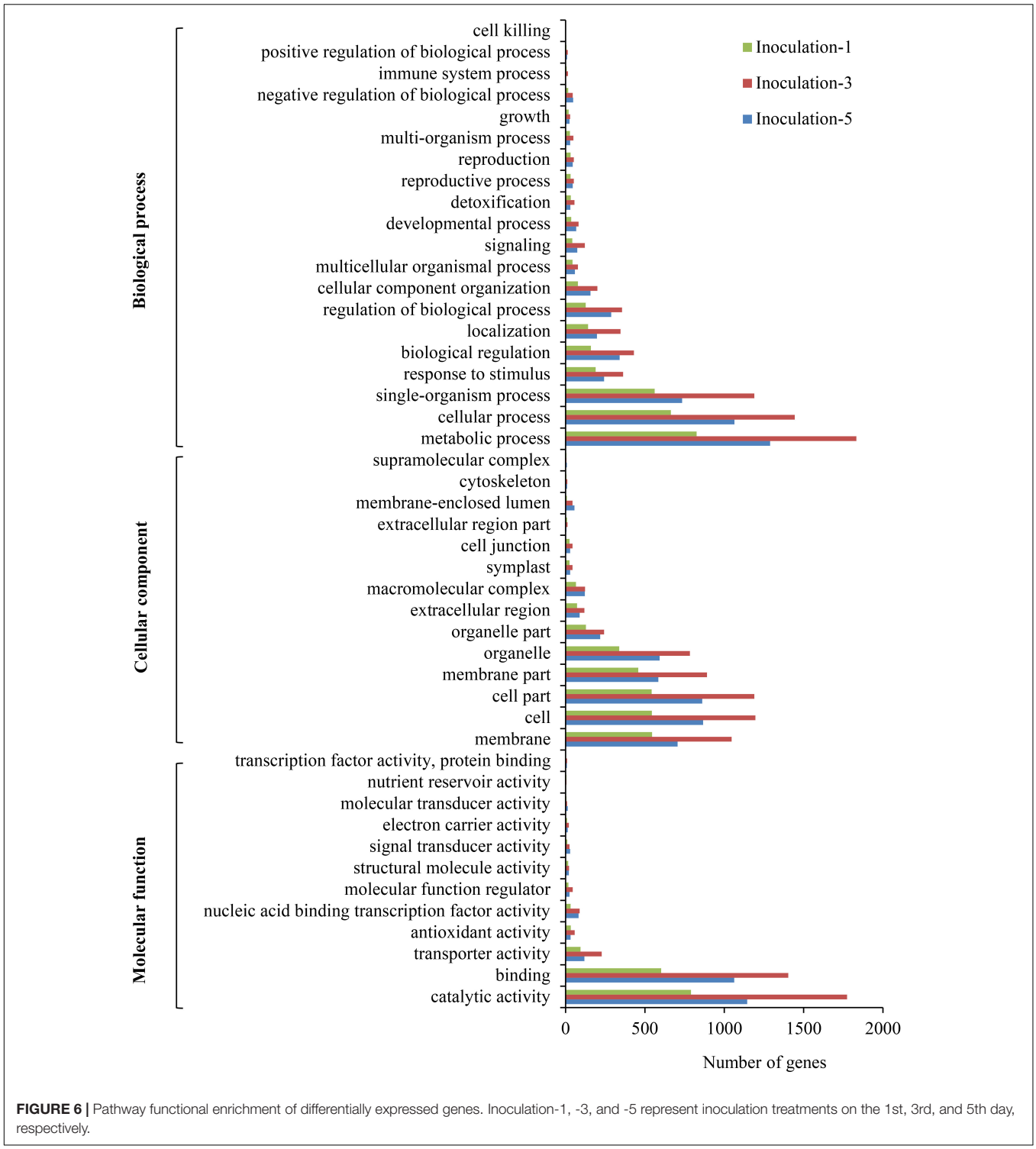

FIGURE 6 | Pathway functional enrichment of differentially expressed genes. Inoculation-1, -3, and -5 represent inoculation treatments on the 1st, 3rd, and 5th day, respectively.

using a TaqMan fluorescent probe. The forward and reverse primers were F: 5′-GCAGGCGGTTTGCTAAGACC-3′ and R: 5′-GCTTTCGTGCATGAGCGTCA-3′. The probe sequence was 5′-CGGGCTCAACCTGGGAACTGC-3′. The qRT-PCR reaction condition as: 2 × SYBR Green I Master Mix (TaKaRa, Dalian, China) 10 μL, 0.5 μL each of the 10 μM primers and probe, DNA template 5 μL, and ddH_2O supplemented to 20 μL. Reaction conditions are as follows: pre-denaturation at 95°C for 30 s; denaturation at 95°C for 5 s, annealing at 60°C for 30 s for 40 cycles. Following the completion of the process, the strain copy number in the roots was determined using a standard curve generated by the GXS16 16S rRNA gene plasmid.

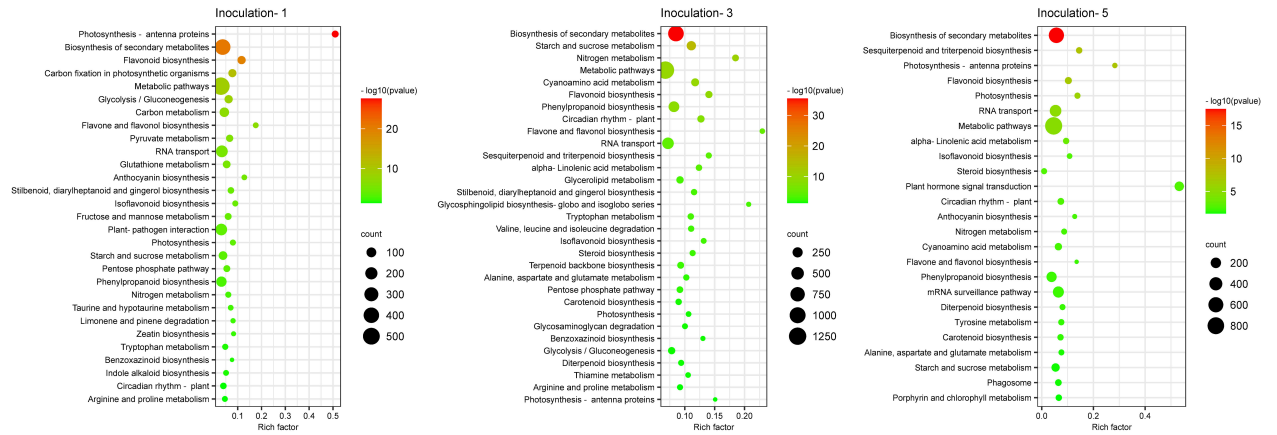

FIGURE 7 | Gene Ontology classification of differential expression genes.

De novo Assembly and Transcriptome Sequencing

BGI Technology Services Co., Ltd. (Shenzhen, China) created the RNA-Seq libraries for the various root samples, which were then sequenced using the BGI SEQ-500 platform. The raw data of the libraries have been uploaded to the National Genomics Data Center with the accession number CRA004951. After quality control, low-quality sequencing reads were filtered out from the raw data, and Bowtie (Langmead and Salzberg, 2012) was used to compare the filtered reads from each sample with the reference database. HTSeq (Anders et al., 2015) was used to calculate gene expression, which is indicated by the fragments per kilobase of transcript per million mapped fragments (FPKM) value. DESeq2 (Love et al., 2014) was used to analyze the gene expression differences between samples. The multiple of difference in expression as $|\log_2$ Fold Change$| \geq 1$, and the adjusted P-value ≤ 0.05, i.e., the gene expressed a significant difference between samples.

Gene Function Annotation

BLAST was used to compare the differentially expressed genes (DEGs) with NR, Swiss-Prot, the KEGG (Kyoto Encyclopedia of Genes and Genomes), and NT databases (threshold value is e-value $< 1e^{-5}$) for gene function annotation. Blast2GO (Conesa et al., 2005) was used for Gene Ontology (GO) annotation, and WEGO was used for GO classification statistics. The ggplot2 package of R language was used to map the bubble diagram for pathway enrichment analysis.

Validation of RNAseq Results by Quantitative Real-Time PCR

Sugarcane *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was selected as the reference gene. Among the DEGs screened, 10 that were involved in ethylene or ABA metabolic pathways were randomly selected, and specific primers were designed (**Supplementary Table 1**). The transcriptome RNA from related root samples was extracted and converted into cDNA, and gene expression was confirmed using quantitative real-time PCR (qRT-PCR). The reaction condition is as follows: 10 μL 2 × All-in-One qPCR Mix (Gene Copoeia, Rockville, MD, United States), 2 μL cDNA, and 1 μL each of the 4 μM primers and ddH_2O up to 20 μL. The reaction proceeds as follows: pre-denaturation at 95°C for 10 min; denaturation at 95°C for 10 s, annealing at 60°C for 20 s, extension at 72°C for 20 s of 40 cycles. Relative expression quantification of the selected DEGs to control *GAPDH* was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Data Analysis

Data were analyzed using standard ANOVA followed by the Duncan's Multiple Range Test (DMRT). The SPSS statistical software was used to accomplish all analyses (SPSS Version 23, SPSS Inc., Chicago, IL, United States). Differences were considered significant at the $p < 0.05$ level. All experiments were carried out in replicate, and the results are given as mean values.

RESULTS

Colony Morphology and Colonization of Green Fluorescent Protein Tagged Strain GXS16

Figures 1A,B depicts the colony characteristics as well as the strain's corresponding GFP-tagged microscopic features. Images taken using a fluorescent confocal microscope indicated that the GFP-labeled strain had infiltrated the roots of sugarcane plantlets (**Figure 1**). We discovered tagged bacteria penetrate the root surface and elongation zones 1 day after inoculation (**Figure 1D**). 3 days after inoculation tagged strain GXS16 was attached to roots alone or in clusters (**Figure 1E**). Furthermore, GFP-tagged bacterial cells were seen as clusters and patches in the root maturation zone on the 5 days after inoculation (**Figure 1F**). Colonization of GFP-tagged bacteria was readily visible as little specks of green fluorescence.

Nitrogenase Activity of Strain GXS16

The strain has increased nitrogenase activity in the early testing. As a result, it was employed for further characterization under various physiological and nutritional settings. The nitrogenase activity of the strain was substantially more significant at 30 and 45°C as compared to 20°C, with the maximum at 30°C (**Figure 2A**), whereas the greatest nitrogenase activity was obtained at pH 8 (**Figure 2B**). In addition, sucrose and glucose were used as carbon sources. The strain had increased nitrogenase activity with 5% sucrose, whereas glucose had comparable tendencies with various concentrations (1–10%), however, the higher sucrose content had a negative effect on nitrogenase activity (**Figure 2C**). In terms of nitrogen sources, the maximum nitrogenase activity was seen at 4 and 6 g/L of $(NH4)_2SO_4$ and KNO_3, respectively (**Figure 2D**).

De novo Assembly and Transcriptome Sequencing

After assembling all samples together and filtering the abundance, we got 181,345 Unigenes, the total length, average length, N50, and GC content of Unigenes are 243161,718 bp, 1340 bp, 2077 bp and 49.37% respectively. And then annotate Unigenes by aligning with 7 functional databases, finally, 116233 (NR:64.09%), 129265 (NT:71.28%), 72209 (Swissprot:39.82%), 79,321 (KOG:43.74%), 84775 (KEGG:46.75%), 49965 (GO:27.55%), and 74188 (InterPro:40.91%) Unigenes are annotated. Venn diagram show the annotation result of NR, KOG, KEGG, SwissProt, and InterPpro (**Supplementary Figure 1**).

Change of Strains Number in Sugarcane Roots

The sugarcane roots at different inoculation times were sampled, and the copy number of GXS16 in the roots was determined using absolute quantitative real-time PCR. copy number calculation formula obtained from the 16S rRNA gene plasmid reaction result was as follows: $Y = -3.406x + 37.05$ [y: *Ct* value; x: Log_{10} (copy number)]. As shown in **Table 1**, the number of GXS16 strains in the roots rapidly increased with the extension of inoculation time.

Changes in Physiological Parameter in Sugarcane Roots

On the first day after inoculation, the contents of H_2O_2 in the roots increased by 15.8% as compared to the control, with the difference reaching a significant level; however, on the third and fifth days after inoculation, the contents of H_2O_2 in the roots increased by 4.1 and 1.9%, respectively, compared with the control, with no significant difference (**Figure 3A**). The whole treatment period, there was no significant variation in the content of Pro in the roots (**Figure 3B**). On the first day after inoculation, the content of MDA in the roots increased by 6.4% compared with the control, but on the third and fifth days after inoculation, the content of MDA did not change significantly from the control (**Figure 3C**). On the first day after inoculation, the ethylene content in roots increased by 29.9%, while on the third day, it decreased by 23.3%, with significant differences. In addition, on the fifth day after inoculation, the ethylene contents in the two treatments did not differ significantly (**Figure 3D**). On the first, third, and fifth days after inoculation, the contents of ABA increased by 91.9, 43.9, and 18.7% compared with those of the control, respectively, with significant differences (**Figure 3E**). In contrast to the changing trend of ABA, the contents of GA_3 decreased by 12.9, 28.5, and 45.2% compared with the control, respectively, with significant differences (**Figure 3F**).

Changes of Antioxidant Enzymes Activities in Sugarcane Roots

The activities of CAT significantly increased by 31.3 and 26.6%, respectively, on the first and fifth days after inoculation. Still, there was no significant difference in CAT activity between the two treatments on the third day (**Figure 4A**). There were no substantial changes detected in POD activity within 5 days of inoculation (**Figure 4B**). On the first day after inoculation, the SOD activity of the roots was significantly lower than that of the control. On the third day after inoculation, the SOD activity increased by 11.6%, with significant differences (**Figure 4C**). The activities of PPO and APX were significantly higher than those of the control on the first and third days after inoculation, increasing by 15.2 and 19.9% and 8.4 and 9.9%, respectively, but on the fifth day after inoculation, there was no significant difference in these activities compared with the control (**Figures 4D,E**). On the third day after inoculation, the activity of GR increased by 13.7%, with a significant difference (**Figure 4F**).

Identification of Differentially Expressed Genes

The DEseq2 algorithm was used to detect DEGs in the samples at each inoculation period compared with the control. The total numbers of DEGs on the first, third, and fifth days after inoculation were 2,437, 6,678, and 4,586, respectively, and the highest number of DEGs was on the third day after inoculation. The numbers of up and downregulated DEGs in different sampling days were 1,403 and 1,034; 2,826 and 3,852; and 2,033 and 2,553, respectively. Additionally, the DEGs were preferentially induced in the early inoculation stage (first day), and the downregulated expression of genes played a dominant role in the latter stage (fifth day) (**Figure 5A**). Notably, there were 601 genes significantly expressed overlapped in all three sampling conditions (**Figure 5B**).

Gene Ontology Classification of Differentially Expressed Genes

The GO annotations for DEGs were submitted to the Web Gene Ontology Annotation Plot (WEGO) for GO classification. In summary, the DEGs were assigned to 46 GO categories (**Figure 6**). Among them, 20, 14, and 12 categories belonged to biological processes, cellular components, and molecular functions, respectively. In the biological process category, the metabolic process, cellular process, and single-organism process comprised the top three terms with respect to the number of DEGs. For cellular components, the cell, cell part, and membrane part categories were the top three with respect to the number

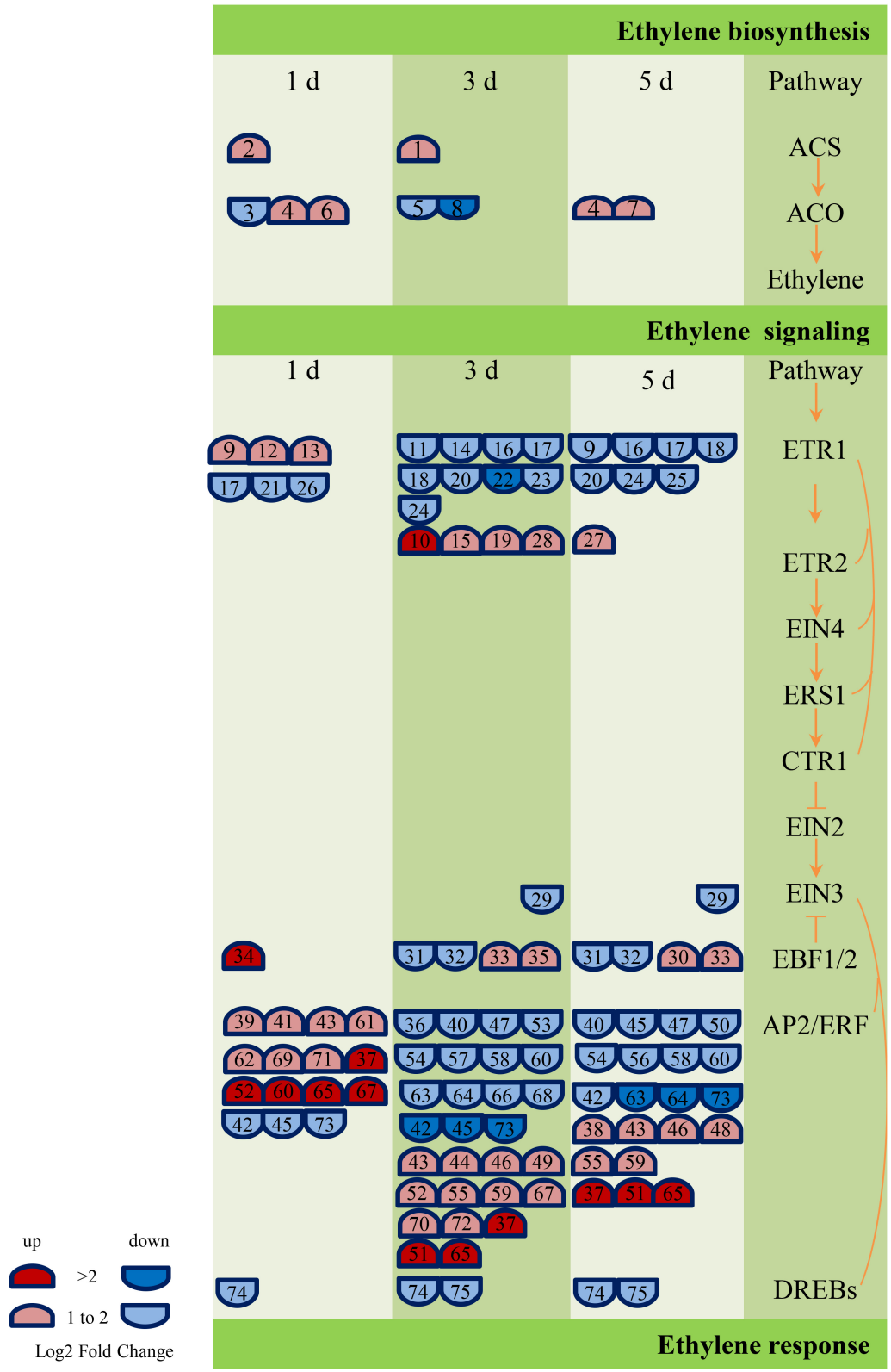

FIGURE 8 | Ethylene biosynthesis and signaling-related genes responses to GXS16 colonization in sugarcane roots. Gene expression levels (log2 fold change) are represented as colors and symbols. Red stands for upregulated and blue stands for downregulated genes. The DEGs are identified with numbers which are illustrated in **Supplementary Table 5**.

of genes. The DEGs were mainly involved in catalytic activity, binding, transporter activity, antioxidant activity, and nucleic acid binding transcription factor activity.

Pathway Classification of Differentially Expressed Genes

A KEGG database comparison showed that the DEGs could be annotated into five level-1 or 19 level-2 metabolic pathways. In the specific metabolic pathways, on the first, third, and fifth days after inoculation, a total of 1,932, 4,852, and 3,400 DEGs were annotated into 120, 131, and 127 metabolic pathways, respectively (**Supplementary Table 2**), and there were 29, 31 and 25 metabolic pathways with DEGs that were significantly enriched ($P \leq 0.05$), respectively, in each sampling days (**Figure 7**), among which 12 metabolic pathways were improved considerably in each inoculation period, including the biosynthesis of secondary metabolites, circadian rhythm-plant, flavone and flavonol biosynthesis, flavonoid biosynthesis, isoflavonoid biosynthesis, nitrogen metabolism, metabolic pathways, phenylpropanoid biosynthesis, photosynthesis, photosynthesis-antenna proteins, rRNA transport, and starch and sucrose metabolism.

Plant Hormone Related Genes Analysis

According to the annotation results of GO and KEGG, the genes related to ABA, GA, and ethylene metabolic pathway were screened further, and 17 (**Supplementary Table 3**), 42 (**Supplementary Table 4**), and 75 (**Supplementary Table 5**) DEGs were obtained, respectively. These genes are engaged in biochemical activities such as biological processes of biosynthesis, metabolism, transport, or signal transduction of these plant hormones. However, the number of genes related to ethylene metabolism was much higher than GA and ABA. In addition, most of the genes related to the ABA metabolic pathway (NCED, PP2C, and SNRK2) were downregulated, and most of the GA signal transduction gene DELLA was upregulated, while the other signal transduction genes (GID1 and PIF) were primarily downregulated. The largest number of DEGs (38) encoded the ethylene signaling gene AP2/ERF (Ethylene-responsive transcription factor). The AP2/ERF genes were predominantly upregulated on the first day of inoculation (12 genes were upregulated, and three genes were downregulated), and the number of upregulated and those of the downregulated genes were equal on the third and fifth days of inoculation. **Figure 8** and **Supplementary Table 5** show the details of the number and expression fold changes of the ethylene signal transduction genes.

Validation of RNA-Seq Data by Quantitative Real-Time PCR

To verify the results of transcriptome sequencing analysis, 10 DEGs occupied in the ethylene and ABA metabolic pathways (**Table 1**) were randomly selected for qRT-PCR detection. The correlation coefficient R^2 of data on the change of gene expression obtained by the two methods reached 0.95 (**Figure 9**), which indicates that the transcriptome sequencing data in this study are reliable and repeatable.

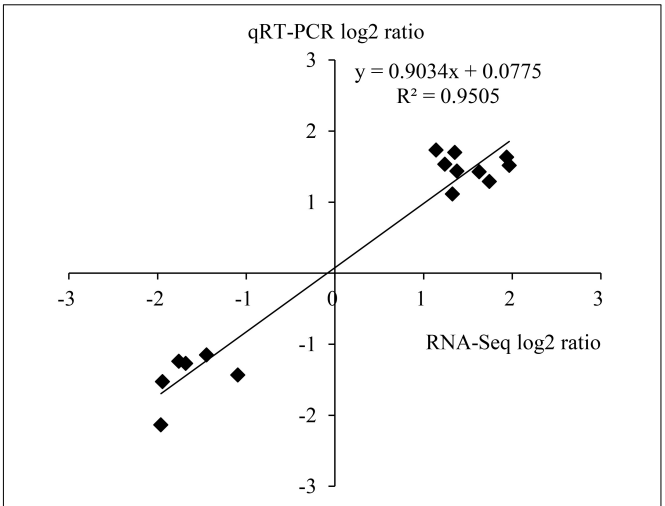

FIGURE 9 | Correlation between RNA-seq and qRT-PCR.

DISCUSSION

In the traditional methods of cultivating sugarcane in China, the excessive application of nitrogen fertilizer poses a severe problem (Yang et al., 2014) that leads to high costs of sugarcane production and further reduces the international competitiveness of its sugarcane industry. Furthermore, due to the low rate of nitrogen use in sugarcane production (about 14.5–24.7%), a considerable portion of the nitrogen is volatilized, leached, and absorbed by the soil. Resulting in the loss of a large amount of nitrogen, which causes severe waste and becomes an essential non-point source pollution factor after entering the environment (Li and Yang, 2015). Making full use of sugarcane's biological nitrogen fixation characteristics and reducing the amount of nitrogen fertilizer applied are effective ways to minimize fertilization and increase benefits. It is also highly significant in promoting the sustainable development of the sugarcane industry in China (Li and Yang, 2015). The roots have been shown to have an essential role in the biological nitrogen fixation of sugarcane. Compared with other tissues and organs, the roots provide better carbohydrates for the function of nitrogenase, and the partial pressure of oxygen in the roots and their surroundings is lower, which can effectively prevent the oxidation of nitrogenase. Therefore, it contributes to a higher percentage of nitrogen fixation (Hu et al., 2017). Close interaction between the NFB and sugarcane roots is the basis for NFB to fix nitrogen effectively. The present study discovered that GFP-tagged bacteria successfully colonize the sugarcane root and bacteria enter root maturation zone at fifth day. The number of GFP-tagged bacteria rose as the inoculation period increased, as seen by microscopic characteristics, and this is confirmed by quantitative real-time PCR. The quantity of gene copies rose as the inoculation time increased. Wei

et al.,2014 used fluorescence microscopy to colonize a GFP-tagged *Klebsiella* strain in the interior of the roots and aerial portions of micropropagated sugarcane plantlets. *Burkholderia anthina* MYSP13 strain was also found in lateral root emergence locations indicating that colonization occurred in sugarcane root and shoot (Malviya et al., 2019). Recently endophytic plant growth promoting strain *Enterobacter roggenkampii* ED5 labeled with GFP had been effectively colonized the sugarcane tissues (Guo et al., 2021).

In this study, we established that GXS16 has great nitrogen fixation capability in a somewhat alkaline atmosphere and strong nitrogenase activity at temperatures of 40°C, allowing it easier to survive and play a role in nitrogen fixation in sugarcane tropical and subtropical environment. According to Luna et al. (2000), the carbon supply is sometimes more significant than the nitrogen source for *Acetobacter diazotrophicus* strains with nitrogen fixation capabilities. In this investigation, sucrose outperformed glucose for the investigated strain GXS16. Xing et al. (2016) discovered that The nitrogenase activities of *Stenotrophomonas maltophili* and *Agrobacterium tumefaciens* rose with rising concentrations of $NH4^+$ and $NO3^-$ in a limited range, but when the nitrogen concentration exceeded the critical values, the nitrogenase activity of the two strains declined. This is consistent with the current study's findings. Actinobacteria strain also showed similar nitrogenase activity at varying temperatures, pH levels, and carbon and nitrogen sources, according to Wang et al. (2016).

The RNA-seq approach was employed in this work to investigate the molecular basis of interaction between sugarcane cultivars in response to an N-fixing endophytic strain inoculation, to observe the physiological and hormonal changes in sugarcane plant roots. The defense response plays a vital role in the perception of bacterial invasion by plants. When foreign bacteria invade a plant, even if the phenotype of the plant has no noticeable change, the internal tissues and cells of the plant will undergo programmed death owing to the invasion of the bacteria, and a series of ROS and similar compounds are generated to kill the invading microorganisms (Alqueres et al., 2013; Zhou et al., 2015). As signal molecules, a low concentration of ROS regulates the expression of genes in cells. In contrast, a high concentration of ROS will cause severe damage to cell membranes and proteins, with lipid peroxidation of the cell membranes as the most typical case. The content of MDA indicates the severity of injury (Singh et al., 2016). By using sugarcane variety RB86-7515 as tissue culture seedlings, this study examined the effects of inoculation of the dominant endogenous NFB GXS16 on the physiological and biochemical products and expression of genes of the sugarcane roots. H_2O_2 and MDA varied consistently in the roots. They were significantly higher than the control in the early stage of inoculation. Still, They showed no significant change during the middle and later stages, which indicates that GXS16 only triggers the root defense response in the early stage of inoculation and has no significant effect in the middle and later stages. It also confirms the results of previous studies (Vandenkoornhuyse et al., 2015), which have shown that the response of plants to stress can initiate the protective enzymatic clearance system (Zhang et al., 2019), the non-enzymatic clearance system (Zhan et al., 2021), and other approaches to ensure the normal function of their own cells. The clearance of active oxygen in plants is completed collaboratively by the protective system of antioxidant enzymes and compounds. In this study, the activities of CAT, SOD, PPO, APX, and GR in the roots increased significantly following inoculation with GXS16, which could be the reason why the contents of ROS and MDA in the middle and late stages (third and fifth days after inoculation) did not differ significantly from those in the control. In addition, ROS at low concentrations can be used as signal molecules to regulate the expression of genes in root cells, which is conducive to the colonization of roots by nitrogen-fixing strains. The change in the copy number of GXS16 strains in roots shows that the number of cells in colonized roots increases with the extension of inoculation time. It has also been demonstrated that proline, as an osmoregulatory substance, also affects the production and clearance of ROS (Gerona et al., 2019). In this study, there was no significant difference in proline content between the roots inoculated with GXS16 and the control, indicating that antioxidant enzymes also play a dominant role in the elimination of ROS.

Plant hormones play important role in the resistance response to endophytic bacterial colonization (Ibort et al., 2018). In this study, the content of ethylene in the roots increased first and then decreased after inoculation with GXS16. Although the increment gradually reduced, the content of ABA was significantly higher than that of the control during the whole treatment period, and the trend in the change of GA_3 was opposite to that of ABA. Previous studies have shown that ABA-dependent metabolic pathways play vital role in the response of plants to abiotic stress (Li et al., 2015, 2019). The content of endogenous ABA in plants under stress increased rapidly. In this study, 17 DEGs related to ABA metabolism were obtained by transcriptomic sequencing, including the NCED gene, which is associated with ABA biosynthesis, and the PP2C and SnRK2 genes that are related to ABA signal transduction. Under normal conditions, PP2C combines with SnRK2 to dephosphorylate SnRK2 and deactivate it (Chen et al., 2020). ABA combines with a receptor protein and then acts on PP2C to change its conformation and inhibit its enzyme activity. The activity of SnRK2 negatively regulated by PP2C can be released to activate the expression of target genes in response to ABA signals and control the reaction of plants to stress conditions (Chen et al., 2020). However, whether sugarcane roots respond to the colonization of NFB GXS16 through this pathway remains to be clarified.

Different plant hormones can exert their biological functions synergistically or antagonistically through complex and cross-connected network-like regulatory pathways to regulate the process of plant development (Berens et al., 2017). As a pair of classical hormone combinations, GA and ABA have antagonistic biological effects on seed dormancy, taproot development, seedling growth, and stress responses (Lin et al., 2015; Shu et al., 2018). This could be why the trend in variation of GA_3 content

in this study was opposite to that of ABA. Studies indicate that ethylene biosynthesis and its signal transduction components play an essential role in the adaptation of plants to colonization by bacterial endophytes (Ibort et al., 2018; Nascimento et al., 2018). In this study, there were 75 DEGs involved in ethylene biosynthesis (*ACS* and *ACO*) or signal transduction (CTR1, ENI3, EBF1/2, AP2/ERF, and DREB). The diversity of both gene number and expression is much higher than that of DEGs related to the metabolism of ABA and GA, indicating that ethylene could play a more important role in the response of sugarcane roots to GXS16 colonization. When the plants are subjected to stress, ethylene will be produced, and high levels of ethylene can inhibit the elongation of plant roots and promote the aging and abscission of plant organs (Pan et al., 2019; Singh et al., 2021). 1-Aminocyclopropane-1-carboxylic acid (ACC) is a direct precursor of ethylene biosynthesis (Pattyn et al., 2021). Studies have shown that under adverse conditions, some bacteria that have ACC deaminase activity and colonize plants can absorb the ACC secreted by plant cells and catalyze the ACC deamination reaction through ACC deaminase to generate α-ketobutyric acid and NH_3 as carbon and nitrogen sources for bacterial growth (Barnawal et al., 2017; Liu et al., 2021), respectively, reducing the concentration of ACC in plant cells and reducing the production of ethylene, which alleviates the inhibition of accumulation of ethylene on plant growth. Our previous study showed that the NFB GXS16 have high ACC deaminase activity (Nong et al., 2021). However, more research is needed to clarify whether it is involved in the regulation of plant hormones in the response of roots to colonization by GXS16 and whether it is correlated with the number of DEGs and the diversity of gene expression.

CONCLUSION

The plant hormones GA, ABA, and ethylene all play important roles in sugarcane roots' response to GXS16 colonization. GXS16 inoculation increases the amount of H_2O_2, MDA, and ABA in sugarcane roots while decreasing the concentration of GA3. The ethylene concentration rises at first, then falls. The activity of antioxidant enzymes in the roots increases dramatically, which effectively clears the formation of excess ROS and lowers the buildup of MDA-induced membrane lipid peroxidation products.

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

CL and Y-RL initiated and designed the research. QN, MM, LL, JX, ZM, ZW, X-PS, and XH performed the laboratory experiments together. QN, CL, MM, and MS wrote the manuscript. MS, AS, SR, and Y-RL revised the manuscript. All authors reviewed the manuscript and approved it for publication.

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Differential response of bacterial diversity and community composition to different tree ages of pomelo under red and paddy soils

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Rhizosphere soil microbial communities substantially impact plant growth by regulating the nutrient cycle. However, dynamic changes in soil microbiota under different tree ages have received little attention. In this study, changes in soil physicochemical properties, as well as bacterial diversity and community structures (by high-throughput Illumina MiSeq sequencing), were explored in pomelo trees of different ages (i.e., 10, 20, and 30 years) under red and paddy soils cultivated by farmers with high fertilizer input. Moreover, soil factors that shape the bacterial community, such as soil pH, AP (available phosphorous), AK (available potassium), and AN (available nitrogen), were also investigated. Results showed that pH significantly decreased, while AP, AK, and AN increased with increasing tree age under red soil. For paddy soil, pH was not changed, while AP was significantly lower under 10-year-old pomelo trees, and AK and AN contents were minimum under 30-year-old pomelo trees. Both soil types were dominated by *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* and showed contrasting patterns of relative abundance under different tree age groups. Bacterial richness and diversity decreased with increasing tree age in both soil types. Overall, bacterial community composition was different under different tree ages. RDA analysis showed that soil pH, AP, and AN in red soil, and pH and AP in paddy soil showed the most significant effects in changing the bacterial community structure. A random forest model showed *Sinomonas* and *Streptacidiphilus* in red soil, while *Actinoallomurus* and *Microbacterium* in paddy soil were the most important genera explaining the differences among different age groups. The ternary plot further revealed that genera enrichment for Age_30 was higher than that for Age_10 and Age_20 in red soil, whereas specific genera enrichment decreased with increasing tree age under paddy soil. Co-occurrence network revealed that bacterial species formed a complex network structure with increasing tree age, indicating a more stable microbial association under 20 and 30 years than 10-year-old pomelo trees. Hence, contrasting patterns of changes in soil physicochemical properties and soil

microbial communities were recorded under different tree ages, and tree ages significantly affected the bacterial community structure and richness. These findings provide valuable information regarding the importance of microbes for the sustainable management of pomelo orchards by optimizing fertilizer input for different ages of trees.

KEYWORDS

pomelo orchard, tree ages, bacterial community, environmental factors, red and paddy soils

Introduction

Soil microbes, especially bacteria, which represent the most abundant group accounting for ≈80% of total microbiota in the terrestrial ecosystem (Gans et al., 2006), play central roles in various biochemical and ecological processes, including carbon and nitrogen cycles and decomposition of organic matter (Gougoulias et al., 2014; Diao et al., 2020). Various factors, like soil pH, available phosphorous (AP), and carbon-to-nitrogen ratio (C:N ratio), may influence soil bacterial diversity (Li et al., 2021; Sheng et al., 2021). It is generally known that tree age and species can affect soil microbial diversity and community composition directly or indirectly by changing the circulation of soil nutrients (Sun et al., 2014; Du et al., 2018). The variation in soil microbial population reflects the dynamics of nutrient cycling and soil health. Hence, it is imperative to figure out how the bacterial population changes in response to varying tree ages.

Previous studies have shown that soil microbial communities vary under different stand ages. For example, *Robinia pseudoacacia* and *Pinus tabuliformis* have distinctive soil bacterial populations (Dang et al., 2017; Liu et al., 2018). These differences in bacterial community and diversity could result from an increase in the tree canopy density with increasing tree age, which in turn reduces the light availability and increases the soil moisture content, leading to structural changes of the microbial community (Qu et al., 2020). So far, various studies have examined the changes in bacterial diversity and community composition across different tree ages in the forest tree species (Wu et al., 2015; Zhou et al., 2017; Zhu et al., 2019). However, few studies have explicitly focused on how the host age could affect the soil microbial diversity and community composition of the orchards, especially in the citrus orchard.

Citrus, an evergreen fruit tree grown in tropical and subtropical regions, is the leading fruit crop globally and contributes to many countries' economies and fruit production (Liu et al., 2012). China is among the world's top citrus producers, with an annual production of 4,406 × 10^4 tons (Huang et al., 2021). Pomelo (*Citrus grandis*) is China's third most popular citrus type after *Citrus reticulata* and *Citrus sinensis* (Li et al., 2015). Hence, the land-use change results in diverse management practices, including fertilization, cultivation methods, and irrigation, which significantly affect the soil quality and may lead to changes in soil microbial diversity and community composition (Guo et al., 2019). Therefore, understanding the underlying differences in microbial communities in the red and paddy soils is of great interest. Moreover, it has also been documented that as the stand age increases, soil nutrient status is also changed, and as a result, soil microbial communities change, including forest and orchard systems (Kyaschenko et al., 2017; Zhang et al., 2017; Antisari et al., 2018; Wu et al., 2020). Citrus belongs to perennial tree species that frequently undergo progressive growth and changes year by year, and plant age substantially affects rhizosphere bacterial composition (Ji et al., 2019). Many earlier research studies have emphasized the effects of planting on soil physicochemical properties. By contrast, little attention has been paid to how the changes in planting age could affect the soil microbial communities.

In this study, we used high-throughput Illumina MiSeq sequencing technology to investigate the bacterial community structures in pomelo trees of different ages under the red and paddy soils. This study aimed to determine (a) bacterial diversity and community composition under pomelo trees of different ages, (b) the environmental factors influence the bacterial community composition, and (c) the key species of bacterial communities residing in the red and paddy soils. These research findings could lead to future sustainable management of pomelo trees.

Materials and methods

Sampling site and collection of soil samples

This study was conducted in Pinghe County (24°02′-24°35′N, 116°54′-117°31′E), Fujian Province, southern China, which belongs to a subtropical monsoon climate with an annual average temperature of 25.38°C and humidity 1,600 mm (Yan X. et al., 2021). The soil samples were collected from red and paddy soils of the pomelo orchards of Pinghe County. These soil samples were collected from pomelo trees of different ages, that

is, 10 years (Age_10), 20 years (Age_20), and 30 years (Age_30). A total of 57 soil samples were collected from red soil (i.e., topsoil 0–20 cm), and 10-, 20-, and 30-year-old trees had 20, 22, and 15 samples. In the case of paddy soil, 32 soil samples were collected from topsoil, and 10-, 20-, and 30-year-old trees had 15, 11, and six samples because of the limited availability of trees in the selected groups of ages. Soil samples were transported to the laboratory on ice. Soil samples were sieved through a 2-mm-diameter mesh, and the straw residues and fine roots were removed manually. The soil samples were divided into parts for determining soil properties (stored at 4°C) and molecular analysis (stored at −80°C).

Determination of soil physicochemical properties

In order to determine soil pH, we prepared a soil/water suspension in the ratio of 1:2.5 (*w/v*), and it was measured by using a pH meter (ORION A215STAR, Thermo Ltd., USA) (Zhang et al., 2021). Available nitrogen (AN) was determined by sodium hydroxide (NaOH) hydrolysis. In short, soil was treated with NaOH, hydrolyzed-N was converted into NH_3, and then absorbed by H_3BO_3. At the final stage, hydrolyzed-N was titrated with H_2SO_4 (Zhou et al., 2022). Available phosphorous (AP) was extracted using 0.5 mol L^{-1} of sodium bicarbonate ($NaHCO_3$) at pH 8.5 and quantified by a SpectraMax M4 spectrophotometer (Molecular Devices, Ca, USA) (Yan et al., 2022). Available potassium (AK) was extracted with ammonium acetate solution ($NH_4CH_3CO_2$) and quantified by flame photometry (FP6410, INESA, China) (Guo et al., 2022).

Soil DNA extraction

For total soil DNA extraction, 0.5 g of soil sample was used for each soil type of red and paddy soils by following the instructions of the soil DNA extraction kit (MO-BIO Laboratories, Carlsbad, CA-USA). To check DNA purity and concentration, agarose gel electrophoresis and a Nanodrop-2000 spectrophotometer (Thermo-Scientific) were used. An adequate sample quantity was collected in a centrifuge tube, diluted with sterile water to 10 ng/μl, and stored at −40 °C for further analysis.

PCR analyses and high-throughput sequencing

To amplify the soil DNA, specific primers of 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAGT-3′) were used, which cover the bacterial 16S V4–V5 amplification region. PCR was performed with a 25 μl mixture containing PCR buffer (1×), $MgCl_2$ of 1.5 mM, deoxynucleoside triphosphate of 0.4 mM, and 0.5 U TaKaRa Ex-Taq. The concentration of soil genomic DNA was 10 ng, and each primer was 1.0 μM. The PCR was performed with an initial denaturation step at 94°C for 3 min, followed by 30 cycles at 94°C for 40 s, 56°C for 60 s, and the final extension at 72°C for 10 min. After each sample's PCR amplification, the two PCR results were combined (Li et al., 2015). Gel electrophoresis containing 1% agarose was used to run both PCR products, and the targeted bands of DNA were extracted and purified with a gel extraction kit. To quantify PCR products, a NanoDrop (Thermo Scientific NanoDrop-2000) spectrophotometer was used. For sequencing, the samples were prepared according to the manufacturer's instructions of the TruSeq DNA kit. Qubit and qPCR were used to analyze the constructed library and then sequenced on the Illumina Miseq system (Bobett Biotechnology Co., Ltd., Sichuan, China).

Sequencing data processing

The raw sequences were processed with the default parameters and UPARSE workflow using the Quantitative Insights Into Microbial Ecology (QIIME v1.9.0) tool. The low-quality reads, primers, and barcode sequences were all excluded from the analysis. At 97% sequence similarity, the operational taxonomic units (OTUs) were grouped. The sequencing data are available at the NCBI BioProject SRA database under accession number PRJNA779204.

Data analyses

Species richness and alpha diversity were calculated based on alpha diversity indices, including Observed, Chao1, and Shannon. We used KW (Kruskal–Wallis) tests to determine the significant difference in the alpha diversity index between different tree age groups in the red and paddy soils. The variation in bacterial beta diversity among different tree ages was assessed using principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity. The permutational multivariate analysis of variance (PERMANOVA) method was also used to investigate whether bacterial populations differed significantly between different tree ages. Random forest analysis was applied to identify the most important bacterial taxa in red and paddy soils by using a random forest package. To further investigate which bacterial community significantly increased or decreased under different tree ages, ternary plot analysis was performed in R language-based package DESeq2, ggplot2, and grid to investigate the enriched and depleted bacterial community among different tree age groups. To visualize the overlapping and unique enriched genera among different age groups, we

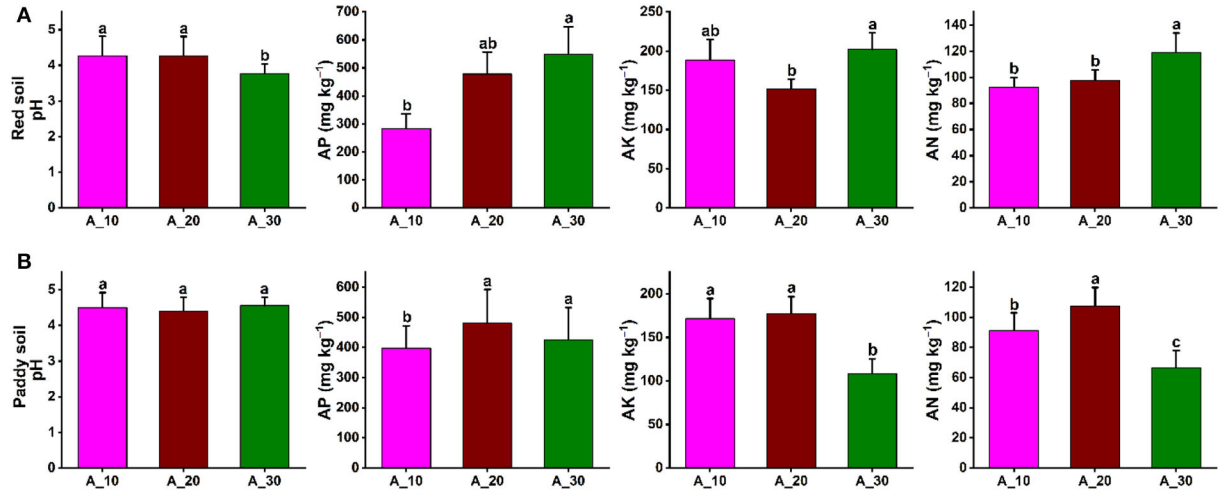

FIGURE 1
Variations in soil properties under different tree ages. Variation in soil physicochemical properties (soil pH, AP, AK, and AN) under different groups of tree ages for **(A)** red soil **(B)** paddy soil. Here, AP, AK, and AN represent available phosphorous, available potassium, and available nitrogen, respectively. Different lowercase letters indicate the significant differences ($p < 0.05$) among different pomelo trees age groups of Age_10 (10 years old), Age_20 (20 years old), and Age_30 (30 years old).

used Venn diagrams (Venny 2.1.0) (Pang et al., 2021, 2022). Based on Spearman's correlation, we explored the network of co-occurrences of bacterial communities. The OTUs were selected based on significant correlation ($P < 0.01$, $\rho > 0.7$). Taxonomic abundances were analyzed pairwise, resulting in an extremely complex network in which each node represents a phylum and the internodes represent significant associations. Gephi V0.9.2 was used to visualize and modularize co-occurrence. All these statistical analyses were performed using the R package "microeco v0.2.0" (Liu et al., 2020).

Results

Changes in soil physicochemical properties under different tree ages

In red and paddy soils of pomelo orchards, the soil physicochemical properties were significantly affected by increasing tree age. In red soil, soil pH was significantly decreased with increasing tree age, that is, 4.26, 4.27, and 3.76 under 10-, 20-, and 30-year-old trees, respectively. By contrast, AP, AK, and AN significantly increased with increasing tree age, and maximum contents of 549, 202, and 119 mg kg^{-1} were recorded, respectively (Figure 1A). In the case of paddy soil, tree age also had a significant effect on soil physicochemical properties. The soil pH was not significantly changed with increasing tree age, and the average soil pH of 4.48 was recorded. The AP was significantly lowest for 10-year-old trees. The AK and AN contents also decreased significantly with increasing tree age, and minimum contents were observed in 30-year-old pomelo trees. AK contents were 172, 177, and 108 mg kg^{-1}, while for AN, 91, 107, and 67 mg kg^{-1} contents were recorded for 10-, 20-, 30-year-old trees, respectively (Figure 1B). These results showed contrasting patterns of changes in soil physicochemical properties under different tree ages in the red and paddy soils.

Variation in the bacterial community structure under different tree ages

Overall, 24,327 OTUs were obtained after removing chimeras and resampling. Red soil was dominated by bacterial profiles of *Proteobacteria* (44.03%), *Acidobacteria* (16.51%), *Actinobacteria* (13.19%), *Chloroflexi* (8.67%), *Bacteroidetes* (3.16%), *AD3* (1.50%), *Gemmatimonadetes* (1.24%), and *Firmicutes* (0.97%). In different groups of tree ages, the average relative abundance of *Proteobacteria* was highest in Age_10 (41.26%), Age_20 (47.90%), and Age_30 (46.96%) compared to other phyla. However, relative abundance of *Proteobacteria* increased with increasing tree age. The second most abundant phylum was *Acidobacteria*, with average relative abundances of 17.76, 15.14, and 18.68% in Age_10, Age_20, and Age-30, respectively. The relative abundance of *Acidobacteria* was highest in Age_30 (Figure 2A). The Venn diagram revealed 4,765 shared OTUs by different age groups, and the shared OTUs were characterized by 90% of total reads (Figure 2B).

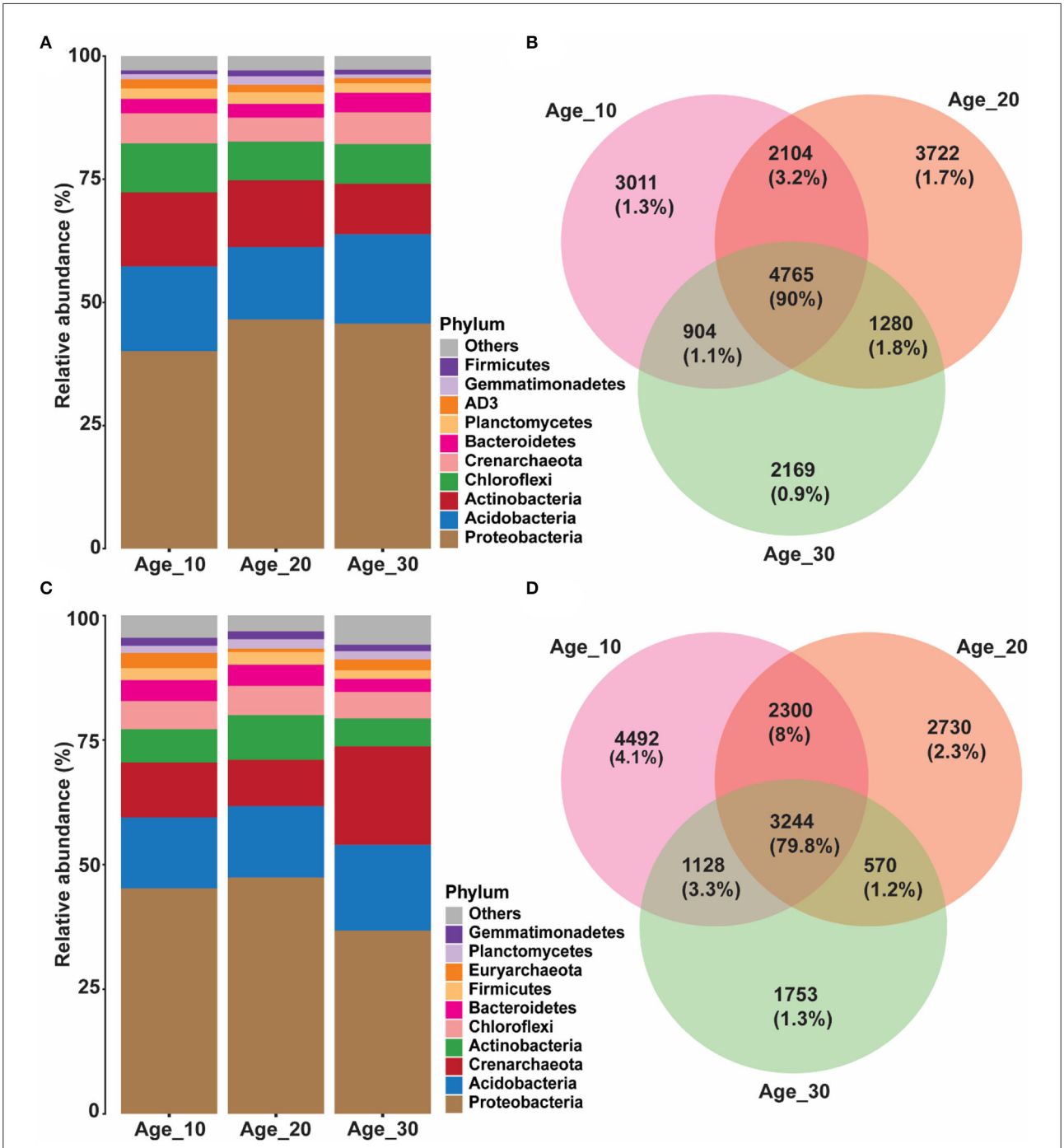

FIGURE 2
Variations in relative abundance of soil bacterial community under different tree ages. Changes in relative abundance and comparison of bacterial community were observed under different tree ages; **(A,B)** red soil; and **(C,D)** paddy soil. Relative abundance has been shown of top 10 bacterial community at phylum level. The different lowercase letters indicate the significant differences ($p < 0.05$) among different pomelo trees age groups of Age_10 (10 years old), Age_20 (20 years old), and Age_30 (30 years old).

Similarly, the paddy soil was also dominated by the bacterial communities of *Proteobacteria* (44.39%), *Acidobacteria* (14.83%), *Actinobacteria* (7.27%), *Chloroflexi* (5.65%), *Bacteroidetes* (3.93%), *Firmicutes* (2.32%), *Planctomycetes* (1.63%), and *Gemmatimonadetes* (1.52%). The average relative abundance of *Proteobacteria* was high compared to other

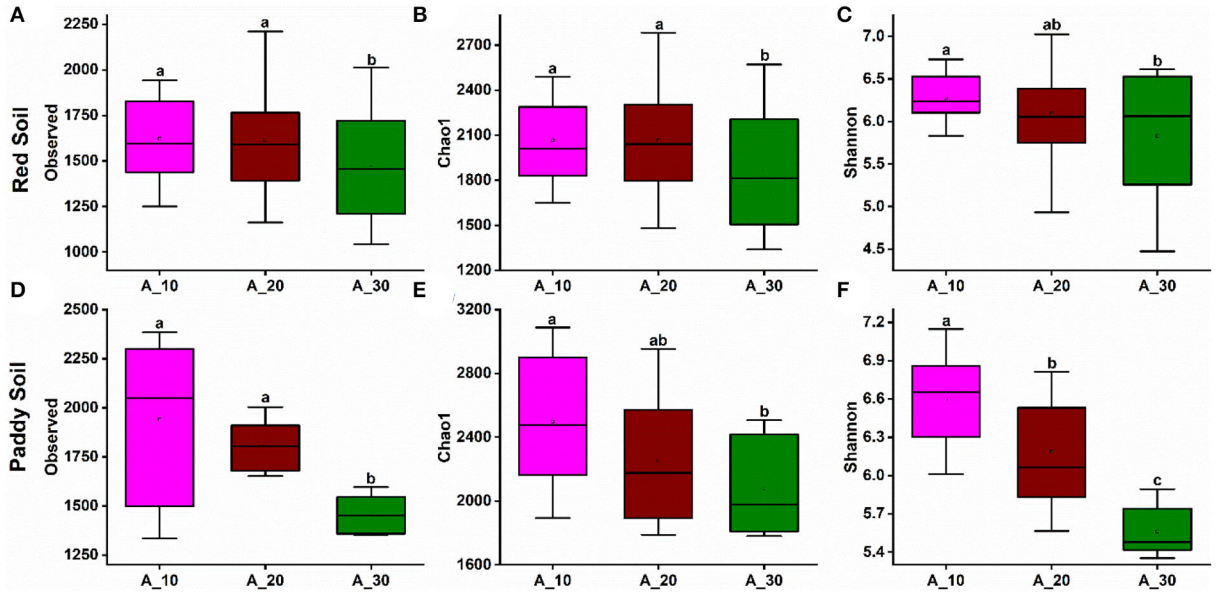

FIGURE 3
Alpha diversity indexes under different tree ages. Changes in alpha diversity indices, including observed, Chao-1, and Shannon indices, were observed under different tree ages for **(A–C)** red soil and **(D–F)** paddy soil. Here, Age_10, Age_20, and Age_30 denote the different pomelo tree ages of 10, 20, and 30 years, respectively.

bacterial phyla in Age_10 (45.23%), Age_20 (47.40%), and Age_30 (36.75%). Hence, in contrast to red soil, the relative abundance of *Proteobacteria* was decreased at Age_30 in paddy soil. In paddy soil, the second most abundant phylum was *Acidobacteria*, with average relative abundances of 14.22, 14.34, and 17.21% at Age_10, Age_20, and Age-30, respectively, and with the highest relative abundance under Age_30 (Figure 2C). However, an average relative abundance of *Actinobacteria* was low under paddy soil compared with red soil. The Venn diagram showed that different age groups shared 3,244 OTUs, accounting for 79.8% of total reads (Figure 2D). It showed that the similarity of the bacterial community of varying tree ages under red and paddy soils had a substantial degree of similarity, and their bacterial profiles had a generally stable composition for ecological functioning.

Bacterial richness and diversity under different tree ages in red and paddy soils

Alpha diversity indexes evaluated bacterial richness and diversity, such as observed OTU number, Chao1, and Shannon. In red soil, bacterial species richness indices, such as the observed number of OTUs (Figure 3A) and Chao1 (Figure 3B), decreased with increasing tree age. The bacterial diversity, that is, Shannon diversity index, also decreased with increasing tree age (Figure 3C). Similarly, the bacterial richness and diversity also decreased with increasing tree age under paddy soil (Figures 3D–F). It suggests that younger trees (i.e., 10 and 20 years) harbor higher bacterial richness and diversity.

Changes in bacterial communities with different tree ages in red and paddy soils

The principal coordinate analysis (PCoA) was performed to evaluate the bacterial community composition across different tree ages under red and paddy soils. PCoA results showed that bacterial community under different tree ages was different from each other in red soil, where PCo1 and PCo2 accounted for variations of 13.6 and 8.9%, respectively. PERMANOVA also suggested that overall bacterial communities under different trees was highly significantly different ($Pr(>F) = 0.001$) (Figure 4A). Furthermore, PERMANOVA revealed significant bacterial community differences for Age_10 vs. Age_20 ($P = 0.029$), and Age_10 vs. Age_30 ($P = 0.002$), while non-significant differences were recorded for Age_20 vs. Age_30 (Table 1). Similarly, under paddy soil, the bacterial community was different under different tree ages, where PCo1 and PCo2 accounted for variations of 13.6 and 10.6%, respectively, and PERMANOVA analysis also showed significant differences ($Pr(>F) = 0.01$) (Figure 4B). Furthermore, PERMANOVA revealed significant bacterial community differences only for Age_10 vs. Age_30 ($P = 0.016$), while non-significant differences were recorded for Age_10 vs. Age_20 and Age_20

TABLE 1 Significance test of differences among fungal communities in different treatments using permutational analysis of variance based on Bray–Curtis distance.

Soil type	Groups	Measure	Permutations	R^2	*P* value	Significance
Red soil	Age_10 vs. Age_20	Bray	999	0.038	0.029	*
	Age_10 vs. Age_30	Bray	999	0.06	0.002	**
	Age_20 vs. Age_30	Bray	999	0.034	0.157	
Paddy soil	Age_10 vs. Age_20	Bray	999	0.056	0.06	
	Age_10 vs. Age_30	Bray	999	0.081	0.016	*
	Age_20 vs. Age_30	Bray	999	0.893	0.071	

Star represents significance (*, $P \leq 0.05$; **, $P \leq 0.01$; while no star represents non-significance).

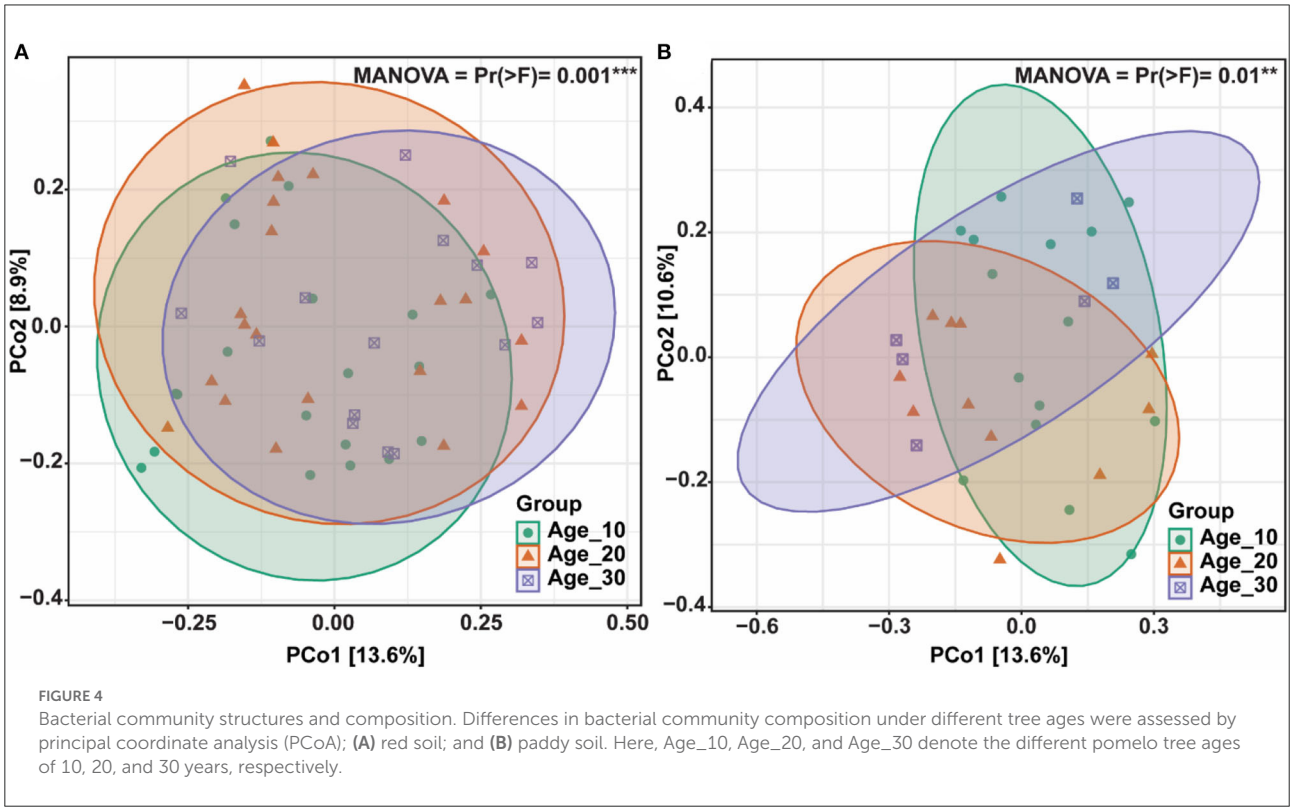

FIGURE 4
Bacterial community structures and composition. Differences in bacterial community composition under different tree ages were assessed by principal coordinate analysis (PCoA); **(A)** red soil; and **(B)** paddy soil. Here, Age_10, Age_20, and Age_30 denote the different pomelo tree ages of 10, 20, and 30 years, respectively.

vs. Age_30 (Table 1). These results showed that tree age significantly affected bacterial community composition for red and paddy soils.

Soil physicochemical properties correlation with the bacterial community with different tree ages

The effects of environmental factors, including soil pH, AP, AK, and AN, were examined on the bacterial population under red and paddy soils (Figure 5). RDA analysis showed that environmental factors, including soil pH, AP, and AN, were the most critical factors controlling the bacterial community under red soil (Figure 5A, Table 2). Similarly, in paddy soil, environmental factors such as soil pH and AP showed an empirical role in driving the bacterial community under the paddy soil (Figure 5B, Table 2). Moreover, at the phylum level, *Actinobacteria*, *Chloroflexi*, and *AD3* were negatively correlated, while Planctomycetes and Gemmatimonadetes showed a positive correlation with soil pH and AP, AK, and AN in red soil. In addition, *Proteobacteria* and *Firmicutes* were negatively correlated with soil pH, while positively correlated with other environmental factors in red soil (Figure 5C). *Proteobacteria*, *Actinobacteria*, and *Chloroflexi* were negatively associated with pH in the paddy soil. By contrast, *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, *Planctomycetes*, and *Gemmatimonadetes* showed a positive correlation with soil pH (Figure 5D).

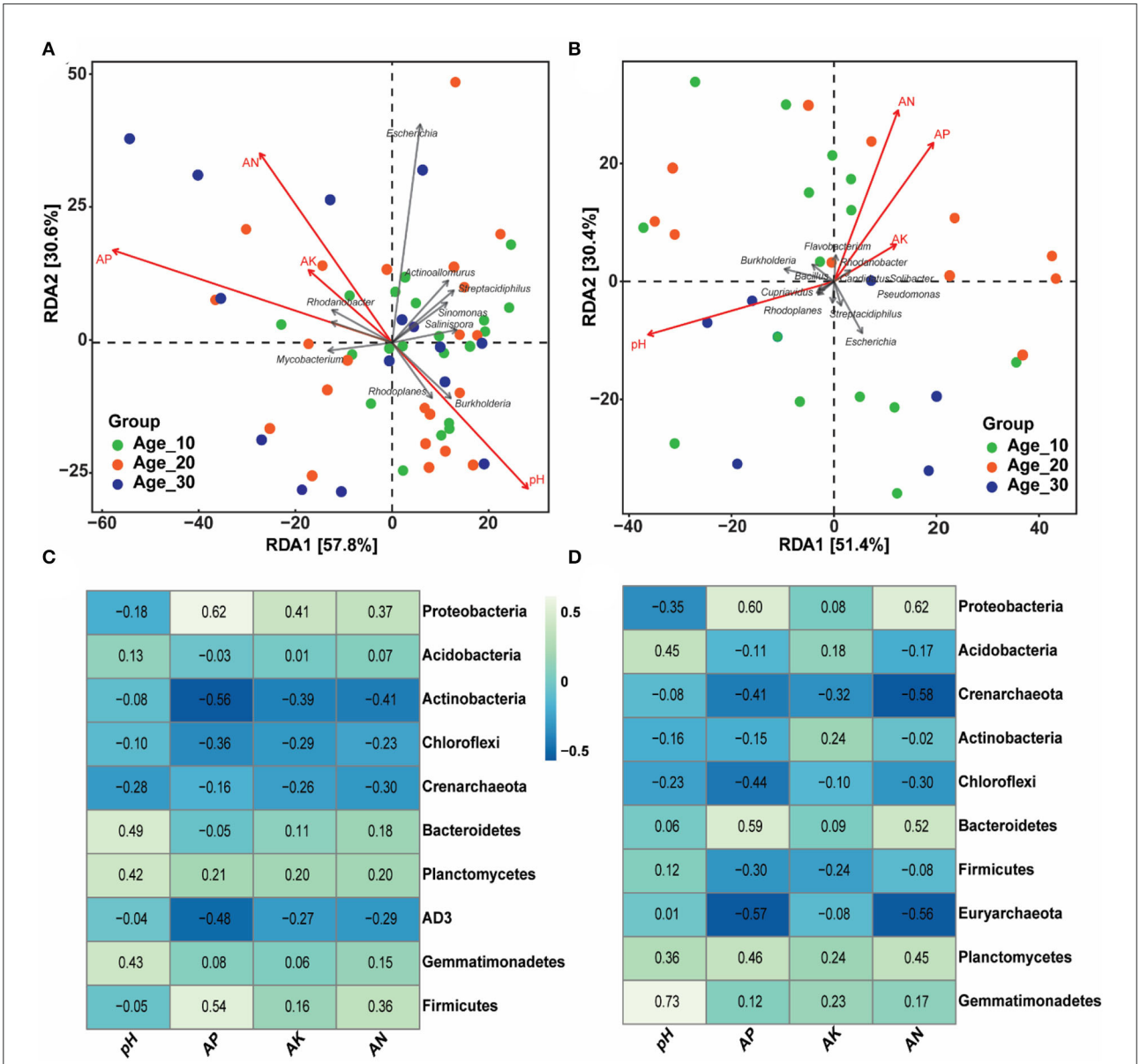

FIGURE 5
Influence of soil physicochemical characteristics on bacterial community. **(A,B)** Community–environment relationship using the RDA ordination plot of bacterial communities in red and paddy soils, respectively. **(C,D)** Heatmap showing the strength of correlation between the soil properties and bacterial community for red and paddy soils, respectively. Here, Age_10, Age_20, and Age_30 denote the different pomelo tree ages of 10, 20, and 30 years, respectively.

The random forest model determined the most important genera that classified the samples into different age groups. Results revealed that *Sinomonas*, *Streptacidiphilus*, *Burkholderia*, *Edaphobacter*, and *Phenylobacterium* were the most important genera under red soil and found significant differences in their abundances under different age groups (Figure 6A). In the case of paddy soil, the most important genera were *Actinoallomurus*, *Microbacterium*, *Nocardioides*, *Rathayibacter*, and *Streptacidiphilus*, and their abundances were significantly affected among different tree age groups (Figure 6B). Moreover, ternary plot analysis was performed to identify the specific enriched and depleted bacterial genera under different tree ages. The results showed that the pomelo tree of Age_10 in red soil was significantly enriched with *Streptacidiphilus*, while *Mycobacterium* and *Leptolyngbya* were depleted significantly. Under Age_20, we did not observe any significant enriched or depleted genera. By contrast, the pomelo tree of Age_30 was significantly enriched with *Rhodanobacter*, *Paenibacillus*, and *Stenotrophomonas*, while depleted with *Burkholderia*, *Sinomonas*, and *Actinomycetales*. Overall, genera enrichment

TABLE 2 Pearson correlation between the Bray–Curtis dissimilarity score and soil characteristics using the Mantel test.

	Variable name	Corr-method	Corr_res	p_res	Significance
Red soil	pH	Pearson	0.369	0.001	***
	AP (Available Phosphorous)	Pearson	0.203	0.002	**
	AK (Available Potassium)	Pearson	0.001	0.437	
	AN (Available Nitrogen)	Pearson	0.131	0.033	*
Paddy soil	pH	Pearson	0.251	0.003	**
	AP (Available Phosphorous)	Pearson	0.233	0.009	**
	AK (Available Potassium)	Pearson	−0.048	0.676	
	AN (Available Nitrogen)	Pearson	0.073	0.215	

Star represents significance (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$, while no star shows non-significance).

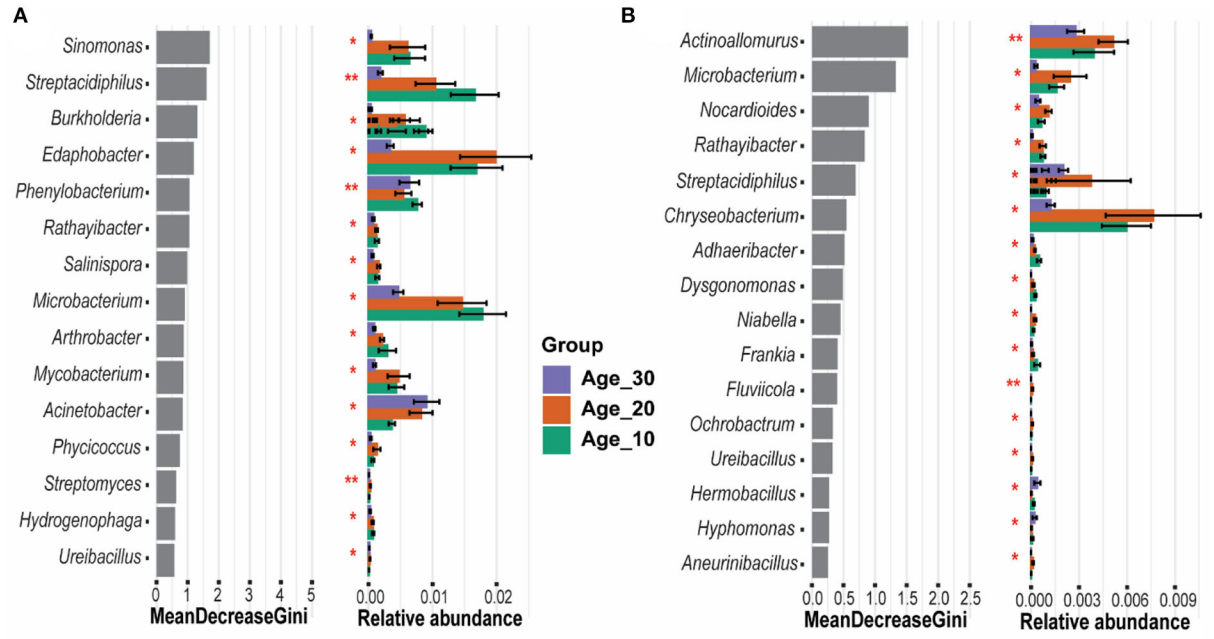

FIGURE 6
Random forest classification for evaluation of important genera. Gini index was used to determine the most significant genera, which exhibited the significance of each genus in distinguishing different age groups of **(A)** red soil and **(B)** paddy soil. Here, Age_10, Age_20, and Age_30 denote the different pomelo tree ages of 10, 20, and 30 years, respectively.

for Age_30 was higher than that for Age_10 and Age_20 (Figure 7A). For paddy soil, *Cupriavidus*, *Pseudomonas*, and *Salinispora* were significantly enriched under Age_10, while *Pedosphaera* was enriched under Age_30. Hence, generally, genera enrichment decreased with increasing tree age under paddy soil (Figure 7B).

Co-occurrence network analysis

A non-random co-occurrence network pattern of phylotypes was studied to investigate how the interactions between phylotypes respond to variations in tree ages under red and paddy soils. The values of the modularity index ranged from 0.71 to 0.77 under red soil, while 0.52–0.81 under paddy soil (Figure 8). The modularity index value greater than the proposed threshold value, that is, 0.40, indicated that all networks were modularly structured. The number of nodes increased with increasing tree age in red soil, and the lowest number of nodes was found in Age_10. However, the number of edges, that is, 1,603 and 1,404; the average degree (the tendency of nodes to cluster together), that is, 4.57 and 4.90; and the average clustering coefficient under (degree of nodes connectivity), that is, 0.40 and 0.53 under red soil in Age_20 and Age_30, respectively, were higher than Age_10 edges (1,273), average degree (4.45), and average clustering coefficient

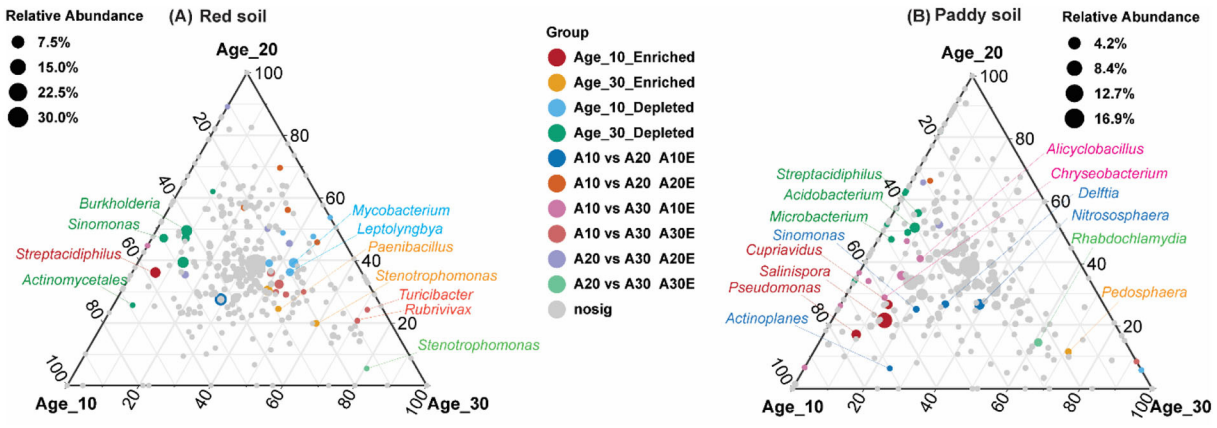

FIGURE 7
Ternary plot analysis. Ternary plot represents the soil bacterial communities with significant differences in relative abundance in different tree ages at the genus level in **(A)** red soil and **(B)** paddy soil. Here, Age_10, Age_20, and Age_30 denote the different pomelo tree ages of 10, 20, and 30 years, respectively. Each point represents species, and the size of the point corresponds the relative abundance of each species. "Enriched" denotes the significant increase in relative abundance compared with others two, while "depleted" represents the significant lower relative abundance of a group than other two groups, and non-sig represents the non-significant differences among different age groups.

(0.36). The bacterial species *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Chloroflexi* represented the most dominant bacterial community in red soil (Figures 8A–C). Similarly, under the paddy soil, the number of nodes increased with increasing tree, and the highest nodes were recorded under Age_30. Moreover, the number of edges (1,064), average degree (14.69), and average clustering coefficient (0.99) under Age_30 were also higher than those under Age_10 and Age_20. Similar to red soil, in the paddy soil, the bacterial species *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, and *Bacteroidetes* were the most dominant bacterial community (Figures 8D–F). However, these findings revealed that bacterial species tended to interact more closely and formed more complex network structures with increasing tree age, for example, Age_20 and Age_30 compared to Age_10 tree.

Discussion

Soil microbes are the key components of soil ecosystems and play a significant role in maintaining a healthy and stable environment for tree growth and development. In this study, we examined the changes in soil physicochemical properties, bacterial diversity, and community structure under different tree ages in the red and paddy soils. The soil properties, bacterial diversity, and community structure differed significantly among pomelo trees of different ages in red and paddy soils.

Soil physicochemical properties are an important index to evaluate the soil quality that ultimately affects the soil fertility and microbial community (Shao et al., 2020; Yin et al., 2021). Various studies have indicated that tree age substantially impacts soil physicochemical properties (Sharma et al., 2009; Yin et al., 2021). For instance, soil pH is one of the most critical determinants of soil quality. At present, contrasting results have been reported on the effects of tree age on soil pH (Figure 1). Some studies have found that soil pH is increased significantly with an increase in tree age (Malchair and Carnol, 2009), while others reported that the pH value of the soil steadily drops as the tree age increases (Bormann and DeBell, 1981; Sharma et al., 2009), indicating a trend of soil acidification. In this experiment, the pH value was significantly decreased with increasing tree age in red soil, consistent with previous findings (Bormann and DeBell, 1981; Sharma et al., 2009). The possible reason could be the reduced base saturation caused by H^+ production in the nitrification process, with H^+ substituting the base cations leached from the soil with NO_3^- (Binkley and Sollins, 1990). Additionally, leaching of base cations with water, organic matter accumulation, atmospheric acid deposition, and soil microbial respiration may all contribute to a reduction in soil pH (Hinsinger and Jaillard, 1993; Wong et al., 2008). While, in the paddy soil, there was no significant difference in pH between different tree ages, the availability of soil nutrients was significantly affected, and the same results have been reported in a previous study (Yin et al., 2021). The concentration of phosphorous availability (AP) was increased with a decrease in soil pH because it has been reported that a decrease in pH may result from increasing the activity of proton-coupled solute transporters and enhancing the anion uptake (White, 2011; da Silva Cerozi and Fitzsimmons, 2016; Yin et al., 2021). The availability of potassium (AK) and nitrogen (AN) increased in red soil while decreasing with increasing tree age in paddy soil. The soil pH significantly affects nutrient availability (Zhao et al., 2011). Most plant nutrients are available in soil that is slightly acidic to slightly alkaline. Various plant nutrients are unavailable

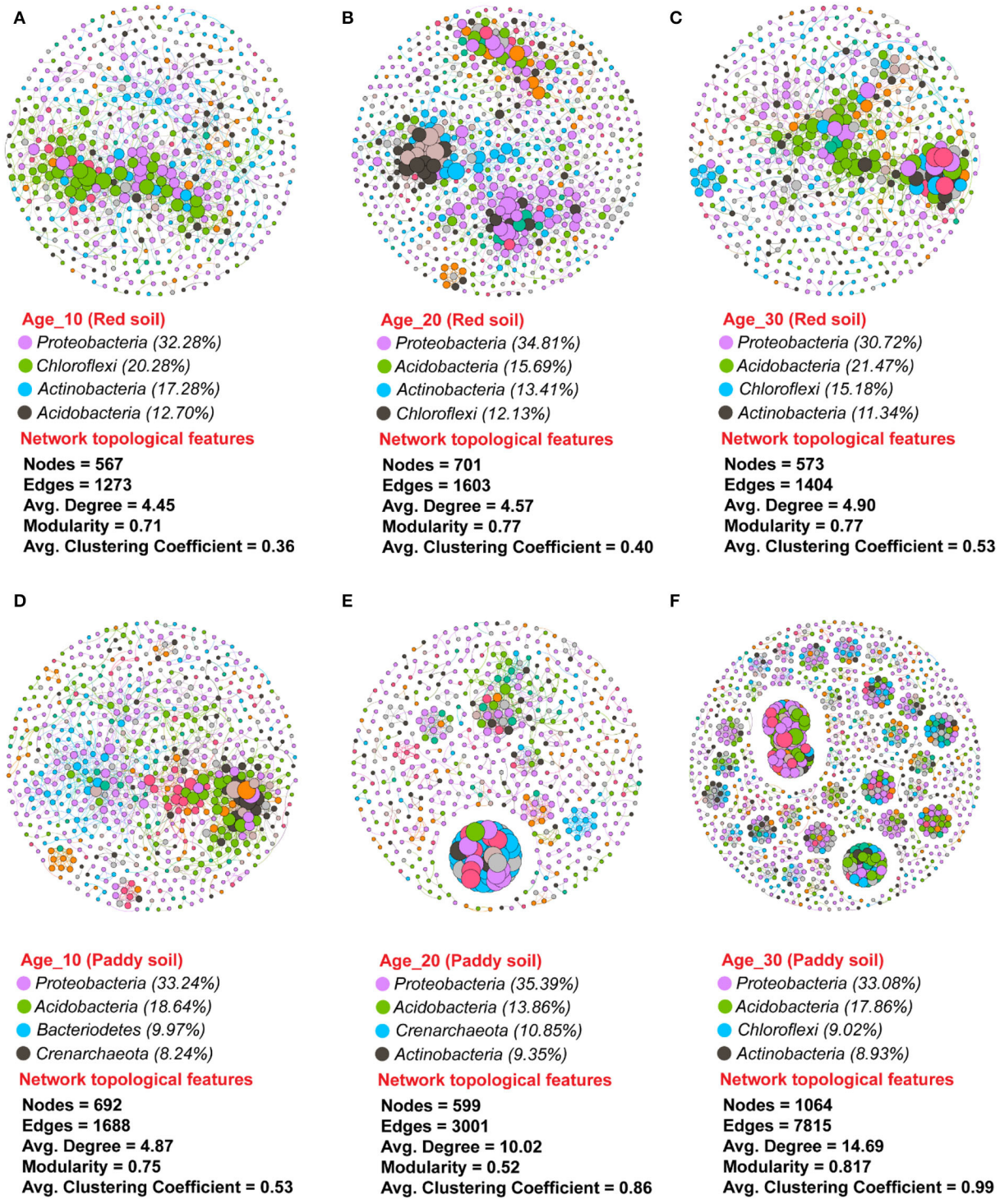

FIGURE 8
Dynamic changes in bacterial co-occurrence networks under different groups of tree ages. Co-occurrence network was investigated for **(A–C)** red soil and **(D–F)** paddy soil. Each node in the network has a different color based on its phylum. The size of each node indicates the taxa's relative abundance. The connections are significant ($P \leq 0.05$) and strongly correlated (Spearman's $\rho \geq 0.6$). Here, Age_10, Age_20, and Age_30 denote the different pomelo tree ages of 10, 20, and 30 years, respectively.

in highly acidic or alkaline soils owing to chemical reactions in the soil that fix nutrients and transform them into a form that is unavailable to plants. Hence, tree ages have a substantial impact on soil physicochemical properties.

The bacterial community in red and paddy was dominated by *Proteobacteria* and *Acidobacteria* (Figure 2), which is consistent with the previous studies on soil microbiome (Zhou et al., 2015; Wang et al., 2016). However, the relative abundance of *Proteobacteria* and *Acidobacteria* was increased in 20- and 30-year-old trees compared to 10-year-old trees in red soil. This is because *Proteobacteria* belongs to the copiotroph group, and its relative abundance increases with an increase in resources (Eilers et al., 2010), as in the present study, we found AP, AK, and AN increased with increasing tree age in red soil. By contrast, in paddy soil, *Proteobacteria* relative abundance decreased because of the nutrient-poor environment, for example, a decrease in AK and AN under Age_30. *Acidobacteria* relative abundance increased with tree age, and the highest relative abundance was found under Age_30 in red and paddy soils. This is because *Acidobacteria* favors the acidic environment because it was isolated from an acidic mineral environment (Kishimoto et al., 1991), and Jones et al. (2009) found that the relative abundance of *Acidobacteria* increased under low soil pH, consistent with our findings that relative abundance of *Acidobacteria* increased under low soil pH. These findings imply that soil pH plays a vital role in changing the relative abundance of *Acidobacteria* across the age sequence of the pomelo plantation.

Under both red and paddy soils of the present study, the bacterial richness and diversity index values decreased substantially with increasing tree age (Figure 3), which concurs with the results of previous studies showing a high level of diversity in 10- and 18-year-old trees, while diversity decreased in 30-year-old trees (Zhou et al., 2017), and some other studies also found that bacterial diversity decreased with increasing plantation age (Wu et al., 2015; Zhu et al., 2019). It has been widely accepted that tree development affects the soil microbiota through various pathways (Mitchell et al., 2012; Dang et al., 2017). For example, tree cover has a direct impact on the amount of light available as well as the composition of the understory, which affects the microbial population in the soil. As a result, differences in the tree canopy of different tree ages may indirectly impact soil bacterial diversity *via* understory litter substrate and root system (Dang et al., 2017). The other possible explanation for the decrease in bacterial richness and diversity could be the low soil pH because we found that with increasing tree age, soil pH was significantly decreased, and it has been found that soil acidification significantly decreases the bacterial diversity (Yun et al., 2016). These findings suggest that tree age significantly shifts the underlying soil bacterial communities, in line with the study of Wu et al. (2015), who found that plant age had a substantial effect on microbial communities in *Pinus elliottii*.

Changes in tree age significantly affect soil physicochemical properties, which in turn affect the soil bacterial community structure (Qu et al., 2020; Yin et al., 2021). For example, soil pH has been recognized as the most influential factor that drives the variation of soil bacterial community assembly (Siciliano et al., 2014; An et al., 2019). In the current study, we also found that soil pH, AP, and AN effects were significant on the bacterial community (Figure 5, Table 2) and consistent with various studies (Liu et al., 2014; An et al., 2019). This may explain the changes in the bacterial community under different tree ages. *Proteobacteria* was positively correlated with AP, AK, and AN because it is categorized into a copiotroph group that prefers high-nutrient soil resources (Eilers et al., 2010). *Acidobacteria* was positively correlated with soil pH and consistent with previous findings (Wang et al., 2018; Muneer et al., 2022). Various studies have reported inconsistent results of soil pH on *Acidobacteria*, where some studies also have shown a negative correlation (Jones et al., 2009; Navarrete et al., 2013). It is possible due to a better suitable environment for their adaptation. For example, it has been found that some *Acidobacteria* usually prefers high acidic conditions (i.e., pH $\sim$ 2–3) compared to other environments (Kleinsteuber et al., 2008). Despite the substantial (positive or negative) association with pH, it is still unknown whether pH affects *Acidobacteria* directly or is dependent on other environmental factors that fluctuate with soil pH. Overall, we found positive and negative correlations between bacterial community and soil pH. The researchers have proposed two possible hypotheses explaining the relationship between soil pH and bacterial community. First, soil pH exerts a physiological restraint on the bacterial community, affecting the competitive outcomes or limiting the net growth of taxa that cannot live if the soil pH goes beyond a particular range. The majority of bacterial intracellular pH is close to neutral, and hence, the severe pH level may cause substantial pressure, and as a result, some taxa can withstand better than others (Lauber et al., 2009). Second, soil pH may not have a direct effect on the bacterial community, but rather serve as an integrating factor that offers a comprehensive assessment of soil environments. Various soil properties (e.g., nutrient availability, organic carbon, and soil moisture) are directly or indirectly related to soil pH, and these factors may attribute to changes in the bacterial community (Bissett et al., 2011; Zhou et al., 2017).

Random forest analysis showed that the most important genera in red soil belonged to phylum *Actinobacteria* (i.e., *Sinomonas* and *Streptacidiphilus*) and *Proteobacteria* (i.e., *Burkholderia* and *Phenylobacterium*) (Figure 6). *Sinomonas* has been reported to help in nitrogen fixation and plant growth (Lee et al., 2015; Susilowati et al., 2015), while *Streptacidiphilus* has an important role in biodegradation (e.g., chitin and lignocellulose), secondary metabolite production, and plant growth-promoting potential (Bentley et al., 2002). *Burkholderia* and *Phenylobacterium* are ubiquitous and found in a variety of soils owing to their functions in nitrogen fixation, degradation of organic compounds, and their interaction with plants,

including beneficial and pathogenic (Compant et al., 2008). Likewise, another important genus, *Edaphobacter*, belonging to the phylum *Acidobacteria*, has been found mostly in acidic and calcareous soils and could play a key role in the regulation of nutrient cycling and plant growth promotion (Kalam et al., 2020). Similarly, in paddy soil, the most important identified taxa, including *Actinoallomurus*, *Microbacterium*, *Nocardioides*, *Rathayibacter*, and *Streptacidiphilus*, belonged to *Actinobacteria*. These have several important functions, including cycling of carbon, nitrogen, phosphorous, and several other elements in soil, and decomposition of organic matter (Holmalahti et al., 1994; Zhang et al., 2019). According to the results of ternary plot analysis (Figure 7), under red soil, the relative abundance of specific genera, including *Rhodanobacter* and *Paenibacillus*, significantly increased with increasing tree age, and these bacterial communities play an active role in N-cycling (Liu et al., 2019; Peng et al., 2022), while *Stenotrophomonas* was found to contribute to N and S cycles and could play a substantial role in improving plant growth (Ryan et al., 2009). Likewise, in paddy soil, the N cycling-related bacterial community of *Cupriavidus* and *Pseudomonas* (Mirza et al., 2006; Wang et al., 2020) was significantly higher under Age_10, and the specific genera enrichment decreased with increasing tree age. However, these findings highlight the important taxa in different soils of pomelo orchard and provide a reference that could be useful for understanding their ecological significance under global changes.

In general, soil microorganisms do not function as individuals, but instead establish complex interaction networks. As a result, co-occurrence network analysis is an effective method for determining how related soil microbiota interact with environmental changes. The co-occurrence networks revealed that the majority of nodes belonged to *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Chloroflexi* across all tree ages (Figure 8). This finding was consistent with that of a previous study (Yan L. et al., 2021), further demonstrating that *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, and *Chloroflexi* are the most dominant phyla in pomelo orchards of red and paddy soils. The highly connected nodes revealed strong bacterial association under 20 and 30 years old trees compared to 10 years old in red and paddy soils, and these taxa are known as keystone taxa (Guo et al., 2019). These keystone taxa have prime importance for sustaining the co-occurrence network structure (Faust and Raes, 2012). As a result of the complex network connectivity, the bacterial community under 20- and 30-year-old trees could be more tolerant to environmental changes as it is generally documented that bacterial communities with higher interconnectivity are more resistant to environmental changes than simple networks with less connectivity (Santolini and Barabási, 2018).

Note that in this study, we only focused on how the dynamic changes in tree age could affect the soil bacterial diversity, community composition, and functioning. However, the impact of root exudates on the soil microbial population was not considered owing to the key focus of tree age. Indeed, the root exudates affect the microbial diversity and community composition. Hence, further studies are needed to understand the composition of the exudate in the orchard system and to better link the tree age, the root exudate, and soil microbiota.

Conclusion

This study revealed that tree age has a significant effect on changing the soil physicochemical properties, and contrasting patterns of changes in soil physicochemical properties were recorded under different tree ages in the red and paddy soils. Moreover, tree age also substantially affected soil bacterial diversity and community composition. Bacterial richness and diversity decreased with increasing tree age in both soil types, while tree age formed the distinct bacterial community structures under red and paddy soils. Soil pH, AP, and AN in red soil, while pH and AP in paddy soil were the key environmental variable contributing to the shift in the bacterial community structure. However, the co-occurrence network revealed that bacterial species formed a complex network structure with increasing tree age, indicating a more stable microbial association under 20- and 30-year-old than 10-year-old pomelo trees. Moreover, the random forest model identified the most important genera in red soil, for example, *Sinomonas* and *Streptacidiphilus*, which play a key role in nitrogen fixation, plant growth, biodegradation, etc., while paddy soil was dominated by *Actinoallomurus Microbacterium*, etc., which have key functions including cycling of carbon, nitrogen, phosphorous, and several other elements in soil, and decomposition of organic matter. These findings provide valuable information regarding the importance of microbes for the sustainable management of pomelo orchards by optimizing fertilizer input for different ages of trees.

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/, PRJNA779204.

Author contributions

CZ: conceptualization and writing—original draft. KK and YZ: data curation and software.

WY and LW: investigation, data curation, and conceptualization. MZM and BJ: writing—review and editing. MAM: writing—original draft, software, visualization, and writing—review and editing. All authors contributed to the manuscript 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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Modulation of extracellular *Penicillium expansum*-driven acidification by *Papiliotrema terrestris* affects biosynthesis of patulin and has a possible role in biocontrol activity

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The active regulation of extracellular pH is critical for the virulence of fungal pathogens. *Penicillium expansum* is the causal agent of green-blue mold on stored pome fruits and during its infection process acidifies the host tissues by secreting organic acids. *P. expansum* is also the main producer of patulin (PAT), a mycotoxin found in pome fruit-based products and that represents a serious health hazard for its potential carcinogenicity. While it is known that PAT biosynthesis in *P. expansum* is regulated by nutritional factors such as carbon and nitrogen and by the pH, the mechanistic effects of biocontrol on PAT production by *P. expansum* are not known. In this work, we assessed how optimal and suboptimal concentrations of the biocontrol agent (BCA) *Papiliotrema terrestris* LS28 affect both extracellular pH and PAT biosynthesis in *P. expansum*. In wounded apples, the optimal and suboptimal concentrations of the BCA provided almost complete and partial protection from *P. expansum* infection, respectively, and reduced PAT contamination in both cases. However, the suboptimal concentration of the BCA increased the specific mycotoxigenic activity by *P. expansum*. *In vitro*, the rate of PAT biosynthesis was strictly related to the extracellular pH, with the highest amount of PAT detected in the pH range 4–7, whereas only traces were detectable at pH 3. Moreover, both *in vitro* and in apple wounds the BCA counteracted the extracellular *P. expansum*-driven acidification maintaining extracellular pH around 4, which is within the pH range that is optimal for PAT biosynthesis. Conversely, in the absence of LS28 the pathogen-driven acidification led to rapidly achieving acidic pH values (<3) that lie outside of the optimal pH range for PAT biosynthesis. Taken together, these results suggest that pH modulation by LS28 is important to counteract the host tissue acidification and, therefore, the virulence of *P. expansum*. On the other hand, the buffering of *P. expansum*-driven acidification provided by the BCA increases the specific rate of PAT biosynthesis through the extension of the time interval at which the pH value lies within the optimal range for PAT biosynthesis. Nevertheless,

the antagonistic effect provided by the BCA greatly reduced the total amount of PAT.

KEYWORDS

Papiliotrema terrestris, patulin, *Penicillium expansum*, pH, mechanisms of biocontrol

Introduction

The modulation of extracellular pH plays a fundamental role during the attack on host plant tissues by many fungal pathogens (Prusky et al., 2001; Fernandes et al., 2017; Vylkova, 2017). For some of them the molecular mechanisms of pH sensing and adaptation have been elucidated. A key role is played by the Pac/Rim alkaline response pathway, which regulates the expression of genes involved in the adaptation to environmental pH (Antonio et al., 2010; Selvig and Alspaugh, 2011; Franco-Frías et al., 2014; Ost et al., 2015; Wu et al., 2016). On the other hand, fungi can actively modify the environmental pH by secreting acids or alkali. In the majority of necrotrophic phytopathogens, acidification of the extracellular environment is achieved through the secretion of acidic compounds such as butyrate, oxalate, malate, citrate, gluconate, and succinate, by removing ammonium ions from ammonium sulfate salt, and/or by excreting hydrogen (H^+) ion as a byproduct of ammonium (NH^{4+}) assimilation (Fernandes et al., 2017; Vylkova, 2017).

Penicillium expansum is an important post-harvest pathogen of many deciduous fruits worldwide. It is the etiological agent of the blue mold disease of pome fruits and is responsible for significant losses during post-harvest handling and storage of these commodities. As an infection strategy, *P. expansum* enhances its virulence by locally modulating the host pH through the production of organic acids, mainly citric and gluconic, and the efflux of H^+ as a result of NH^{4+} utilization. The accumulation of these chemical species in the decaying fruit tissue leads to a reduction of pH by 0.5 to 1.0 units, which fosters the activity of polygalacturonase enzymes and the transcription of genes involved in fungal virulence (Prusky and Yakoby, 2003; Hadas et al., 2007; Prusky et al., 2007; Barad, Espeso et al., 2016). In agreement with these findings, it has been reported that *P. expansum* virulence and disease severity are much higher in apple cultivars characterized by low pH of the fruit tissue, or in fruits treated with citric acid (Marín et al., 2006; Kumar et al., 2018). Conversely, local alkalinization with sodium bicarbonate ($NaHCO_3$) reduces decay development (Prusky et al., 2007). Furthermore, during the host tissue attack, *P. expansum* produces a large number of secondary metabolites, with patulin (PAT) being the most represented (Andersen et al., 2004). Patulin is a dangerous mycotoxin that contaminates stored fruits and derived products, representing a serious health hazard, especially for children. It has shown to be genotoxic on human cells *in vitro* (Glaser and Stopper, 2012; Pinedo et al., 2018) and possesses tumorigenic activity on mouse skin cells (Wright, 2015). Patulin can induce ROS-dependent damage by rapidly reacting with Glutathione, the depletion of which perturbates the cell redox homeostasis (Castoria et al., 2011; Ianiri et al., 2016). Therefore, maximum tolerable contamination levels of PAT have been set up (Commission Regulation, EC 1881/2006, 2006). The level of PAT synthesis varies considerably in different fungal strains and its biosynthesis and accumulation are influenced by environmental factors. Types of carbon sources and their concentration strongly influence PAT production by *P. expansum*, with glucose and glucose-containing sugars facilitating its production, while higher sugar concentrations reducing its accumulation (Barad et al., 2012; Prusky et al., 2014). Patulin production is also related to nitrogen availability, with organic nitrogen inducing higher PAT production than inorganic sources. In particular, ammonium sulfate was found as the most unfavorable nitrogen source for PAT biosynthesis (Rollins and Gaucher, 1994; Miyara et al., 2010; Zong et al., 2015). Another relevant factor affecting PAT biosynthesis is the extracellular pH. For the majority of *P. expansum* strains, acidic conditions promote higher PAT production than alkaline environments (Jimdjio et al., 2021). Nevertheless, the optimum of PAT biosynthesis is achieved in the pH range 4.0–5.0, while its production decreases at higher and lower pH values (Zong et al., 2015). Accordingly, PAT accumulation also depends on fruit cultivars and their degree of ripeness. For example, the tissue acidity of some apple varieties, such as Granny Smith (pH 2.90), allows lower PAT accumulation than less acidic varieties like Golden Delicious (pH 3.30; McCallum et al., 2002; Marín et al., 2006; Morales et al., 2007; Snini et al., 2016). Moreover, during apple maturation, the reduction of malic acid content and the decreasing fruit firmness increase susceptibility to *P. expansum* infection and PAT accumulation (Kumar et al., 2017).

In recent years, several studies evaluated the effect of control measures of mycotoxigenic fungal pathogens on mycotoxin biosynthesis. The effect of antifungal compounds on PAT biosynthesis is not unique because some of these induce downregulations of genes of the PAT biosynthetic pathway (Wang et al., 2018; Martínez-Culebras et al., 2021), while others (Delgado et al., 2022) stimulate its production and accumulation, especially if one considers the lower fungal biomass that develops in the decaying host tissue. Regarding the effect of microbial competitors of mycotoxigenic fungal pathogens on PAT biosynthesis and accumulation, such as yeasts acting as biocontrol agents (BCAs; De Curtis et al., 2004), a few studies report a reduction both *in vitro* and *in vivo* (i.e., in the fruit; Kabak et al., 2007;

Castoria et al., 2008; Mahunu et al., 2016; Zheng et al., 2017; Nešić et al., 2021). The possible mechanisms involved are prevention of PAT contamination and detoxification. The prevention of PAT contamination is mainly based on controlling *P. expansum* through the reduction of conidial germination, germ tube elongation and inhibition of fungal growth, with a consequent decrease of PAT biosynthesis and accumulation (Yu et al., 2020). Conversely, PAT degradation consists of enzymatic degradation operated by several bacterial and yeast species leading to the formation of the end-products desoxypatulinic acid, (Z)-ascladiol, and hydroascladiol (Castoria et al., 2011; Ianiri et al., 2013; Mahunu, 2017; Tannous et al., 2017; Wei et al., 2020; Awuchi et al., 2021; Wang et al., 2022). Other studies show that some yeast species are also able to decrease PAT concentration through its adsorption to the cell wall (Caridi, 2007; McCormick, 2013; Nešić et al., 2021). However, the impact of these mechanisms on the decrease in PAT contamination *in vivo* has not yet been well understood. On the other hand, recent studies report that some BCAs, such as the basidiomycete yeasts *Rhodotorula mucilaginosa* strain 3617 and *R. kratochvilovae* strain LS11 increase the specific rate of PAT biosynthesis in *P. expansum* both *in vitro* and *in vivo*; however, the fungal growth, disease incidence and severity, and overall PAT accumulation were drastically reduced (Zheng et al., 2017).

In the present study, the effect of the interaction between the biocontrol yeast *P. terrestris* strain LS28 and *P. expansum* on environmental pH and PAT biosynthesis were investigates, with the aim of clarifying the factor(s) that influence the toxigenic activity and virulence of the fungal pathogen and the protection exerted by the BCA.

Materials and methods

Strains and growth conditions

The microorganisms used in this study were the mycotoxigenic postharvest pathogen *P. expansum* strain 7015, deposited in the "Toxigenic Fungi Culture Collection" of the Institute of Sciences of Food Production of the National Research Council (ISPA CNR—Bari, Italy), and the basidiomycetous yeast *P. terrestris* strain LS28, already known as a post-harvest biocontrol agent (Lima et al., 1999; Kheireddine et al., 2021). The fungal inoculum was prepared by culturing *P. expansum* on Potato Dextrose Agar medium (PDA) for 10 days at 25°C in the dark. The surface of the fungal culture was rinsed off with 10 ml of sterile distilled water containing 0.005% (v/v) Tween 80, and the concentration of the resulting conidial suspension was adjusted with a hemocytometer, as required for subsequent experiments. The BCA *P. terrestris* LS28 was routinely cultured in YPD medium (yeast extract at 10 g L^{-1}, peptone at 20 g L^{-1}, dextrose at 20 g L^{-1}, and agar at 20 g L^{-1} for solid medium) at 28°C in shaking cultures at 150 rpm. Following incubation, cultures were centrifuged at 7,000 rpm for 5 min, washed twice with sterile distilled water and determination of cell concentration was performed using a hemocytometer.

Measurements of pH

The effect of *in vitro* microbial interactions on pH trend and PAT accumulation was evaluated in apple mimicking medium (AMM), which was prepared by boiling 200 g of Golden Delicious apples for 30 min and filtration through sterile gauze to remove solid parts. The liquid volume was adjusted to 1 L that was autoclaved at 121°C for 20 min. For solid AMM, 18 g L^{-1} of agar was added.

To evaluate the role of the pH on PAT biosynthesis, pH of AMM was adjusted to 3.0, 4.0, 5.0, and 6.0 by using a buffer solution consisting of citric acid (0.1 mol L^{-1}) and disodium phosphate Na_2HPO_4 (0.2 mol L^{-1}), as reported by Furné et al. (2008). The *in vitro* experiments in liquid and solid AMM were carried out by inoculating *P. terrestris* at a concentration of 10^6 cells ml^{-1} and *P. expansum* at a concentration of 10^3 conidia ml^{-1}. Cultures were incubated in rotary shakers at 150 rpm and 25°C for 7 days.

P. expansum conidia were washed twice with sterile distilled water and inoculated in 250 ml of different fresh media at a final concentration of 10^3 conidia ml^{-1}. Subsequently, 10^6 cells ml^{-1} of *P. terrestris* LS28 were added to *P. expansum*-inoculated medium, and the culture was incubated at 25°C in agitation at 150 rpm. Cultures containing either *P. expansum* or LS28 alone were used as controls. To record the pH dynamics in liquid AMM, aliquots of the culture supernatant were withdrawn and subjected to pH measurement every 6 h using a pH-meter. Experiments were performed three times with three replicates for each experiment.

For a visual evaluation of the pH changes during microbial interactions, a 5-µl drop of conidial suspension of *P. expansum* at 10^8 ml^{-1} was spotted onto 9-cm ⌀ Petri dishes containing 20 ml of agarized AMM and allowed to dry. Subsequently, 20 µl of suspension of LS28 at a concentration 10^8 cells ml^{-1} were spotted 3 cm distant from the fungal spot and allowed to dry. After 5 days of incubation at 25°C, plates were overlaid with a 0.833 µM solution of the pH indicators bromocresol green or purple. The two microorganisms grown individually were used as controls. Experiments were performed at least four times, with two replicates in each experiment.

In-vivo assessment of pH trend was carried out on Golden Delicious apples purchased from a local market (Campobasso, Italy). The average values of fruit firmness and soluble solid concentrations were 3.4 kg and 14° Brix, respectively. Experiments included the time-course assessment of mesocarp pH and biocontrol assays in which LS28 was challenged by *P. expansum*. In the biocontrol experiments, LS28 was grown overnight in liquid YPD medium, the yeast cell suspension was washed twice with sterile distilled water and adjusted to concentrations of 5×10^6 cells ml^{-1} (sub-optimal) and 10^8 cells ml^{-1} (optimal). Apples were superficially sterilized by 1 min immersion in a sodium hypochlorite solution [1% (v/v) of active chlorine], rinsed twice with sterile distilled water and dried at room temperature. Four wounds (3 mm wide × 3 mm deep) were made on each fruit around the petiole using a cork borer. Immediately after wounding, 30 µl of the suboptimal or optimal LS28 cellular

suspension was inoculated into each apple wound. After 30 min, 15 µl of a *P. expansum* suspension 2×10^4 conidia ml^{-1} was added into the same artificial wounds. Mesocarp pH was measured at 0, 6, 12, 24, 48, 72, 96, 120, 144, and 176 h by placing a micro pH electrode directly on the exposed inoculated tissues, as previously described by Prusky and Yakoby (2003). For each treatment and each time point, measurements were repeated three times on 3 different fruits.

Patulin extraction and HPLC quantification

The mycotoxigenic activity of *P. expansum* strain 7015, alone or co-incubated with the BCA *P. terrestris* LS28, was evaluated both *in vitro* and *in vivo*.

In vitro quantification of PAT was performed after 7 days of incubation in liquid AMM inoculated with the fungal pathogen as previously described. At the same time point (after 7 days of incubation), 20 ml of each culture was withdrawn and centrifuged at 6,000 rpm for 20 min: the pellet was heated at 65°C for 2 days to obtain the mycelial dry weight, while the supernatants were filtered with cellulose nitrate 0.45 µm filters and frozen at −20°C for successive mycotoxin quantification. Patulin was extracted from each supernatant with the same volumes of ethyl acetate for three times, according to AOAC (2000). The collected ethyl acetate (60 ml for each sample) was cleaned up with sodium carbonate solution (1.5% Na_2CO_3), dried with anhydrous sodium sulfate (Na_2SO_4), and then evaporated using a rotavapor system at 40°C. The dry residue was redissolved in 0.5 ml of mobile phase [H_2O (acidified with 1% acetic acid) and methanol 95:5 (v/v)] and used for high-performance liquid chromatography (HPLC) determination as described below. For each treatment, three biological replicates were carried out.

Analyses of *in vitro* PAT production by *P. expansum* co-incubated with *P. terrestris* LS28 was carried out in solid AMM. To this aim, dual culture plates were set up as described above with some modifications. Briefly, a sterile cellophane paper was placed on top of the agar AMM medium, then the two microorganisms were spotted 3 cm apart as described in Section "Measurements of pH" and incubated for 10 days at 25°C in the dark. Afterwards, the cellophane with the fungal and yeast biomass was removed from the Petri dishes. The agarized AMM of each plate was finely shredded with a scalpel, transferred into a glass tube containing 20 ml of acidified distilled water, then PAT was extracted from this suspension three times with 15 ml of ethyl acetate (acidified with HCl). The solvent layer (45 ml) was dried as described above and redissolved in 0.5 ml of H_2O (acidified with 1% acetic acid) and methanol 95:5 (v/v) for HPLC analysis. The experiments were repeated three times.

In vivo PAT production by *P. expansum* in rotting apple tissues, alone or co-inoculated with the BCA LS28, was assessed as previously described (Zheng et al., 2017) with slight modifications. Samples of rotting apple tissue for PAT quantification were collected 7 days after inoculation. Rotting tissues of the same technical replicate plus 1 cm of surrounding healthy tissue were withdrawn with a sterile scalpel, pooled, and stored at −80°C. To increase efficacy of extraction of both PAT and gDNA extraction (the latter for fungal biomass measurement described in Section "Measurement of *P. expansum* Biomass in Wounded Apple Tissue"), all the collected samples were lyophilized. Subsequently, 1 g of the lyophilized sample was grounded with a pestle, re-suspended in 5 ml of sterile distilled water, and PAT was extracted three times, each time with 5 ml of acidified ethyl acetate. The collected ethyl acetate layers (15 ml for each sample) were dried and redissolved in 0.5 ml of mobile phase (1% acetic acid solution and methanol 95:5, v/v), as described above. The samples were centrifuged, filtered through a 0.22 µm filter, and 20 µl were injected into an HPLC apparatus.

In all cases, HPLC analysis was carried out with a Dionex (Sunnyvale, CA, United States) analytical system consisting of a P680 solvent delivery system, and a 20 µl injector loop (Rheodyne, Cotati) was used for PAT determination. Detection was performed by measuring the absorbance of UV light at 276 nm by means of a UVD170 detector (Dionex, Sunnyvale, CA, United States) connected to a data integration system (Dionex Chromeleon, version 6.6). A Zorbax analytical column (SBC18 250 mm × 4.6 mm, 5 µm, Agilent Technologies) was used. The mobile phase, H_2O (acidified with 1% acetic acid) with methanol 95:5 (v/v), was used with a flow rate of 1 ml/min and isocratic mode. Detection was performed by measuring the absorbance of UV light at 276 nm. Prior to sample analysis, a calibration curve was drawn by injecting triplicates of PAT standards at different concentrations (0, 4, 25, 50, 75, and 100 ppm). The standard solutions were prepared from a stock solution of 2,000 µg ml^{-1} of pure PAT (Sigma-Aldrich) solubilized in acidified ethyl acetate. The value of R^2 was higher than 0.99. Each experiment consisted of three replicates per time point and each replicate consisted of three apples with four wounds each. Rotting wounds, collected from each replicate were pooled before extraction. Chromatographic analysis was performed in duplicates. Data from the three experiments, which were expressed as µg/ml of PAT, were similar as resulting from ANOVA analysis and were pooled. Bars in the figure represent mean values ± standard deviations.

Measurement of *Penicillium expansum* biomass in wounded apple tissue

Real-time quantitative PCR (RT qPCR) was used to quantify the biomass of *P. expansum* strain 7015 grown in wounded apple tissues when inoculated alone or co-inoculated with the BCA *P. terrestris* LS28. To this aim, total DNA extraction and purification were performed as described by Zheng et al. (2017) from 0.5 grams of the same samples of lyophilized apple tissue that were withdrawn for PAT analyses (see above). RT qPCR was performed by using specific primer pairs designed on the *PatF* gene, patF_F (ATGAAATCCTCCCTGTGGGTTAGT) and patF_R (GAAGGATAATTTCCGGGGTAGTCATT), reported by Tannous et al. (2015). Reaction mixtures contained 10.0 µl of SYBR Premix Ex TaqTM (Takara), 0.4 µl of each primer (20 mM) and 2 µl of total DNA extracted from the apple samples in a final volume of 20 µl.

Two simultaneous replicated amplifications were carried out. Amplification reactions were performed in 96-well microtiter plates (Twin.tec PCR plate, Eppendorf) and a thermal cycler apparatus (Mastercycles EP gradient S, Eppendorf) by using the following cycling protocol: an initial denaturation step (30 s at 95°C) followed by 40 cycles of 5 s at 95°C, 20 s at 55°C, and 20 s at 72°C. Afterwards, a melting curve program was run in which measurements were made at 0.5°C T increments every 10 s within a range of 60–95°C. The DNA concentration of each sample was extrapolated from a standard curve which was developed by plotting the logarithm of known concentrations (10-fold dilution series from 10 ng to 1 pg., 25 µl reaction) of *P. expansum* genomic DNA.

Determination of the specific mycotoxigenic activity *in vitro* and *in vivo*

The specific rate of patulin biosynthesis by *P. expansum* strain 7015 in liquid AMM medium was calculated by referring the mean amount of patulin (µg) to the weight (g) of dry fungal mycelium or mL of cultural broth, depending on the experiment; in agarized AAM assays, the amount of patulin was referred to the respective fungal biomass expressed as cm of fungal radial growth. In *in vivo* experiments, the specific mycotoxigenic activity by *P. expansum* was expressed as ng patulin per µg of fungal DNA, as already reported in the work by Zheng et al. (2017).

Statistical analysis

Data were submitted to factorial analysis of variance (one-way ANOVA) followed by Uncorrected Fisher's LSD comparison test. Differences were considered statistically significant for $p < 0.05$. The percentages of infected wounds were converted into Bliss angular values (arcsine $\sqrt{\%}$) before statistical analysis. The comparisons between two groups were carried out using a two-tailed unpaired Student's *t*-test. Statistical analyses were performed using the GraphPad software 8.

Results

Effect of *Papiliotrema terrestris* on patulin accumulation by *Penicillium expansum* and mycotoxigenic activity *in vivo*

The effects of *P. terrestris* LS28 was evaluated *in vivo* on green blue mold disease development and toxigenic activity of *P. expansum* strain 7015 by analyzing the percentage of infected wounds and PAT biosynthesis during the tri-trophic interaction between the host (apple fruit), the pathogen, and the biocontrol agent. The BCA *P. terrestris* LS28 was applied at optimal and sub-optimal cell concentrations. As expected, and in agreement with previous data (Lima et al., 1998, 1999, 2005; Castoria et al., 2003, 2021) after 7 days of incubation the application of the BCA at the optimal cell concentration results in a significantly lower percentage of wounds infected by *P. expansum* as compared to the fungal pathogen applied alone (Figures 1A,B). Furthermore, the high antagonistic activity of *P. terrestris* LS28 at the optimal cell concentration is associated to a substantial reduction of PAT accumulation in rotting tissues as compared to the positive control, both in terms of µg of PAT per gram of lyophilized apple tissues (Figure 1C), and ng of PAT per µg of fungal DNA (Figure 1D). In the control treatment, *P. expansum* inoculated alone is able to produce approximately 150 µg of PAT per gram of dry apple tissue (Figure 1C), and 2 ng of PAT per µg of fungal DNA (Figure 1D). Conversely, the (lower) suboptimal concentration of the BCA LS28 causes a significant reduction in the disease severity induced by *P. expansum*, although at a lesser extent than the optimal BCA concentration (Figure 1A) but provided almost no protection from *P. expansum* infection (Figure 1B). Moreover, when considering the amount of PAT (µg) per gram of dry apple tissue, the lower protection operated by the BCA resulted in a higher level of PAT contamination as compared to the treatment with the optimal yeast concentration, although lower than the positive control (Figure 1C). Strikingly, the suboptimal concentration of the BCA LS28 strongly stimulates PAT biosynthesis, as shown by the specific toxigenic activity of *P. expansum* (~8 ng of PAT per µg of fungal DNA; Figure 1D), despite the overall contamination level being lower than the control (Figure 1C). In particular, the co-incubation of the pathogen with the suboptimal concentration of *P. terrestris* cells leads to an approximately fourfold increase of the specific mycotoxigenic activity of *P. expansum* as compared to the positive control (*P. expansum* alone), with 7.8 and 1.8 ng of PAT per µg of fungal DNA, respectively.

In vitro the BCA stimulates patulin accumulation

Patulin biosynthesis by *P. expansum* during the interaction with the biocontrol agent *P. terrestris* LS28 was evaluated *in vitro* co-culturing the two microorganisms in liquid and solid AMM. In liquid assays, after 7 days, the fungal pathogen alone is able to accumulate 525 µg of PAT per ml^{-1} of broth. Co-incubation with LS28 strongly stimulates mycotoxin biosynthesis, with a PAT content of 1,270 µg ml^{-1} of cultural broth (Figure 2A). A similar approach was carried out on solid AMM. The dual culture plates were kept in incubation until the fungal colony covered the entire Petri dish (Figure 2C) in the control condition (fungal alone); at this time point, PAT was extracted and quantified, and the mycotoxin content was expressed as µg per cm of radial growth (Figure 2B). As in the case of the experiments in liquid medium, PAT biosynthesis by *P. expansum* is much higher in the presence of the BCA (113.9 µg per cm of fungal radial growth) as compared to the fungal pathogen alone (20.5 µg per cm of fungal radial growth).

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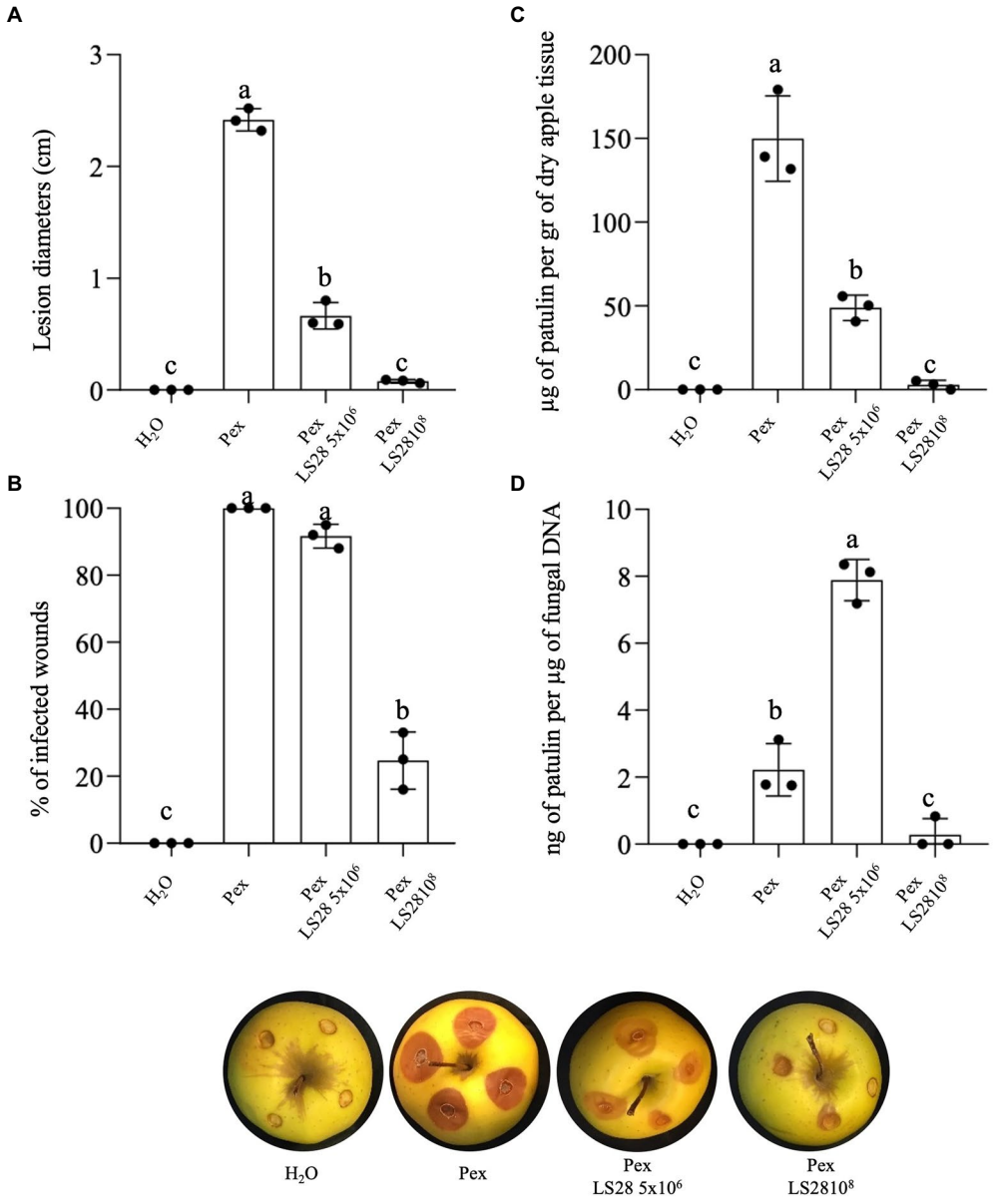

FIGURE 1
Effect of optimal ($1x10^8$CFUml^{-1}) and suboptimal ($5x10^6$CFUml^{-1}) concentrations of the biocontrol yeast *Papiliotrema terrestris* LS28 after 7 days of incubation on **(A)** lesion diameter; **(B)** percentage of infected wounds; **(C)** patulin accumulation; **(D)** specific mycotoxigenic activity, in apples inoculated with *P. expansum* strain 7015 (Pex). Columns with common letters are not significantly different according to one-way ANOVA followed by Uncorrected Fisher's LSD comparison test ($p<0.05$). Data shown are the mean values ± SD from three independent experiments (black dots).

Penicillium expansum toxigenic activity is pH-dependent *in vitro*

To elucidate the effect of extracellular pH on the specific mycotoxigenic activity of *P. expansum* strain 7015, the pathogen was cultured in liquid AAM at pH values ranging from 3 to 7. After 7 days of the inoculation, PAT content in the cultural broth was determined and expressed in relation to the fungal dry biomass of each condition. As reported in Figure 3, PAT production in AMM is strongly related to pH value. In particular, the highest specific mycotoxigenic activity is achieved at pH 5, whereas it decreases at both higher and lower pH values, being the lowest at pH 3.

Influence of *Papiliotrema terrestris* on *Penicillium expansum*-induced acidification *in vitro*

As already known for *P. expansum* (Prusky and Yakoby, 2003; Hadas et al., 2007; Prusky et al., 2007; Barad, Espeso et al., 2016;

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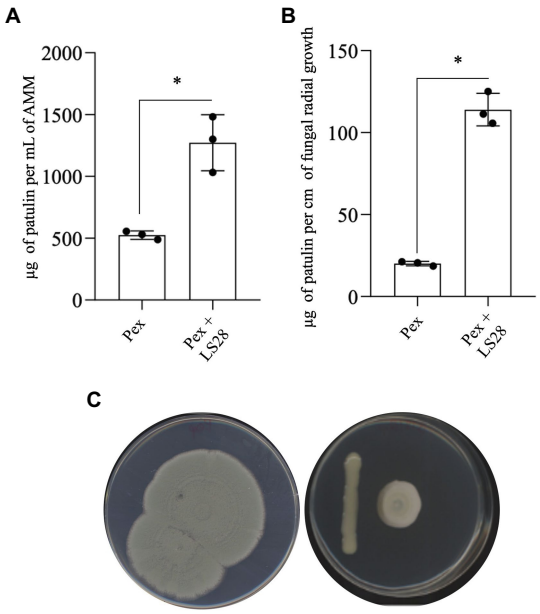

FIGURE 2
Patulin quantification in dual cultures of *Penicillium expansum* strain 7015 (Pex) and the biocontrol agent *Papiliotrema terrestris* LS28 in **(A)** liquid AMM (µg patulin ml^{-1} of medium) and **(B)** agarized AMM (µg patulin cm^{-1} of fungal radial growth). Patulin was extracted and quantified 7days after the inoculation. Data are presented as mean values ± SD from three independent experiments (black dots). *p<0.05 vs. Pex according to two-tailed, unpaired Student's t-test.

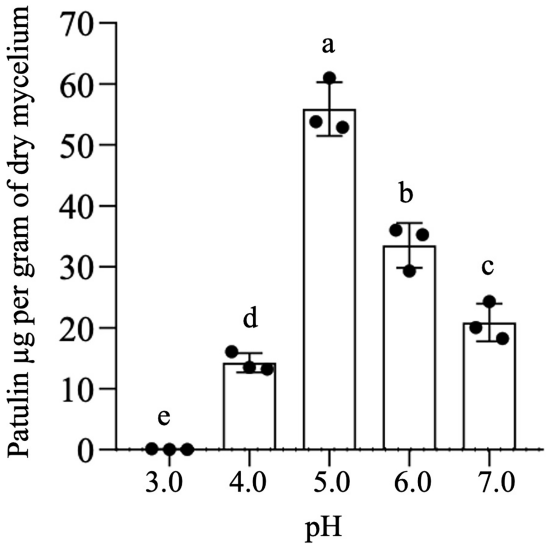

FIGURE 3
In vitro patulin production at fixed pH values. Patulin was extracted from a 7-day-old culture of *Penicillium expansum* strain 7015 in liquid AAM in which the pH was kept unvaried at fixed values. Data shown are the mean values ± SD from three independent experiments (black dots). Columns with common letters are not significantly different according to one-way ANOVA followed by uncorrected Fisher's LSD comparison test (p<0.05).

Barad, Sionov et al., 2016; Tannous et al., 2018), and as confirmed for the strain 7015 used in this study (Figure 3; see above), PAT biosynthesis is strongly influenced by environmental pH.

To investigate the involvement of this mechanism in the interaction between *P. terrestris* strain LS28 and the fungal pathogen, the extracellular pH was monitored over time *in vitro*, both in solid and liquid AAM media, either with the microorganisms grown alone or in co-incubation. As shown in Figure 4A, *P. expansum* alone quickly acidifies the extracellular environmental pH from 4.2 to 3.6 at 48 h. The tissue acidification driven by the fungal proliferation gradually continues until it reached pH 2.6 at 144 h of incubation. Conversely, after the same time interval, *P. terrestris* LS28 increases by approximately 0.8 unit (pH 5.0) the extracellular pH compared to the first time point (pH 4.2). Interestingly, in dual culture the biocontrol agent induces an increase of the extracellular pH from 4.2 to 4.5 after 96 h of incubation, thus apparently counteracting the rapid acidification caused by *P. expansum* (Figure 4A). At the latter time points, the pH slightly decreases to reach a value of 4.2 at 144 h of incubation.

The effect of *P. terrestris* LS28 on extracellular pH was also investigated on agar AMM by using the pH indicators green and purple bromocresol, which allowed to visualize pH variations during the dual interaction. The pictures in Figure 4B clearly show that after 7 days from the inoculation, *P. expansum* 7015 strongly acidifies the agar medium by producing a yellow halo surrounding the fungal colony in the presence of both purple and green bromocresol. Conversely, *P. terrestris* LS28 grown alone on AMM leads to a slight color change in the presence of bromocresol purple, while no color variations are observed with bromocresol green. In the dual culture, the biocontrol agent LS28 apparently counteracts the acidification produced by the pathogen so that no color change occurs in the presence of bromocresol green.

In vivo influence of *Papiliotrema terrestris* on *Penicillium expansum*-induced acidification of apple wounds

For assessing the *in vivo* pH trend, *P. expansum* and two different concentrations (optimal and suboptimal) of the biocontrol yeast were inoculated in apple wounds, and wound pH monitored over time (Figure 5). In *P. expansum*-inoculated wounds, as expected there was a rapid acidification with pH value of 3.5 at 48 h of incubation; pH values further decrease to reach 2.8 at 168 h of incubation. *P. terrestris* LS28 alone causes only slight pH changes in apple wounds, which display a trend like that of uninoculated healthy wounds: the inoculum with lower suboptimal yeast cell concentrations induces a slight pH increase during incubation, whereas the higher optimal concentration of

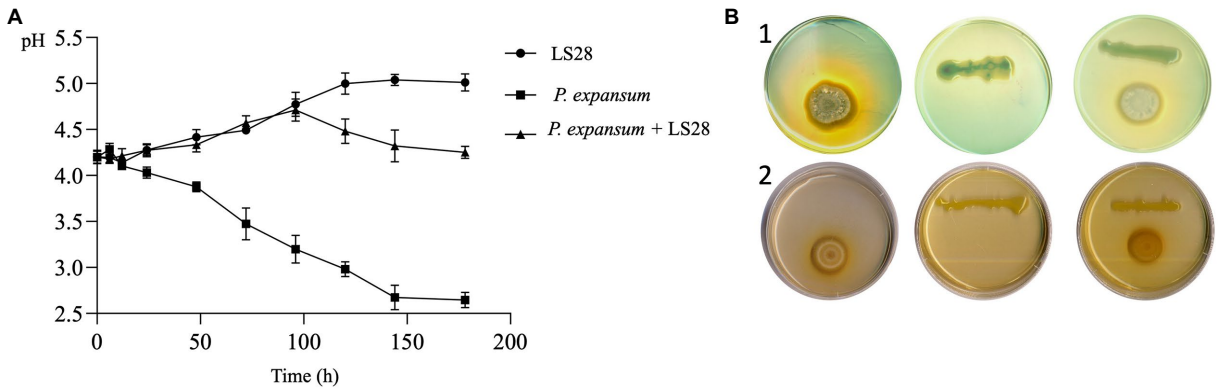

FIGURE 4
(A) *In vitro* extracellular pH trend of *P. expansum* co-inoculated with LS28 in liquid AMM. pH was measured at 0, 6, 12, 24, 48, 72, 96, 120, 144, and 178h after co-inoculation. Data shown are the mean values ± SD from three independent experiments. **(B)** Representative pictures showing *P. terrestris* strain LS28 and *P. expansum* strain 7015 grown alone or in dual cocultures for 7days in AMM plates containing the pH indicators bromocresol green (yellow, pH<3.8; blue, pH>5.4) or purple (yellow, pH<5.2; purple, pH>6.8). In the plates amended with the pH indicator bromocresol green (b-1), the extracellular acidification produced by *P. expansum* growth is visible as a clear halo, while this pH changing is counteracted by the biocontrol yeast; No color changes occur in the plates with the pH indicator bromocresol purple (b-2).

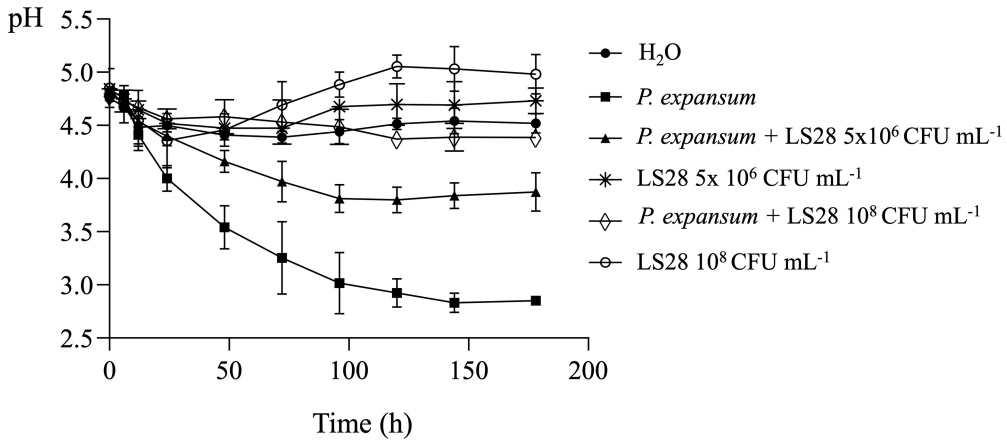

FIGURE 5
In vivo extracellular pH trend in apple wounds inoculated with optimal (10^8CFUml^{-1}) and suboptimal (5×10^6CFUml^{-1}) concentrations of the biocontrol yeast *Papiliotrema terrestris* LS28 and with the fungal pathogen *Penicillium expansum* strain 7015. pH was measured at 0, 6, 12, 24, 48, 72, 96, 120, 144, and 178h after inoculation. Data shown are the mean values ± SD from three independent experiments.

the BCA induces a more pronounced alkalinization, with pH value of 5.0 after 168 h of incubation. As in the case of *in vitro* experiments (Figure 4), in apple wounds co-inoculated with LS28 and *P. expansum*, the BCA appears to counteract the acidification produced by the fungal pathogen with an efficacy that is correlated to the concentration of the yeast cells: the co-incubation of 5×10^6 CFU ml^{-1} of LS28 and *P. expansum* produces a lower acidification than the pathogen alone, stabilizing the pH at 3.8 after 96 h from inoculation. Conversely, the optimal concentration of LS28 keeps the pH unchanged during the incubation with a trend similar to H_2O-treated healthy wounds, in which the pH remains stable over time, ranging from pH 4.7 to 4.5 for the whole duration of the experiments.

Discussion

The ability to perceive and adapt to the environment is a key aspect for which both eukaryotes and prokaryotes invest in terms of energy. Many cellular responses intimately depend on a plethora of external variables and, among these, pH emerges as a crucial parameter which all microorganisms influence and depend on (Kemmitt et al., 2006; Falkowski et al., 2008; Bethke et al., 2011; Lennon and Jones, 2011; Amend et al., 2013; Maguffin et al., 2015; Zhalnina et al., 2015). The pH is also a crucial factor in the infection process. Several fungal plant pathogens are able to finely change the pH in the infection site, thus achieving the optimal condition for the expression of virulence-related genes through a process driven

by different modulators (Rodriguez-Moreno et al., 2018; Li et al., 2020; Laethem et al., 2021). *Penicillium expansum* is the causative agent of green-blue mold on stored pome fruits. This fungus secretes a combination of organic acids to acidify the infection site, thus modulating the expression of virulence-related genes and creating a favorable condition for the activity of polygalacturonases, enzymes that are responsible for cleavage and depolymerization of pectin, a major component of middle lamella. During its infection process *P. expansum* produces also the mycotoxin PAT, although its active role in pathogenesis is controversial (Prusky and Yakoby, 2003; Hadas et al., 2007; Prusky et al., 2007; Puel et al., 2010; Sanzani et al., 2012; Barad et al., 2013; Barad, Sionov et al., 2016; Li et al., 2020; Bartholomew et al., 2021). Although many aspects of PAT biosynthesis have been elucidated, to date little is known about the effect of pH modulation on PAT biosynthesis *in vivo*, i.e., on apple fruits infected by *P. expansum* (Moake et al., 2005; Jimdjio et al., 2021). Similarly, no reports exist on the effect of biocontrol agents on host tissue pH when they counteract the attack on fruits by *P. expansum*.

In the present study, the effect of an optimal and a suboptimal concentration of the biocontrol agent *P. terrestris* LS28 on disease incidence and severity, and on PAT biosynthesis in apples inoculated with *P. expansum* was initially investigated. In agreement with our previous results (Lima et al., 1998, 1999, 2005; Castoria et al., 2003, 2021) the optimal concentration of the BCA *P. terrestris* LS28 provides almost full protection of artificially infected apple wounds after 7 days, both in terms of disease severity and disease incidence (i.e., lesion diameters and percentage of infected wounds, respectively; Figures 1A,B). The biocontrol activity of *P. terrestris* LS28 also influences PAT accumulation by reducing the total amount of this mycotoxin in infected apples (Figure 1C). As expected, the lower concentration of the BCA results in a lower biocontrol activity, but determined an increase of the specific mycotoxigenic activity by *P. expansum* (Figure 1D), even if the overall contamination with PAT is reduced also in this case (Figure 1C). Moreover, the increased PAT production by *P. expansum* in the presence of LS28 also occurs in *in vitro* experiments performed both in liquid and solid media (Figure 2), especially when the amount of PAT was referred to a parameter mirroring the fungal biomass in the dual culture (i.e., fungal radial growth; Figure 2B). Data obtained from the above-mentioned *in vivo* and *in vitro* experiments are in agreement with those reported by Zheng et al. (2017) who investigated the effects of two biocontrol yeasts, *R. mucilaginosa* 3617 and *R. kratochvilovae* (syn. *Rhodosporidium kratochvilovae*) LS11, on green blue mold disease and PAT contamination caused by two different strains of *P. expansum* (PY and FS7) in artificially inoculated Fuji apples. While the BCAs significantly reduced the disease symptoms and lowered the development of fungal biomass, they both increased the specific rate of PAT production even though they reduced the total mycotoxin contamination. Similarly, Zhu et al. (2015) reported the effect of the marine yeast *R. paludigenum* on PAT accumulation in apples and pears. The occurrence and severity of apple and pear decay caused by *P. expansum* were significantly inhibited, but the yeast enhanced PAT accumulation as compared to the controls in infected fruits.

Next, we aimed to elucidate the mechanisms responsible for the increased specific mycotoxigenic activity by *P. expansum* in the presence of a suboptimal concentration of the biocontrol agent *P. terrestris* LS28. First, because it is known that PAT production by *P. expansum* is influenced by the pH (Zong et al., 2015), the *P. expansum* strain 7015 specific mycotoxigenic activity was determined in apple mimicking medium in which fixed pH values were kept constant over time. In agreement with Zong et al. (2015), PAT production is closely related to pH, with its biosynthesis occurring at pH between 4 and 7, and with the optimum at pH 5 (Figure 3). Following this observation, we speculated that the biocontrol agent *P. terrestris* LS28 might influence PAT production by *P. expansum* by modifying the pH of the media. To assess this hypothesis, *in vitro* experiments were performed by growing *P. terrestris* strain LS28 and *P. expansum* strain 7015 alone or co-incubated in both liquid and solid artificial apple medium and by monitoring the pH at determined time points. Our results clearly show that *P. terrestris* is able to counteract the strong acidification induced by *P. expansum*, with pH value remaining almost unchanged (4.2–4.5) for the whole duration of the experiments (Figure 4A). This buffering effect of *P. terrestris* against the extracellular acidification induced by the fungal pathogen is also observed *in vivo*, i.e., in apple wounds, and the pH increase induced by *P. terrestris* strain LS28 is proportional to the cellular concentration used, being more prominent when a high number of *P. terrestris* cells were used (Figure 5).

Remarkably, the pH increase determined by *P. terrestris* when coincubated with *P. expansum* falls within or close to the range in which the highest production of PAT by *P. expansum* is detected, clearly suggesting that the buffering effect exhibited by *P. terrestris* against the acidification induced by *P. expansum* accidentally is responsible for the increased mycotoxigenic activity of the fungus. It is likely that the increased PAT production by *P. expansum* during co-cultivation with other BCAs of the genus *Rhodosporidium* (Zhu et al., 2015; Zheng et al., 2017) might be also due to a modulation of the extracellular pH, although this parameter had not been investigated by the aformentioned authors. If this hypothesis is true, the mechanisms of extracellular pH modulation induced by the BCA might be a conserved feature of Basidiomycetous BCAs and its specific role in biocontrol acitivity should be studied in more details. In this regard, we have unpublished evidences that the addition of alkalinizing or acidifying compounds during microbial competition between *P. terrestris* LS28 and *P. expansum* in apple wounds leads to an increase or a decrease in the yeast biocontrol activity, respectively. Nevertheless, in these experiments the compounds that were used to modulate the apple wounds pH were also a source of nutrients both for the BCA and the fungus (e.g., ammonium sulphate, an acidifying compound that is also a source of nitrogen and sulfur), and as a consequence, it was difficult to exclusively determine the role of the pH modulation in the biocontrol activity of *P. terrestris* LS28 (data not shown). Therefore, our data are not conclusive and

further studies are necessary to define the role of the extracellular pH buffering in the biocontrol activity of *P. terrestris* LS28.

It is interesting to note that the buffering activity displayed by *P. terrestris* LS28 is not triggered by the presence of the fungus, since there is a more pronounced pH increase where the BCA was inoculated alone, both *in vitro* and *in vivo* (Figures 4, 5). Although this mechanism is unknown, it can be speculated that the pH increase might be due to the production and secretion by the BCA of an alkaline compound, which then would counteract tissues acidification induced by certain plant pathogens as *P. expansum*. On the other hand, environmental alkalinization due to ammonia production is also a known virulence factor for some other plant pathogens such as *Colletotrichum gloeosporioides,* the cause of anthracnose fruit rot (Shnaiderman et al., 2013), and in this case the buffering activity of *P. terrestris* LS28 might be advantageous for the phytopathogenic fungus.

Besides pH modulation, as noted by Zheng et al. (2017), a contribution to the increase of mycotoxigenic activity by *P. expansum* could also be due to the nutritional stress encountered by this pathogen during competition for nutrients operated by the BCA, since the association between nutritional imbalance and the induction of secondary metabolism of *P. expansum* has recently been reported (Tannous et al., 2018).

Conclusion

Our results show that the biocontrol agent *P. terrestris* LS28 is effective in controlling green blue mold caused by *P. expansum* and in extremely reducing PAT contamination in apple fruits. This reduction occurs even if this biocontrol yeast causes an increase of specific mycotoxigenic activity by the pathogen, as measured as the quantity of PAT per unit of fungal biomass. This increase seems to be associated to the alkalinization of the infection court operated by *P. terrestris* LS28, which keeps the pH value within the range in which *P. expansum* synthesizes the highest level of PAT. Moreover, the ability to counteract the *P. expansum*-driven acidification, a pivotal mechanism for the fungal attack to the host fruit, appears to be also associated to the protection of fruits, suggesting a potential novel biocontrol mechanism operated by *P. terrestris* LS28 to antagonize *P. expansum* infection. While further studies are necessary to validate this hypothesis, to our knowledge this is the first report on the influence of a biocontrol agent on pH of the infection court of a postharvest pathogen, and on the consequent effects on mycotoxin synthesis and the level of fruit protection.

Data availability statement

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

Author contributions

DP, GI, and RC: designed the experiment, analyzed results, wrote, and revised the manuscript. CM: provided suggestion for the experimental design, performed the experiments, analyzed results, and revised the manuscript. IN and PA: performed HPLC analyses, analyzed results, and revised the manuscript. GL and FD: analyzed results and 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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Microbiome and pathobiome analyses reveal changes in community structure by foliar pathogen infection in rice

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Increasing evidence suggests that the plant rhizosphere may recruit beneficial microbes to suppress soil-borne pathogens, but microbiome assembly due to foliar pathogen infection and ecological mechanisms that govern microbiome assembly and functions in the diseased host are not fully understood. To provide a comprehensive view of the rice-associated microbiome, we compared bacterial and fungal communities of healthy rice and those infected with *Magnaporthe oryzae*, the causal agent of blast disease. We found that the soil had a greater diversity of bacterial and fungal communities than plant endospheric communities. There was no significant dysbiosis of bacterial and fungal microbiome diversity due to disease, but it caused a substantial alteration of bacterial community structure in the root and rhizosphere compartments. The pathobiome analysis showed that the microbiome community structure of leaf and grain tissues was changed markedly at the pathogen infection site, although the alpha diversity did not change. Correspondingly, the relative abundances of some bacteria and fungi were clearly altered in symptomatic tissues. We noted an increase in *Rhizobium* bacteria and a decline of *Tylospora, Clohesyomyces,* and *Penicillium* fungi in the symptomatic leaf and grain tissues from both locations. According to the inferred microbial network, several direct interactions between *M. oryzae* and other microbes were identified. The majority of edges in the interaction network were positive in diseased samples; contrastingly, the number of edges was much lower in the healthy samples. With source tracking analysis, we observed a sharp contrast in the source of root endosphere bacteria due to *Magnaporthe* infection. Whereas the majority (71%) of healthy root bacteria could be tracked from the soil, only a very small portion (17%) could be tracked from the soil for diseased samples. These results advanced our understanding and provided potential ideas and a theoretical basis for studying pathobiome and exploiting the microbiome for sustainable agriculture.

KEYWORDS

amplicon sequencing, bacteria, community composition, endosphere, infection site, plant health, network analysis, source tracking

Introduction

The world faces formidable challenges in achieving food security for the ever-increasing population (Mc Carthy et al., 2018; Barrett, 2021). Various biotic and abiotic stressors, such as drought, salinity, disease, and pests affect crop production. In particular, plant pathogens represent an insidious threat to agriculture, and they are accounted for a loss of ∼16% of global crop yield (Oerke, 2006). Numerous studies have been conducted on the intricate interplay between plant and pathogen, including pathogenicity, disease progression, plant immunity, and disease management. However, in most of these studies, the main focus of the investigation has been the binary interactions between plant and pathogen and those under various environmental conditions. The enormous complexity of interactions among plant, pathogen, and other microorganisms and their outcome under diverse conditions have also received attention in the recent years (Bulgarelli et al., 2013; Santos and Olivares, 2021; Trivedi et al., 2022).

Similar to humans and other animals, plants harbor complex microbial communities called the "plant microbiome." There is an increasing interest in understanding the composition and function of the microbiome for harnessing their potential, such as growth promotion and disease resistance of the host, as well as in understanding the basics of host–microbial symbioses (Busby et al., 2017; Song et al., 2020; Zhang et al., 2021). Pathogenic microbes cause changes in plant phenotypes through tissue damage and induction of plant defenses, which can alter the immunity of plants to colonization by microorganisms. Therefore, factors that influence the impact of the pathogen on hosts will likely affect the colonization and growth of plant-associated microorganisms. Various host and environmental factors influence microbiome structure and diversity in the plant (Compant et al., 2019; Dastogeer et al., 2020). Host immunity level is one of the major factors shaping plant microbiome community composition (Dastogeer et al., 2020). Recent studies suggest that similar to the gut microbiome, the plant microbiome can enhance the immune functions of the plant host (Vannier et al., 2019; Ma et al., 2021; Teixeira et al., 2021). It has been shown that plants can recruit selective microorganisms from the soil by exudating compounds in the rhizosphere to positively impact plant growth and health (Reinhold-Hurek et al., 2015; Sasse et al., 2018; Liu et al., 2021).

Accumulating data implies that there remains a constant battle between the host and its microbes to maintain microbiome homeostasis in the host (Paasch and He, 2021). In the human microbiome research, the concept of "healthy microbiome" has gained massive attention, although the definition of "healthy microbiome" is still not very clear-cut (Bäckhed et al., 2012; Shanahan et al., 2021). In addition to modifying host defense, the healthy microbiome maintains ecological stability in the host and thus prevents microbial intruders, such as those protected from pathogen attack. On the other hand, the disease causes a shift in the microbiome termed, "microbiome dysbiosis," a situation in which microbiome homeostasis is disrupted and the organism becomes more vulnerable to potentially harmful microbial invaders. The pathobiome concept arose from human studies, which suggests a shift of "one pathogen–one disease paradigm" to a set of host-associated microorganisms with reduced (or possibly reduced) health status as a result of interactions between members of that set and the host (Defazio et al., 2014; Krezalek et al., 2016). To understand the pathobiome, it is important to clarify the nature of the interactions of the associated microbes among themselves and with the host, in addition to the identity of the community (Bass et al., 2019). It was shown that perturbations in the microbial community by external factors could significantly affect the susceptibility of humans and animals to several diseases (Sekirov et al., 2010; Ferreira et al., 2011; Willing et al., 2011; Vannier et al., 2019). For example, during the progression of mastitis, dysbiosis of the milk microbiome can occur with the increase of opportunistic pathogenic bacteria and reduction of healthy commensal bacteria (Patel et al., 2017). However, it is mostly unclear whether dysbiosis is a cause or consequence of disease (Bäckhed et al., 2012). An in-depth understanding of microbiome dysbiosis may be helpful in the effort to restore a microbial community so as to abate the host damage. There is a paucity of information in the plant system regarding if and to what extent dysbiosis of the microbiome occurs due to pathogen infection in the host. In a study, Kaushal et al. (2020) showed that when *Fusarium oxysporum* infects banana plants, the fungal and bacterial communities had a shift toward a less diverse community. Humphrey and Whiteman showed that bittercress affected by a leaf-mining fly, *Scaptomyza nigrita*, had overall higher bacterial densities than undamaged leaves, mainly due to the increased abundance of typical leaf bacteria, particularly *Pseudomonas* (Humphrey and Whiteman, 2020). Increased bacterial diversity in the damaged tissue is likely caused by increased released nutrients, increased jasmonic acid, and bacterial inoculation by insect secretions (Humphrey and Whiteman, 2020; Smets and Koskella, 2020). Increased understanding of microbiome alterations under stress and functions of microorganisms in improving plant fitness and defenses highlighted the need for the investigation of their role more elaborately across various plant-pathogen-environment studies.

Rice (*Oryza sativa*) is an important crop that constitutes the staple diet of over three billion people worldwide (Skamnioti and Gurr, 2009). Rice blast disease caused by *Magnaporthe oryzae* is a global problem that can cause 10–30% reduction in rice production each year, which could feed 60 million more people (Skamnioti and Gurr, 2009; Kirtphaiboon et al., 2021). Due to a broad host range of the pathogen and the evolution of new pathotypes, blast management is a daunting task (Valent, 2021; Devanna et al., 2022). Also, environmental sustainability necessitates the innovation of natural biocontrol agents in place

of chemical fungicides. Therefore, rice–*Magnaporthe* interaction pathosystem has emerged as a model system to study host–pathogen interaction. The microbiome can play a significant role in host defense and pathogen infection, and the study needs to consider pathobiome for a better understanding of the roles of disease-associated microbes. This study compared the rice microbiomes of healthy and infected rice samples gathered from the same location to gain an insight into the potential role of the pathogens in shaping the microbiome composition. We investigated the microbiome (both bacteria and fungi) structure of non-symptomatic and symptomatic rice plants naturally infected by *M. oryzae*. Symptomatic rice plants were selected based on the morphological symptoms, including typical eye-shaped brown spots in leaves and yellowish lines surrounding the spots. In addition, we demonstrated the composition and assemblage of the naturally occurring microbiome in the bulk soil, rhizosphere, root, leaf, and grain samples. We believe our study is one of the pioneer investigations to describe an inventory of bacterial and fungal communities associated with the components of non-symptomatic and symptomatic rice plants infected by *M. oryzae* in rice.

Materials and methods

Headings collection and processing of soil, rhizosphere, and plant samples

Rice (*Oryza sativa japonica*) samples were collected from two locations, *viz.* Fukushima and Miyagi of Japan. Samples of Miyagi (cultivar: *54-3110*) were collected from a field of Miyagi Prefectural Furukawa Agricultural Experiment Station (38.59652N, 140.91219E) on 9 September 2019, and the Fukushima samples (cultivar: *Hitomebore*) were collected from Nihonmatsu city (37.602373N, 140.586406E) grown in a farmer's field on 21 September 2019. The cultivars were susceptible to blast infection, and there were severe disease symptoms throughout the fields in both locations. In both places, samples were collected at the maturity stage of rice before the ripening phase. Rice plants were dug out with soil from around 20 cm using a shovel. We randomly chose and collected nine healthy-looking plants and nine blast-infected plants in different areas within a plot. Each plant was wrapped separately with a plastic bag and transported to the laboratory in a cooler box containing dry ice to maintain a low temperature to minimize potential microbial community disruptions. In the laboratory, the samples were further kept at 4°C until processed, and processing was completed within 48 h of collection. Sample fractionation into bulk soil, rhizosphere soil, the root, leaf, and grain compartments were performed within 48 h after sampling. At first, plants were cut above the root system. From the shoots, leaf and panicle were separated and washed with water to remove the adhering debris and named "leaf fraction" and "grain fraction," respectively. The roots systems were shaken to collect soil manually, and the soil was labeled as "bulk soil fraction." Large soil clods were broken with a hand-held tiller. All the debris was removed from the soil, placed in the zipper storage bag, and stored at −20°C until use. Using sterilized scissors, rice roots around 6–8 cm in length were cut. The excised roots (cut as needed to fit) were placed in a 50 ml falcon tube containing 35 ml of autoclaved phosphate buffer (6.33 g/L NaH_2PO_4, 8.5 g/L Na_2HPO_4 anhydrous, pH = 6.5, 200 µl/L silwet L-77 as surfactant). The tubes were shaken for 2 min to release the rhizosphere from the surface of the roots (rhizosphere fraction). The roots were taken out with sterilized forceps, blot dried on paper towels, and placed in a new 50 mL falcon tube (root fraction). The roots, leaves, and grains were surface sterilized by washing in 0.25% of NaOCl for 1 min, followed by 70% of EtOH for 40 s and subsequent washing in sterile water thrice. The efficacy of surface sterilization was evaluated by tissue imprint method (Greenfield et al., 2015). We used a cork borer to pierce and collect small tissues from the middle of the symptoms of leaves and named "symptomatic fraction" and from non-symptomatic tissues that did not show any apparent disease symptoms (non-symptomatic fraction). Sterilized tissues were blot dried in autoclaved paper towels, cut into small pieces, and stored at −80°C until further processed. Any plant parts or debris were removed from bulk and rhizosphere soil samples. After suspending in phosphate buffer, the soil was filtered through a sterile 100-µm-mesh cell strainer to remove any small plant parts and debris. Suspended soils were collected by centrifugation at 3000 × g for 5 min, and the pellets were stored at −20°C until DNA extraction. Rhizosphere soils and plant parts from three of the nine plants were bulked to make a biological replicate.

DNA extraction and amplicon analysis

Total genomic DNA from bulk rhizosphere and soil was extracted using NucleoSpin® Soil (Macherey–Nagel, Duren, Germany) and from the plant tissues using DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) according to respective protocols in the manuals. The DNA samples were eluted in 50 µL of nuclease-free water and used for bacterial and fungal community profiling. The quantity and quality of DNA were measured using a Nanodrop 2000, diluted to 100 ng/ml, and stored at −20°C. Bacterial 16S rRNA using 515f/806r primer pair (515f: 5′- GTGCCAGCMGCCGCGGTAA-3′; 806r: 5′-GGACTACNVGGGTW TCTAAT-3 (Caporaso et al., 2011; Apprill et al., 2015) and the fungal ITS2 using ITS1f/ITS2 primer pair (ITS1F: 5′- GTGAATCATCGAATCTTTGAA-3′; ITS2R: 5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990; Turenne et al., 1999) were PCR-amplified. Each sample was amplified in triplicate in a 20 µl reaction volume containing 0.2 µl of Ex-Taq DNA Polymerase, 2.0 µl of 10x Ex buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA), 1 µl of each primer, 13.2 µl of Milli- Q water, and 1 ng of template DNA.

PCR was performed with (94°C/3 min, 94°C/45 s, 50°C/60 s, 72°C/90 s, 72°C/10 min for 35 cycles) for 16S rRNA and (94°C/3 min, 94°C/40 s, 55°C/40 s, 72°C/60 s, 72°C/7 min for 30 cycles) for ITS2. PCR quality was controlled by loading 5 μl of each reaction on a 1% agarose gel and affirming that no band was detected within the negative control. The replicated reactions were combined to make one biological replication, and the barcoded Illumina libraries were sent for paired-end Illumina MiSeq sequencing (2 × 300 bp, Bioengineering Lab. Co., Sagamihara, Japan). The obtained 16S rRNA and ITS amplicon raw reads were deposited into the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the project PRJNA824966.

Sequencing data processing and identification of amplicon sequence variants

Microbiome bioinformatics was performed with QIIME2 2020.8 (Bolyen et al., 2019). The adapter and primers were removed with Cutadapt v2.4 from the raw reads (Martin, 2011). The sequences were demultiplexed using the q2-demux plugin and followed by quality control, length trimming, denoising, chimera, and PhiX removal, and feature table construction by DADA2 with default settings except that "–ptrunc-len-f" and "–p-trunc-len-r" which were set at 250 and 200, respectively for 16S data and at 160 and 200 for ITS data, respectively (*via* q2-dada2) (Callahan et al., 2016). The resulting amplicon sequence variants (ASVs) were aligned with mafft (Katoh et al., 2002) and phylogenetic trees were constructed using fasttree2 (Price et al., 2010). Taxonomy was assigned to ASVs using the QIIME feature-classifier classify-skarn (Bokulich et al., 2018) with the pre-trained naïve Bayes SILVA classifier v132 trimmed to the V4 region of the 16S rDNA gene (Quast et al., 2012) for bacteria and pretrained UNITE ver8 99% database (UNITE Community, 2019), trained on the full reference sequences without any extraction for fungi. Non-bacterial and fungal reads were removed from the obtained ASV table. We normalized the library using scaling with ranked subsampling using the "SRS"-function in the "SRS" with "qiime srs SRS" (Beule and Karlovsky, 2020). The alpha diversity were evaluated with the chao1 estimator (richness) and the Shannon index and Faith PD in qiime2 and Kruskal–Wallis tests were used to compare the diversity. Box plots to display the alpha diversity indices were created using ggplot2 (Wickham et al., 2016) installed in R Core Team (2020). The Bray–Curtis dissimilarity matrix was calculated to assess differences in composition of bacterial and fungal communities and used for the principal coordinate analysis (PCoA) ordination plot in PAST4.04 (Hammer et al., 2001). We compared differences in community composition and structure among sampling sites (α- diversity) using the analysis of similarity (ANOSIM) (Clarke, 1993) and permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2014). Least discriminant analysis (LDA) and random forest were performed using microbiomeanalyst (Chong et al., 2020).

Functional prediction, source tracking, and network analyses of fungal and bacterial community

The marker genes in the samples (16S rRNA and ITS) were inferred for the prediction of functional profiles by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) algorithm software (Douglas et al., 2020). MetaCyc, the comprehensive reference gene function, was mapped to analyze gene functions for bacterial and fungal ASVs (Krieger et al., 2004). Pathways inferred with high-level functions were mapped against the MetaCyc database (Caspi et al., 2020). To predict the ecological function of fungi, we used the FUNGuild database (Nguyen et al., 2016), whereas, for bacteria, we used the FAPROTX (Louca et al., 2016). To estimate the association between bacterial and fungal communities in soil, the rhizosphere, roots, leaves, and grains tissues in healthy and diseased plants, we used SourceTracker2 in python (version 3.7.0) (Knights et al., 2011). We analyzed SourceTracker2 default parameters, and one by one, each environmental sample type was designated as a sink with all other environmental sample types as sources. For example, to investigate source-associated ASVs on leaves of healthy plants, root, rhizosphere soil, and bulk soil of healthy samples were specified as sources, and leaf was designated as the sink. Kruskal–Wallis tests were used to compare the mean percentages assigned to different sources. The percentage value was derived from the statistical average of the results of SourceTracker. We constructed networks of microbiome communities in healthy and infected samples using Pearson correlation with a significance of $p < 0.05$ and correlation coefficient $R > 0.60$. For this, we combined the ASV table of both bacterial and fungal communities. To simplify the network, we extracted only the ASVs that showed a significant correlation with *M. oryzae* and created a subnetwork of interactions between the pathogen and other microbes. Although different DNA extraction kits used for plant and soil samples might cause some variation, we considered the impact to be insignificant for our analysis.

Results

Soil microbial communities are more diverse and distinct from endospheric communities

We analyzed and compared the alpha and beta diversity of bacterial and fungal microbiome of different compartments (i.e., soil, the rhizosphere, root, and leaf) of healthy rice

plants from both locations. As expected, the rhizosphere and bulk soil microbiome were more diverse, followed by the root and leaf endophytic communities in both locations (Figures 1A–H; Supplementary Figures 1A,B). Similar patterns were observed for other diversity indices, such as Chao1 and Faith's phylogenetic diversity (PD) (Supplementary Table 1). Samples from two different locations, i.e., Fukushima and Miyagi, showed variation in the diversity of bulk soil and leaf infosphere communities (Supplementary Tables 1, 2). The PCoA revealed distinct bacterial and fungal assemblages among different compartments in the healthy samples. It was noted that bulk soil and rhizosphere microbial communities overlapped but were separated from endosphere (the root and leaf) communities which were significantly different from each other (Supplementary Figures 1C,G; (Supplementary Table 3). We checked which bacterial groups differed among compartments using LDA. With few others, Delta and Gamma-proteobacteria, Bacteroidia, Verrucomicrobiae, Acidobacteria, and Clostridia were higher in both soil and rhizosphere samples but very low or absent in plant microbiomes (root and leaf). Oxyphotobacteria is the dominant bacteria in the leaf and root microbiome, which were less abundant in the belowground microbiome (Supplementary Figure 1D). We also checked which fungal groups were different among compartments by the illustration of taxa bar plots. Eurotiomycetes, Dothideomycetes, and Agaricomycetes were more abundant in the aboveground compartment (root and leaf), whereas Sordariomycetes and unknown fungal taxa were more in bulk soil and rhizosphere communities (Supplementary Figure 1H).

Disease-caused alteration of bacterial but not fungal community compositions in the rhizosphere and root samples

Since microbiome community composition and diversity were different in different compartments, we compared the bacterial and fungal microbiome of healthy and diseased samples separately from soil, the rhizosphere, root, and leaf. No significant difference was observed for the alpha diversity of bacteria (Shannon, Faith'sPD, and Chao1) between healthy and diseased samples regardless of the microbiome compartments (Figures 1A–D; Supplementary Table 1). PCoA, PERMANOVA, and ANOSIM analyses revealed that the rhizosphere and root compartment had distinctly different bacterial compositions in the diseased plants compared to the healthy samples (Figures 1J,K). The bacterial communities of bulk soil and leaf endophyte did not differ due to disease status. The bacterial community of the two locations was distinctly different regardless of the plant compartment. Several bacterial families were more abundant in the rhizosphere of healthy as compared to the diseased samples, such as *Barnesiellaceae, Desulfobacteraceae, Thermomonosporaceae, Methanosaetaceae, Pirellulaceae,* and *Coriobacteriaceae* in both locations. In contrast, bacteria of the families *Brevinemataceae, Holophagaceae, Paracaedibacteraceae*, and *Solirubrobacteraceae* were highly abundant in the rhizosphere communities of diseased samples (Figure 1Q). In the root communities, members of *Streptomycetaceae*, *Chitinophagaceae*, *Burkholderiaceae*, and *Kineosporiaceae* were present abundantly in the healthy samples, and *Veillonellaceae* were abundant in the diseased samples (Figure 1R). At the genus level, *Turneriella, Pelolinea, Sulfuricurvum, Desulfocapsa, Cuspidothrix,* and *Arenimonas* were more abundant in the healthy rhizosphere, and *Desulfovirga, Roseiarcus,* and *Nakamurella* in diseased samples (Supplementary Figure 2). In roots, *Streptomyces, Methylosinus, Cephaloticoccus, Burkholderia, Paraburkholderia, Caulobacter, Acinetobacter, Geothrix, Ruminiclostridium, Hydrogenophaga,* and *Pleomorphomonas* were more abundant in healthy plants in both locations (Supplementary Figure 2).

In the case of the fungal microbiome, no significant differences were observed for alpha diversity between healthy and diseased samples regardless of the microbiome compartments (Figures 1E–H; Supplementary Table 2). Fukushima samples had a higher diversity of fungal community compared to Miyagi samples which were very prominent in soil communities. However, the richness index (Chao1) did not exhibit any variation between locations except for bulk soil (Supplementary Table 2). From PCoA, we did not observe any influence of blast disease infection on the structure of the fungal community; there were locational variations in the soil as well as endospheric communities (Figures 1M–P; Supplementary Table 2).

Pathobiome analysis of symptomatic and non-symptomatic tissue of diseased plants

We were interested in the microbiome community in symptomatic and non-symptomatic tissues (leaf and grain) from the diseased plants. Shannon alpha diversity analysis indicated no substantial differences in the bacterial and fungal communities between symptomatic and non-symptomatic leaf and grain tissues (Figures 2A–D). PCoA analysis and similarity indices (PERMANOVA, ANOSIM) statistics suggested that there remained clear differences in the bacterial and fungal communities of symptomatic leaves and grains from non-symptomatic tissues and that sampling locations significantly influenced community structuring of leaf and grain communities (Figures 2E–H, Supplementary Tables 4, 5). A further inspection of bacterial group differences using random forest analysis revealed that bacteria belonging to certain families were variably present in non-symptomatic

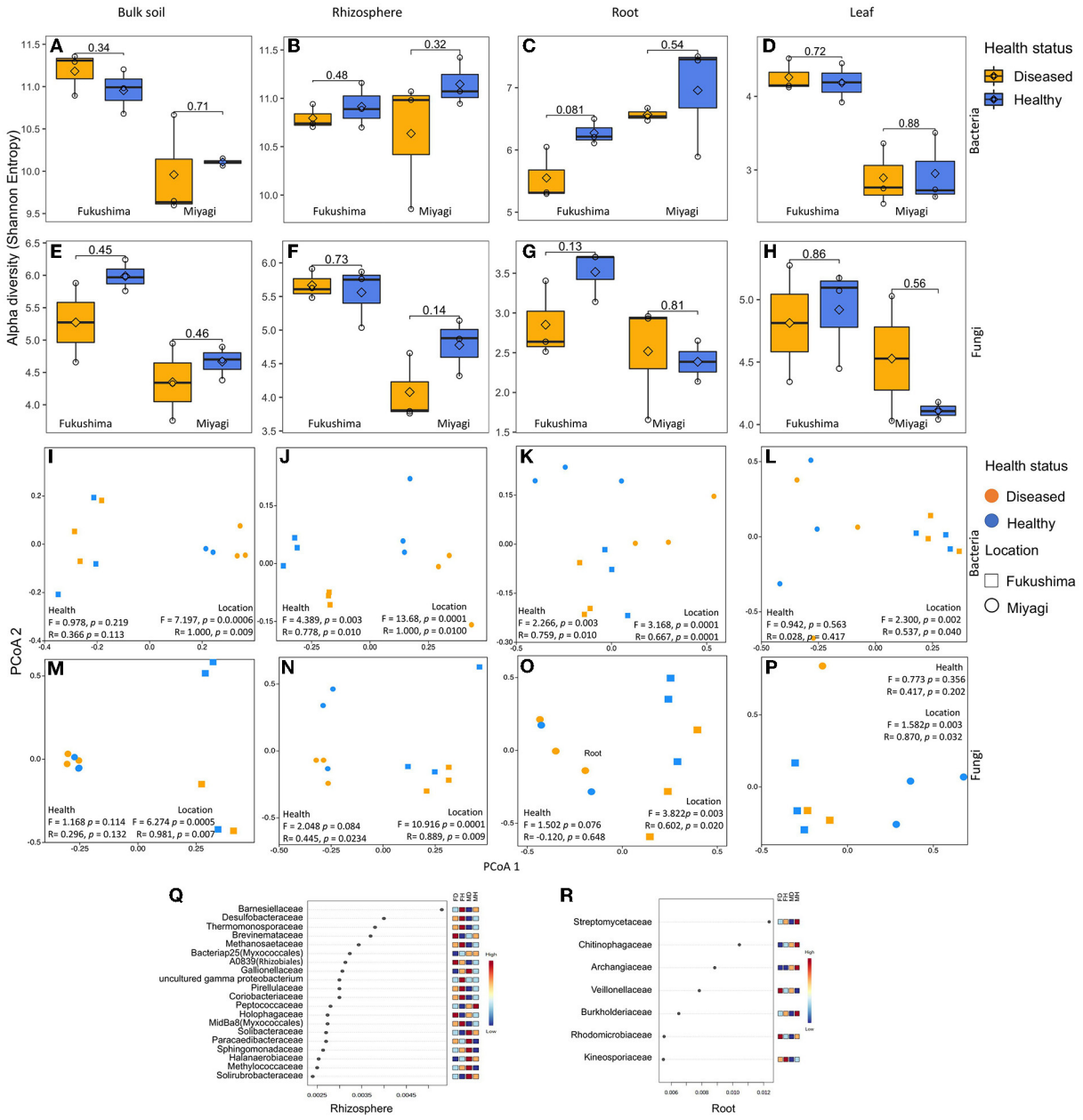

FIGURE 1
Bacterial and fungal microbiome community of rice as influenced by plant health status and location: Alpha diversity (Shannon Entropy) of bacterial **(A–D)** and fungal **(E–H)** community in bulk soil **(A,E)**, rhizosphere soil **(B,F)**, root endosphere **(C,G)**, and leaf endosphere **(D,H)**. Principal co-ordinate (PCoA), analysis of similarity (ANOSIM), and permutational multivariate analysis of variance (PERMANOVA)-based analyses of bacterial **(I–L)** and fungal **(M–P)** microbiome of healthy and diseased (non-symptomatic) samples of rice collected from two locations in Japan. Bray-Curtis coefficient of community similarity index was computed for comparing the bacterial community similarity among groups. The ANOSIM statistic *R* values (up to 1) and PERMANOVA *pseudo-F* indicate similarity/dissimilarity between groups. Ordination plots show the influence of plant health status, and location on microbial community assembly of bulk soil **(I,M)**, the rhizosphere soil **(J,N)**, root endosphere **(K,O)**, and the leaf endosphere **(L,P)**. Differential abundance of bacterial microbiome community of rhizosphere soil **(Q)** and root endosphere **(R)** rice as obtained by random forest analysis. Random forest analysis results of top 20 bacterial family with the highest discriminatory power between diseased and healthy samples of rhizosphere community are shown. Red fields show a high abundance and blue fields a low abundance of the particular bacterial family. FD, Fukushima diseased; FH, Fukushima healthy; MD, Miyagi diseased; MH, Miyagi healthy.

or symptomatic tissues. For instance, the symptomatic leaf tissues had higher abundances of *Xanthomonadaceae, Sphingobacteriaceae, Beijerinckiaceae, Microbacteriaceae,* and *Rhizobiaceae,* and the symptomatic grains were dominated by *Sphingomonadaceae, Burkholderiaceae, and Caulobacteriaceae* families. *Enterobacteriaceae* were found to be highly abundant in the non-symptomatic grains in both the location and the non-symptomatic leaf of Miyagi samples and of the Fukushima samples (Supplementary Figure 3). At the genus level, several bacteria were detected in significantly higher abundance in the non-symptomatic leaves in the Fukushima samples, but they were either lowly present or absent in the Miyagi sample. However, consistently higher abundances of *Stenotrophomonas*, *Pedobacter, Methylobacterium,* and *Rhizobium* were observed in the symptomatic leaves in both locations (Figure 2I). The bacterial genera *Rhizobium, Novosphingobium, Darnella, Brevundimonas, Curtobacterium, Stenotrophomonas,* and *Acinetobacter* had comparatively increased occurrences in the symptomatic grain tissues in both locations. Only *Phenyracillin* was present abundantly in non-symptomatic grain samples irrespective of the sample location (Figure 2J). As for fungi, members of certain families encountered higher in the non-symptomatic tissues. For instance, *Thelephoraceae, Lindgomycetaceae, Atheliaceae, Cantharellaceae,* and *Aspergillaceae* showed increased abundances in leaf and grain non-symptomatic samples (Supplementary Figures 3C,D). The fungal genera, such as *Coniochaeta, Tylospora, Tomentella*, *Clohesyomyces*, and *Penicillium* were found abundantly present in non-symptomatic tissues in both the leaf and grain samples regardless of the sampling location (Figures 2K,L).

Functional prediction: Microbiota functional profiling of microbiome of rice

The potential metabolic functions of bacterial and fungal communities in symptomatic and non-symptomatic tissues were predicted by PICRUSt2. The partial least squares-discriminant analysis (PLS-DA) plots were determined to evaluate the similarity of the microbial functions between the symptomatic vs. non-symptomatic and diseased vs. healthy samples. The PLS-DA analyses revealed an evident clustering among the samples. There was a difference in the predicted functional pathways of bacterial microbiome communities in diseased rhizosphere and root endosphere (Supplementary Figure 4). The number of abundant functional metabolic pathways predicted from the rhizosphere and root microbiome was higher in diseased plants. For example, "ethylmalonyl-CoA pathway," "Amine and Polyamine Degradation," "Amino Acid Biosynthesis," "isopropanol biosynthesis," and several other bacterial metabolic pathways were predicted to be abundant in rhizospheres as well as the root endosphere community of diseased plants as compared to healthy plants (Figure 3A; Supplementary Figure 4). Higher abundance of certain other metabolites was predicted in the microbiome rhizosphere of healthy plants, such as "methyleketone biosynthesis," "Glycan Biosynthesis," Secondary Metabolite Biosynthesis," "Cofactor, Prosthetic Group, Electron Carrier, and Vitamin Biosynthesis," "Glycolysis," but interestingly, these were comparatively more abundant in the roots of diseased plants (Figure 3A, Supplementary Figure 4). Significant differences in the functional pathways in both bacterial and fungal communities were observed in the symptomatic and non-symptomatic leaf and grain tissues (Figures 3B,C, Supplementary Figure 4). The predicted bacterial functional metabolic pathways, such as "TCA cycle," "superpathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass," "fatty acid and lipid biosynthesis," "fatty acid and lipid degradation," "amino acid degradation," and "amine and polyamine biosynthesis" were more abundant in symptomatic tissues in both grain as well as leaf communities (Figures 3B,C). When analyzed the fungal metabolic predictive pathways, certain pathways similar to those of bacterial pathways were found to be more abundant in the symptomatic tissues, such as "fatty acid and lipid degradation" and "amino acid degradation." The pathways, such as "carbohydrate biosynthesis," "pentose phosphate pathways," "TCA cycle," "inorganic nutrient metabolism," and "degradation/utilization/assimilation–other" were more abundant in the fungal metabolites of the non-symptomatic tissues as compared to symptomatic tissues (Supplementary Figure 4). However, some of these pathways predicted from the bacterial community seemed less abundant in the non-symptomatic tissues (Figures 3B,C).

Network of interactions of *M. oryzae* and other microbial members in the rhizosphere, leaf, and grain microbiome

To gain a deeper insight into the interactions of *M. oryzae* with other microorganisms, the networks in healthy and infected plants were visualized. The network structures of rhizosphere communities appeared to be significantly altered in *M. oryzae*-infected rice plants showing higher complexity and connectivity than those of healthy plants (Figures 4A,B). We noted more links (43 links) between *Magaporthe* and other microbial genera in the network of infected rhizosphere compartments than the healthy rhizosphere network (17 links), indicating a shift in the rhizosphere microbiome interactions due to foliar infection. The network of interactions in the infected plants revealed that *M. oryzae* were positively correlated with almost all bacterial genera belonging to Bacteroidetes, Proteobacteria,

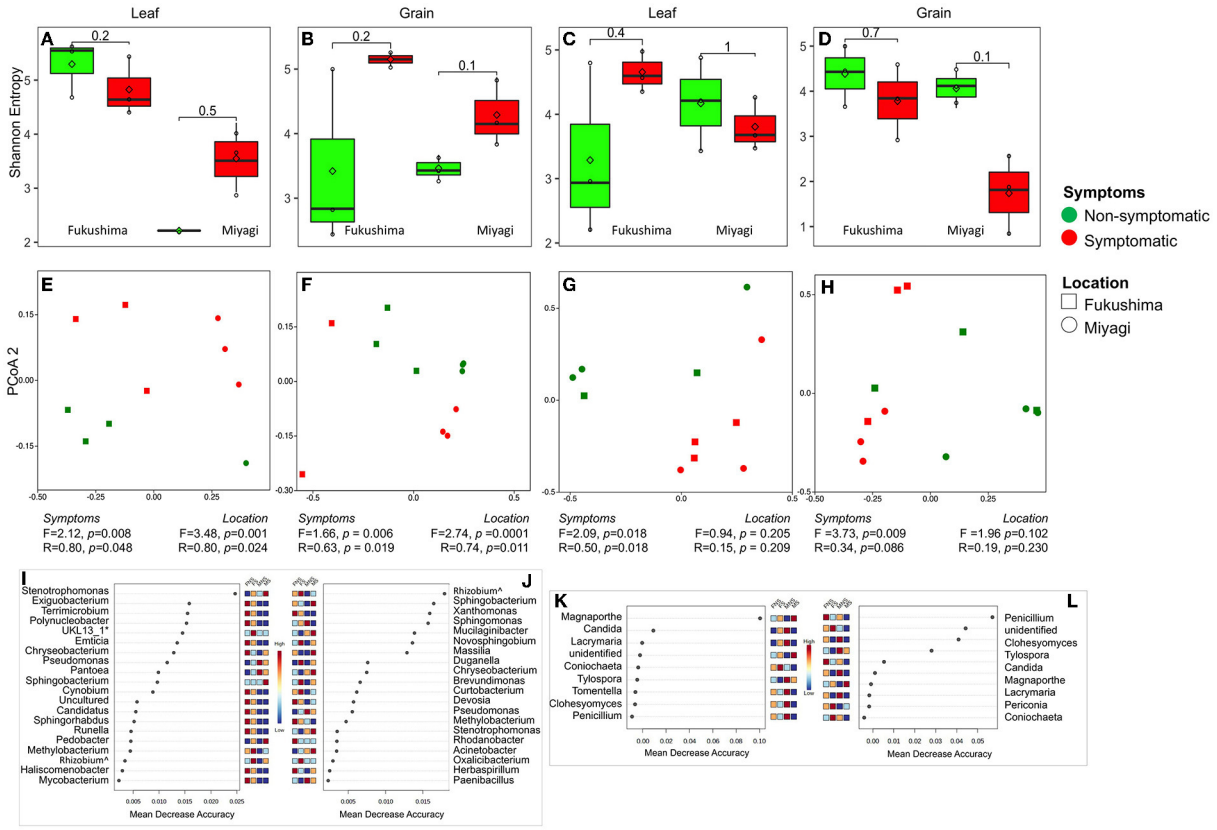

FIGURE 2
Bacterial and fungal microbiome community of rice leaf and grain as influenced by fungal pathogen infection: Alpha diversity (Shannon Entropy) of bacterial **(A,B)** and fungal **(C,D)** community in leaf **(A,C)** and grain **(B,D)** tissues. PCoA, ANOSIM, and PERMANOVA-based analyses of bacterial **(E,F)** and fungal **(G,H)** microbiome of symptomatic and asymptomatic samples of leaf **(E,G)** and grain **(F,H)** samples of rice collected from two locations in Japan. Random forest analysis results of bacterial (top 20) **(I,J)** and fungal **(K,L)** genera with the highest discriminatory power between symptomatic and asymptomatic tissues of leaf **(I,K)** and grain **(J,L)**. Red fields show a high abundance and blue fields a low abundance of the particular bacterial genera. FNS, Fukushima non-symptomatic; FS, Fukushima symptomatic; MNS, Miyagi non-symptomatic; MS, Miyagi symptomatic.

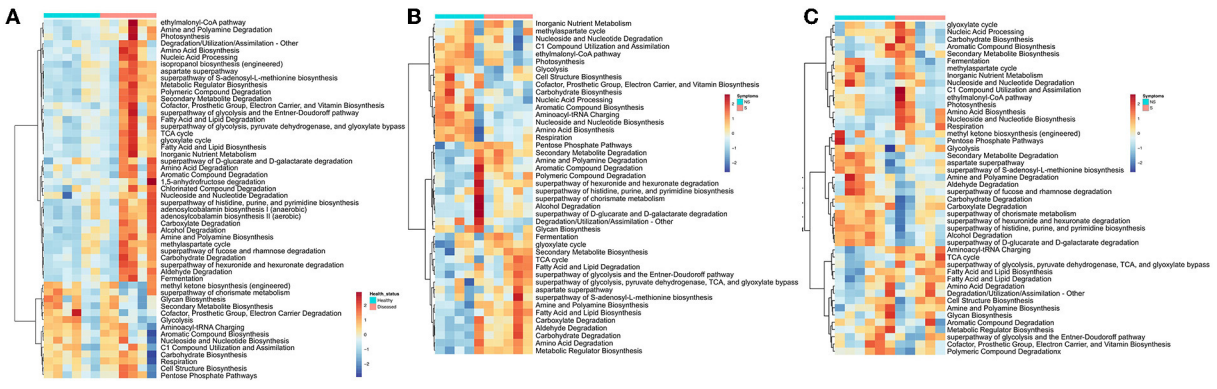

FIGURE 3
Heatmap Clustering the relative abundance of functional signatures of bacterial microbiome of rice from **(A)** the rhizosphere, **(B)** leaf, and **(C)** grain samples as predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) within Meta-Cyc categories. S and NS represent symptomatic and non-symptomatic tissues, respectively.

Firmicutes, Actinobacteria, Spirochaetes, and Fusobacteria, and negatively correlated with *Desulfobacca* (Proteobacteria) and a fungal genus, *Penicillium*. In contrast, most bacterial genera were negatively correlated with *M. oryzae* in diseased plant rhizo-microbiome (Figures 4A,B). When we compared the network structures of symptomatic and non-symptomatic tissues, we observed changes in the correlation pattern of *M. oryzae* and other microorganisms (Figures 4C–F). For instance, we noted that there were more links (22 links) between *Magnaporthe* and other microbial genera in the network of non-symptomatic leaf compartments than the symptomatic network (17 links), indicating a shift in the microbiome interactions due to *M. oryzae* infection. Similar was the case in the grain network, where there were more links (21 links) between *Magaporthe* and other microbial genera in the network of non-symptomatic than in the symptomatic network (11 links). Our observations indicate that pathogen invasion alters microbiome members who might assist in colonization through a mutualistic relationship or come to defend the plant against pathogen during the infection process of disease. In other words, microbial members in the non-symptomatic tissues might play roles in keeping the tissues decreased in the invasion of the pathogen that would otherwise cause disease symptoms.

SourceTracker analysis of microbiome community from soil to other compartments

We utilized the SourceTracker program to study the proportion of bacterial and fungal communities derived from soils. According to the source apportionment results, there were no noticeable differences in the sources of rhizosphere bacterial communities between diseased and healthy plants, and around half of the total were from neighboring bulk soil communities (Figure 5). In healthy samples, the majority of root bacteria community members (71%) were derived from the soil communities (bulk and rhizosphere soils), but rare members (17%) of the root communities of the diseased plant were derived from the soil bacteria communities, indicating there is a clear boundary between the interior and exterior of healthy roots (Figure 5). Very rare members (<10%) of the aboveground (leaf and grain) bacteria were sourced from belowground communities, and the majority were from unknown sources irrespective of the plant health status. Regardless of the symptom expression in grains of diseased plants, most bacteria of gain communities were from leaves (∼75%). Regarding the fungal communities, the rhizosphere soil communities were mainly (∼80%) derived from the bulk soil in both healthy and diseased plants. More than 60% of root endophytic fungal communities were primarily derived from the soil communities (Bulk and rhizosphere). Notably, more than half (60%) of endophytic leaf communities were derived from the belowground in the soil in the healthy plants, which is significantly less (31%) in the non-symptomatic leaf of the diseased plant and very low (11%) in the symptomatic leaf tissues, indicating most of the aboveground fungal species in the healthy plants could be tracked back from the soils but not in the infected plants. Non-symptomatic grain communities derive 40% of fungi from the leaf and 15% from the belowground. In contrast, symptomatic tissues receive the majority (70%) of fungi from the leaf and are very rare (3%) from belowground communities (Figure 5).

Discussion

Rice microbiome composition is shaped by compartments

We present a critical appraisal of bacterial and fungal communities sampled in the bulk soil, rhizosphere soil, the root, leaf, and grain in two different locations of non-symptomatic and symptomatic sample types to provide a comprehensive view of the rice-associated microbiome. Rice-associated bacterial and fungal communities have previously been studied by culture-dependent as well as culture-independent methods (Edwards et al., 2015; Bertani et al., 2016; Kanasugi et al., 2020; Kim and Lee, 2020; Sinong et al., 2020). We profiled the 16S and ITS2 regions to reveal the composition of bacterial and fungal communities. Our results support the concept that distinct plant components play a crucial role in engaging microbial communities, irrespective of the location (Supplementary Figure 1). We demonstrated that the soil (bulk and rhizosphere soil) had a greater diversity of bacterial and fungal communities in the rice plant than endospheric (root and leaf) communities. Bacterial and fungal communities of bulk soil and rhizosphere soil showed no significant difference; however, root endospheric microbial communities differed from leaf communities. The rhizosphere has a diverse and highly populated microbiome and is subject to chemical transformations brought about by the root exudates and metabolites of microbial degradation. The rhizosphere is distinct from the edaphosphere (bulk soil zone), with enhanced microbial activity mainly due to enhanced root exudates deposition (Chaudhary et al., 2012; Reinhold-Hurek et al., 2015; Vieira et al., 2020). However, it is interesting that in our analyses, we observed a slightly higher diversity of rhizosphere bacterial community over bulk soil community in Miyagi but not in Fukushima samples. Although several previous studies reported distinct and more diverse rhizosphere bacterial communities in different crops (Praeg et al., 2019; Hinsu et al., 2021) than in the surrounding bulk soil, we found no distinct separation of the bacterial community of these two zones. This could be due to the fact that the rhizosphere zone is dynamic, and the composition of the microbial community changes as a result of

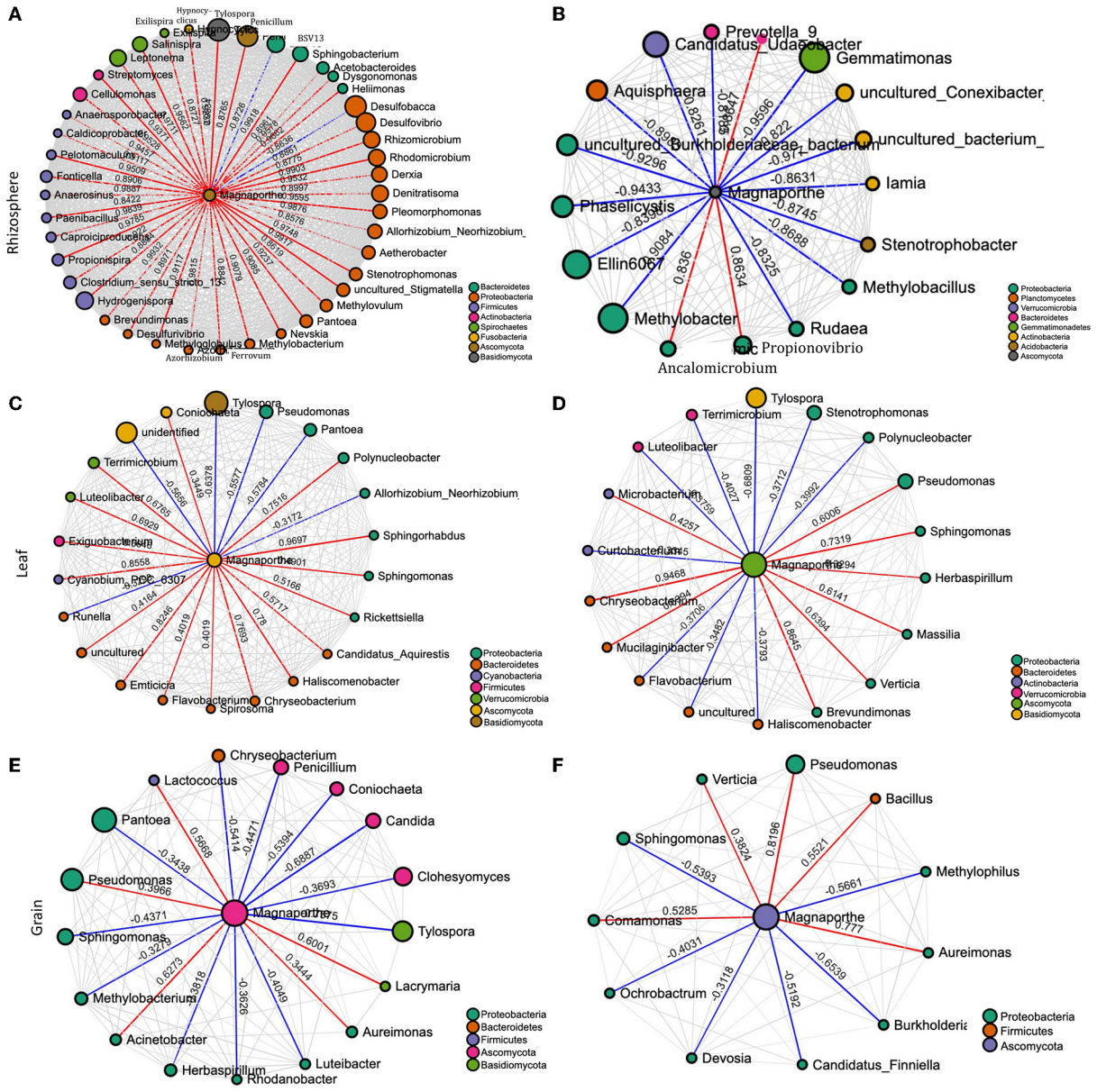

FIGURE 4
Network of interactions between the pathogen (*M. oryzae*) and other species in the rhizosphere of healthy **(A)** and diseased plants **(B)**, non−symptomatic **(C)** and symptomatic leaf **(D)**, and non-symptomatic **(E)** and symptomatic grain **(F)**. The size of the bubbles corresponds to their relative abundances. The red and blue colors of the edge represent positive and negative interaction with *M. oryzae*, respectively.

changing root exudation patterns that vary during the life cycle of plants (Edwards et al., 2015; Qu et al., 2020). We collected the samples at the grain maturity or near ripening stage, and microbial diversity is less than the active growth stage of the plant (Hinsu et al., 2021). Previous studies reported that there was no considerable difference in the bulk soil and rhizosphere soil microbial communities. Several others also reported reduced microbiome diversity in rhizosphere communities compared with the bulk soil community (Schlaeppi et al., 2014; Essel et al., 2019). Therefore, in addition to root exudation, the composition of the microbial community in the rhizosphere is linked with the plant developmental stage and several biological and environmental factors (Schreiter et al., 2014; Essel et al., 2019). Our analysis showed that fungal communities were not influenced by the rhizosphere environment. We assume that root exudate deposition might be selective, or bacteria might respond more strongly to some compound secreted by the rice plant. The lower richness and diversity of endophytic

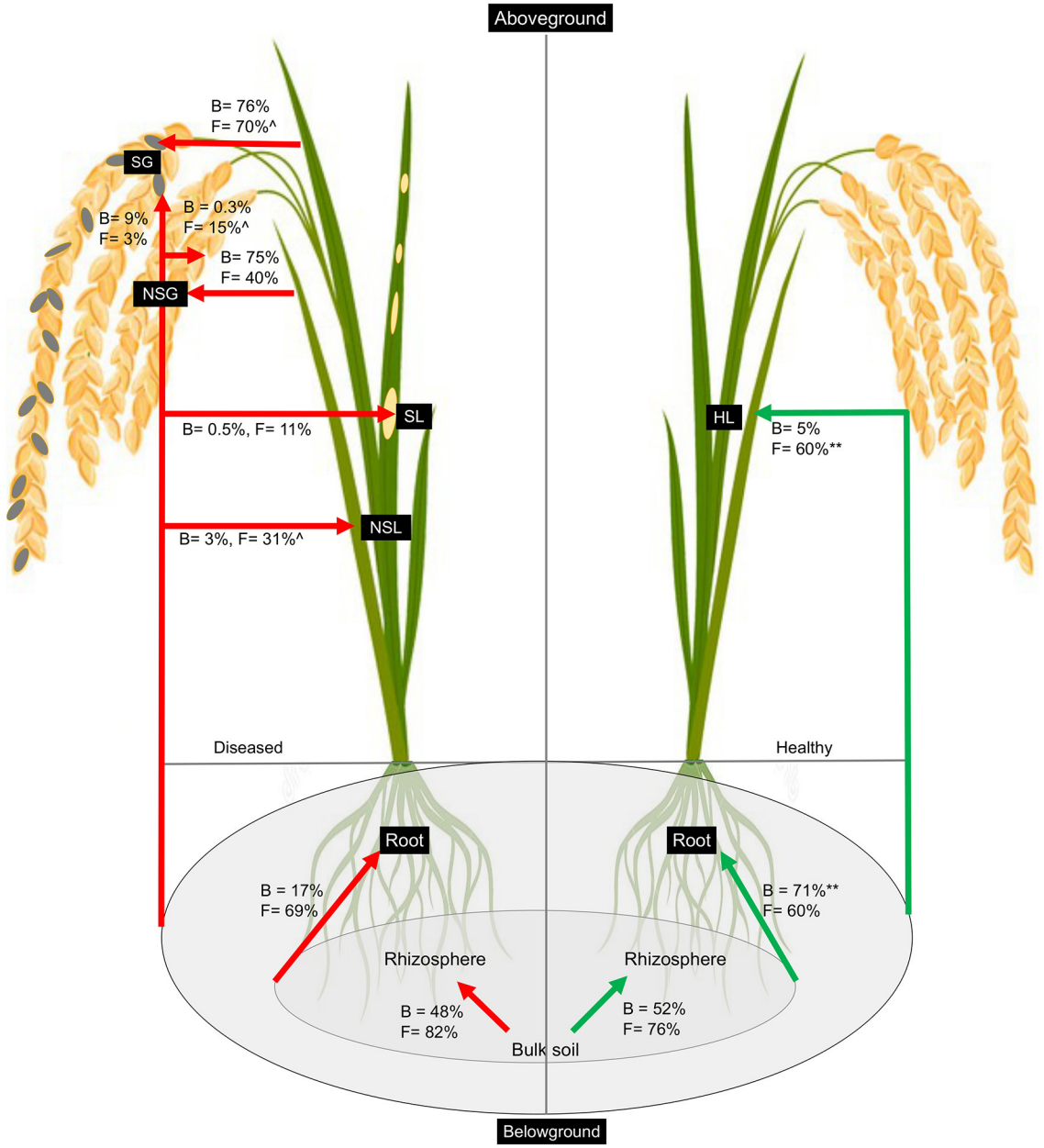

FIGURE 5
Percentage of bacteria and fungi in each sample that was associated with source types as determined by SourceTracker. The asterisk (**) indicates significant higher percentage at $p < 0.01$ in healthy as compared to diseased samples whereas caret (∧) indicates higher percentage at $p < 0.05$ in non-symptomatic tissues as compared to symptomatic tissues of the diseased plants as obtained from Kruskal–Wallis test.

communities (root and leaf) compared to the soil (bulk and rhizosphere) samples are understandable. This finding is coherent with the reports from several plant species, including Arabidopsis, soybean, rice, Agave, and others (Knief et al., 2012; Dong et al., 2019). Intuitively, the root and leaf tissues had different microbial communities, and the diversity of bacteria in the leaf was lower than that in the root communities. Similar results were also reported in rice in another study by Wang et al. (2016) and other plant hosts, including Arabidopsis (Bodenhausen et al., 2013), tomato (Dong et al., 2019), and Agave species (Coleman-Derr et al., 2016). The higher bacterial richness in the root compartment may be attributed to the fact that it is the primary site of the interaction of plants with soil and represents one of the richest microbial ecosystems on earth (Hardoim et al., 2011; Tian and Zhang, 2017). It has been reported that the diversity of bacterial endophytes decreased

as the distance from soil increased (Ottesen et al., 2013; Dong et al., 2019). However, the reverse pattern was found for the fungal diversity, i.e., higher diversity in the leaf than in the root tissues. The previous study also reported higher diversity of fungi in the shoots than in the roots and attributed to the assumption that most endophytic fungi are derived from aerial fungal spores (horizontal transmission), whereas most of the endophytic bacteria are derived from soils (Rodriguez et al., 2009; Wang et al., 2016).

Foliar fungal infection shifted bacterial but not fungal microbiome in the rhizosphere

It was found that the blast-infected samples had different bacterial community structures compared to the healthy plants in the root and rhizosphere, but there was no apparent difference in the fungal community (Figure 1). Our study indicated that bacteria of several families were enriched differentially in the rhizosphere and the roots of the healthy and diseased plants. In particular, members of Streptomycetaceae were found to be significantly more abundant in the root endosphere of healthy plants in both locations (Figure 3). The members of Streptomyces are famous for their ability to produce various bioactive compounds and play essential roles in the agricultural field through their biological control potential against phytopathogens, including *M. oryzae* fungi (Procópio et al., 2012; Ser et al., 2016; Law et al., 2017). Endophytic or N-fixing *Burkholderia* strains show immense potential as a biocontrol, as well as plant growth promotion and bioremediation agent (Compant et al., 2008; Elshafie and Camele, 2021). After risk assessment, these *Burkholderia*-products were withdrawn from the market, as some strains may pose a risk to human health. However, over the last several years, the number of new *Burkholderia* species that show plant-beneficial properties and are associated with clinical patients has increased enormously (Eberl and Vandamme, 2016). The *Caulobacter* genus is often isolated from the endosphere or rhizosphere of various plants (Fitzpatrick et al., 2018; Walters et al., 2018; Luo et al., 2019).

The role of plant-associated microbiota in protection against pathogenic fungi and oomycetes is well documented. Although we did not examine empirically, the differential bacterial assemblage may be linked to the differential compound exudation through the rhizosphere due to plant health status responsible for specific microbial group selection, thus regulating their community compositions. The "cry for help" hypothesis suggests that plants recruit microbial partners to maximize their survival and growth when affected by external stress and is likely a survival strategy conserved across the plant kingdom (Liu et al., 2019, 2020; Gao et al., 2021). However, we do not know if the differential microbiome assemblage is the effect of disease or the cause of disease manifestation. Either hypothesis (cause or effect) or even both simultaneously might be possible, and clarification is warranted with manipulative experiments. We detected the presence of *Magnaporthe* in the rhizosphere of healthy samples. Interestingly, the co-occurrence networks analysis of *Magnaporthe* with other taxa revealed that the rhizosphere of healthy plants had a robust and more complex network with a higher number of nodes and edges than the diseased network. Most edges of healthy rhizo-microbiome networks were positively correlated with *Magnaporthe,* whereas they were primarily negative in diseased plants (Figure 4). Again, there were no fungal taxa correlated with *Magnaporthe* in the diseased plant. Only one ascomycetous genera were positively correlated with it, suggesting the putative importance of bacterial taxa in plant health status under *M. oryzae* attack compared to the fungal microbiome. A previous study also highlighted the higher stability of bacterial than the fungal networks in healthy plants. *Fusarium* infection in Chili pepper decreased the complexity of bacterial networks but increased the complexity of fungal networks (Gao et al., 2021). Our functional prediction analysis indicated that the rhizosphere microbiome of diseased plants putatively carries out more diverse metabolic functions (Figure 3) than those associated with healthy individuals, which may be related to disease-induced microbiome recruitment to defend plants under stress. Metaproteomic and transcriptomic analyses, as well as microbiome manipulation of diseased vs. healthy plants, would provide an increased understanding of those microbial community and their functions.

Pathobiome showed clear separation of the microbiome from those of healthy tissues

The pathobiome concept has been defined as the totality of microbes interacting with a given pathogen species and their influence on pathogenesis (Vayssier-Taussat et al., 2014; Jakuschkin et al., 2016). Characterization of the components of the pathobiome is an important consideration for understanding the pathogenesis, persistence, transmission, and evolution of pathogenic agents (Vayssier-Taussat et al., 2014). Shifts in the composition, richness, and abundance of the microbiome have been shown to occur due to pathogen infection in plants (Jakuschkin et al., 2016; Musonerimana et al., 2020; Mannaa and Seo, 2021). Our results also revealed that the composition of the fungal and bacterial community of rice leaves changed markedly with the *M. oryzae* infection. Through the species classification, we found not only the ASVs assigned to potentially pathogenic *Magnaporthe* were rarely observed abundantly in symptomatic tissues but also from healthy leaf

and grain, albeit with significantly low abundances (Figure 2). We could not technically affirm all those ASVs assigned to *Magnaporthe* are pathogenic by just using ITS sequences. The presence of *Magnaporthe* in the healthy tissue implies either there was a latent infection of the pathogen, or these were non-pathogenic strains of *Magnaporthe*. Correspondingly, the relative abundances of some bacteria and fungi were clearly altered in symptomatic tissues. There was a significant increase in the relative abundance of members, such as *Rhizobiaceae, Microbacteriaceae, Beijerinkiacee,* and *Xanthomonadaceae* in the symptomatic over the non-symptomatic tissues. As for fungi, there were higher abundances of *Thelephoraceae, Lingomycetaceae, Atheliaceae, Catharellaceae,* and *Aspergillaceae* families in the non-symptomatic leaves in both locations. These compositional changes could be a cause or consequence of the pathogen invasion. Species of *Paenibacillus* are well-known pathogen suppressors *in planta*. In many studies, several *Paenibacillus* have been identified and used as biocontrol agents with the ability to secrete antibiotics or other antimicrobial proteins and have been applied to prevent and control various plant pathogens, including *Magnaporthe* (Rybakova et al., 2016; Padda et al., 2017; Yu et al., 2019). In the plant endophytic compartments, the relative abundances of *Paenibacillus*, *Enterobacter*, and several other bacteria that are often considered plant-beneficial microbes showed a significant decrease in abundance compared to the healthy samples. These decreases imply that these endophytic taxa might be excluded due to compromised immune systems locally or outcompeted by more successful colonizers. The shift in the microbiome composition in the symptomatic tissue could be due to the degradation of plant tissue (necrotic/decomposed tissue) by the pathogen that results in colonization with different microbiomes or colonization of bacteria on dead fungal hyphae for their nutrition or bacterial endosymbiont of fungi or pathogen teaming up with commensals (Lundberg et al., 2012; Venturi and da Silva, 2012; Tláskal et al., 2016). On the other hand, the relative abundances of several bacteria, such as those belonging to *Rhizobiaceae, Xanthomonadaceae,* and *Sphingobacteriaceae* in the symptomatic leaf and grain and a few more only in the grain tissues samples showed a marked increase compared to non-symptomatic samples, suggesting they might be involved in the pathogenesis and have mutualistic relationships with the pathogen, or maybe they are opportunists and could take advantage of different ecological niches created by pathogen invasion (Lundberg et al., 2012; Hu et al., 2020). The relatively higher abundances of *Rhizobium* in the symptomatic tissues implied that this strain could either be involved in mutualism with the *M. oryzae* in the disease process or may remain as saprophyte by adopting an oligotrophic lifestyle (Poole et al., 2018). Legume-rhizobium symbiosis is well documented, and much of the rhizobia research has focused on specific parts of the rhizobia–legume symbioses. Our current understanding of rhizobia interaction with non-legumes and its ecology is not comprehensive. Several studies reported rhizobium from the rhizosphere and endosphere of non-legume crops, including rice, and described their roles in plant growth promotion (Peng et al., 2008; Zhang et al., 2014).

According to the inferred microbial network, several direct interactions between *M.oryzae* and other microbes may occur. In contrast to our expectation, the results suggested that mutualism, facilitation, and commensalism may dominate in the interactions of *Magnaporthe* with other microbes in leaves. Almost all the edges in the interaction network were indeed positive in the symptomatic tissues whereas only about half of the edges were positive in the network of healthy tissues. The positive interactions between microbes and the fungal pathogen may be accounted for by endosymbiosis, or they may also be accounted for by commensalism, where bacteria may use dead hyphae as a source of nutrients (Hoffman and Arnold, 2010; Venturi and da Silva, 2012). Bacteria may also facilitate fungal infection because they benefit from the changes in the plant metabolism induced by the fungal pathogen (Venturi and da Silva, 2012). Several *Pantoea* species have been isolated as endophytes without causing symptoms; some isolates are antibiotic producers and have been developed as biocontrol agents for plant diseases (Walterson and Stavrinides, 2015; Jiang et al., 2019). However, this bacterial genus is highly diverse (Walterson and Stavrinides, 2015) and has also been reported to cause disease in rice (Doni et al., 2019). As per the interaction network, there may be a direct, antagonistic (negative) ecological interaction between *Magnaporthe* and *Pantoea* (Figure 4). This finding suggests the putative roles of *Pantoea* to protect against the blast disease. Manipulative experiments are therefore required to validate the isolation and the antagonistic relationship between the two species and decipher the mechanism underlying the interaction.

Soil microbes have the potential to influence plant immune defense and pathogen invasion. Therefore, understanding how to plant endosphere microbes that interact with the soil communities may provide a "road map" to explain the pathogen infection and disease process. Previous studies used the SourceTracker program to estimate the proportions of the microbiome in a given community that comes from potential source environments and have been used to analyze the relationship between disease-associated endosphere microbial communities and rhizosphere communities (Knights et al., 2011; Hu et al., 2020). In this study, we utilized this program to track the source of plant rhizospheric and endospheric microbial communities in healthy vs. diseased plants. Previous studies showed that bacterial communities in the rhizosphere soils were mainly derived from the bulk soil (Mendes et al., 2013; Hu et al., 2020). We found a somewhat difference in that although most fungal communities of the rhizosphere were from bulk, only half of the total bacteria in the rhizosphere were derived from the nearby bulk soils (~80%). There is a sharp contrast between the

source of root endosphere bacteria resulting from *Magnaporthe* infection. Whereas the majority (71%) of healthy root bacteria could be tracked from soil (rhizosphere and bulk combined), there was only a very small portion for diseased samples (Figure 5). However, it is not clear about the sources of most microbes, especially bacteria, in diseased plants. We assume that early in the pathogen infection, due to potential host immunity or "cry for help," as mentioned before, the microbiome was localized prior to our sampling, and the diseased plant could not keep dynamic recruitment of bacteria from soil whereas the healthy individual could. However, controlled experiments with a defined microbiome (synthetic community) and tracking for microbiome sources could reveal important information.

Taken together, we infer that foliar infection by pathogenic *M. oryzae* causes a shift in the rhizosphere bacteria. *M. oryzae* in symptomatic leaf and grain tissues are associated with interactions with various bacteria, including Bacillus, Enterobacter, and several other bacteria, the role of which in the disease process needs to be clarified. The positively interacting bacteria putatively obtain benefits from invading pathogens, which might lead to the migration of many additional bacterial genera into the plant roots and leaves, eventually causing an outbreak of blast disease. These findings will provide potential ideas and a theoretical basis for isolating biocontrol agents controlling the blast disease in rice with further work.

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/, PRJNA824966.

Author contributions

KD: conceptualization, methodology, investigation, formal analysis, software, writing—original draft, and visualization. MY: conceptualization, methodology, writing—review and editing, and project administration. SO: conceptualization, resources, and writing—review and editing, supervision, project administration, and funding acquisition. All authors contributed to the interpretation of the data, critically revised the manuscript, read, and approved the final version before submission.

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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.2022.949152/full#supplementary-material

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Assembly and dynamics of the apple carposphere microbiome during fruit development and storage

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Microbial communities associated with fruit can contribute to quality and pathogen resistance, but little is known about their assembly and dynamics during fruit development and storage. Three apple cultivars growing under the same environmental conditions were utilized to examine the apple carposphere microbiome composition and structure at different developmental stages and storage. There was a significant effect (Adonis, $p \leq 0.001$) of fruit genotype and its developmental stages and storage times on the fruit surface microbial assemblage and a strong temporal microbial community succession was detected (Mantel test: $R \leq 0.5$, $p = 0.001$) in both bacterial and fungal communities. A set of 15 bacterial and 35 fungal core successional taxa and members exhibiting differential abundances at different fruit stages were identified. For the first time, we show the existence of underlying universal dynamics in the assembly of fruit-associated microbiomes. We also provide evidence of strong microbial cross-domain associations and uncover potential microbe-microbe correlations in the apple carposphere. Together our findings shed light on how the fruit carposphere assemble and change over time, and provide new insights into fruit microbial ecology.

KEYWORDS

apple, assembly, dynamics, microbiome, succession

Introduction

Microbial communities in ecosystems comprise complex assemblages of taxa that can establish beneficial, neutral, or detrimental interactions among themselves and with their host in the ecological niches they occupy (Little et al., 2008; Vorholt, 2012; Nemergut et al., 2014; Ghoul and Mitri, 2016; Hassani et al., 2018). In plant systems, microbial communities

are found on the surface or inside plant tissues and organs, and their interactions with their hosts have impacted their colonization, evolution, and diversity (Ivanov et al., 2012; van Schie and Takken, 2014; Skiada et al., 2020; Delaux and Schornack, 2021). The microbial habitat associated with fruit (carposphere), similar to other plant parts, harbors a wide diversity of bacteria, archaea, fungi, and viruses, and their composition and diversity continues to be explored (Droby and Wisniewski, 2018; Abdelfattah et al., 2020, 2021; Piombo et al., 2020; Kumar et al., 2021). In contrast to the rhizosphere (the soil-root interface), where fluctuations in environmental conditions are often dampened by the bulk soil, above-ground parts of plants, such as the carposphere, represent a unique ecological system where the environment is much more dynamic and unstable (Droby and Wisniewski, 2018). Resident microbes in commercial fruit crops are exposed to large fluxes in abiotic conditions and rapid changes in the resource environment during fruit development and after harvest when the fruit are placed in storage. The carposphere is also prone to additional perturbations during various cultural and plant protection management practices. In this regard, it is unclear if ecological processes and models reported for either the rhizosphere or phyllosphere can be extrapolated to conditions prevailing in the carposphere. Interactions between fruit and its resident microbiota may include antagonistic interactions that result in active or latent infections by pathogenic microbes, as well as commensalistic or mutualistic interactions. Questions about the role and function of these microbial interactions in fruit quality and disease resistance are just beginning to be investigated (Diskin et al., 2017; Kusstatscher et al., 2020; Sare et al., 2021; Zhang et al., 2021; Zhimo et al., 2021; Sangiorgio et al., 2022). However, we still lack fundamental knowledge about the assembly, ecology, and community dynamics of fruit-associated microbiota in horticultural fruit crops, during different phenological and physiological stages or during storage. The acquisition of such information could inform the development of new tools like identification of appropriate antagonist microbes and designing synthetic communities that can be specifically formulated to target specific pathogens as an intervention during pre-harvest or postharvest storage, thereby reducing decays and prolong shelf life of fruits.

Studying community succession (defined here as series of progressive changes in the composition of an ecological community over time) is a fundamental pursuit in microbial ecology research (Fierer et al., 2010; Faust and Raes, 2012; Brislawn et al., 2019; Pascual-García and Bell, 2020). While each microbial community is distinct and subject to their specific environments, some generalized principles exist that shape their assembly and succession (Nemergut et al., 2013; Pascual-García and Bell, 2020). The overall variation in community structure and diversity (i.e., beta-diversity) that occurs during microbial community succession reflects two divergent phenomena: turnover and nestedness (Baselga, 2010). Turnover is an ecological process through which existing taxa are replaced with new ones and may reflect species sorting by environmental shifts through space or time, leading to selective differentiation of taxa pools among assemblages (Baselga, 2010; Victorero et al., 2018; Brislawn et al., 2019; Wang et al., 2019). In contrast, nestedness accounts for taxa loss or gain without replacement, where one community is a subset of another community, and may originate from processes of ordered loss or colonization across temporal and spatial gradients (Victorero et al., 2018; Brislawn et al., 2019; Wang et al., 2019; Liu and Müller, 2020).

Another question to address in fruit-associated microbial communities is the concept of universality in microbiomes, initially reported to exist in humans (Bashan et al., 2016), and recently in some arbuscular mycorrhizal (AM) fungal communities, where the existence of universality is dependent on ecosystem types (Van Geel et al., 2017; Verbruggen et al., 2018). Universality describes the extent to which there are consistent underlying dynamics that govern microbial community assembly across different hosts, sites, and ecosystems. Do universal dynamics govern fruit associated microbiomes in different ecosystems (geographic locations, fruit types, fruit developmental stages, management interventions) or does each community have its own unique individual set of dynamics? Universality can be identified by a signature in which the similarity in community composition across samples is linked to the similarity in abundance profiles of shared species (Bashan et al., 2016). This outcome suggests that there are regularities in community assembly across hosts, and that taxa within these communities interact in a similar manner. If this holds true for fruit-associated communities, it would suggest that a method of microbiome manipulation valid in one system is likely to work in other systems.

The fruit surface of the domesticated apple (*Malus* × *domestica* Borkh.) has been reported to be naturally colonized by numerous microbes that vary in abundance and diversity according to host genotype, geographical location, management practices, and fruit tissue type (Wassermann et al., 2019a,b; Abdelfattah et al., 2020, 2021; Bösch et al., 2021). Little is known, however, about the ecological processes, particularly with regard to selection, that regulate temporal carposphere microbial dynamics. Therefore, we designed an experiment utilizing three commercial apple cultivars growing in the same geographical location and management program, and followed the epiphytic bacterial and fungal communities of the fruit throughout several developmental stages and for a period of time after harvest when the apples were in storage (Figure 1). The three cultivars used in the study "Royal Gala," "Golden Delicious" and "Granny Smith" are among the most popular cultivars grown worldwide with significant economic importance. "Granny Smith" and "Golden Delicious" have different genetic lineages arising from chance seedlings while "Royal Gala" is a product of traditional breeding between "Kidd's Orange Red" and "Golden Delicious." We aimed to address the following objectives: (1) determine the role of host factors, such as genotype, fruit developmental stage and postharvest storage, in shaping the microbial assembly and dynamics of the apple carposphere, (2) explore patterns of community succession and quantify the relative contribution of turnover and nestedness components, and (3)

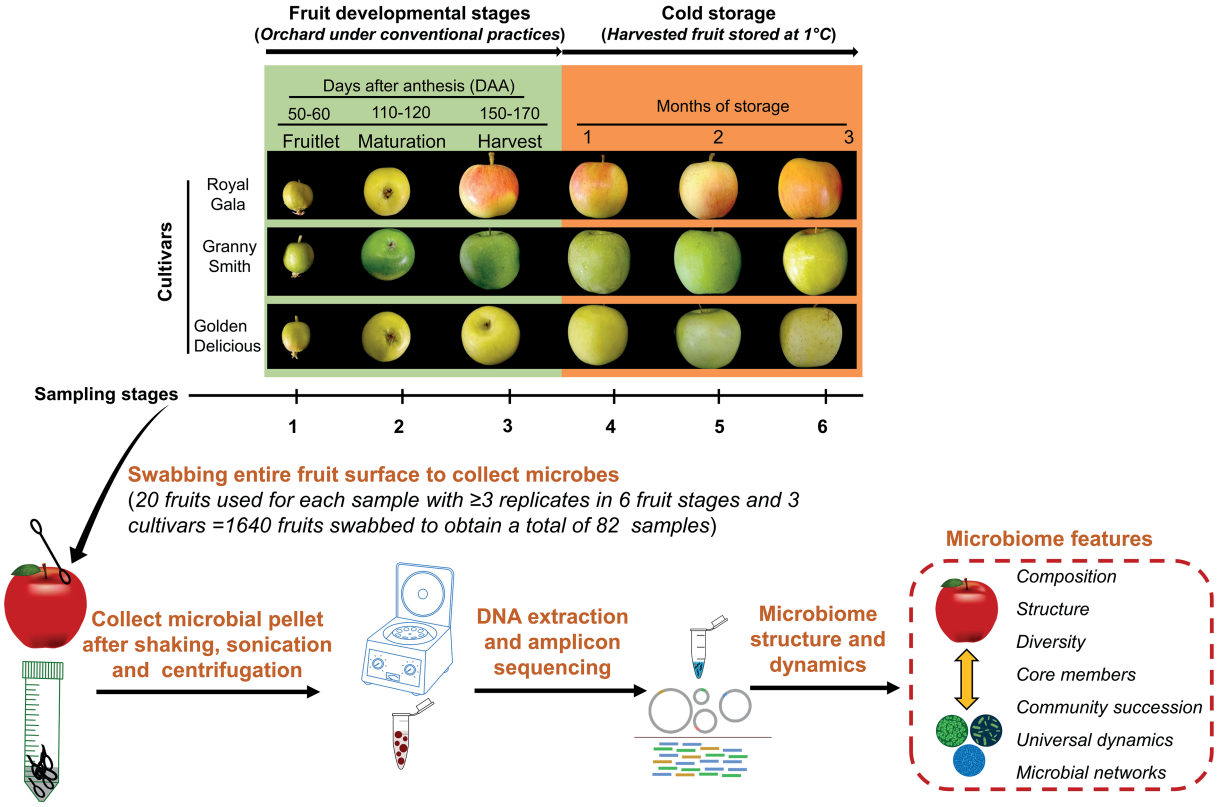

FIGURE 1
Schematic illustration of the experimental set-up. The apple fruit microbiomes were sampled from three cultivars namely "Royal Gala," "Granny Smith" and "Golden Delicious" growing in the same orchard and plot where they received a conventional maintenance program. Sampling was carried out in the field during three developmental stages: fruitlet (50–60 days after anthesis; DAA), maturation (110–120 DAA) and at harvest (150–170 DAA) and another three times from the harvested fruit lot during cold-storage periods (1°C) at monthly intervals. Fruits were swabbed, microbial pellets were collected by washing the swabs and concentrated from 20 fruits each to represent a sample. Genomic DNA was extracted from these samples, sequenced and processed to obtain microbiome features.

elucidate the underlying ecological networks and microbial associations that govern carposphere microbial assembly and dynamics of the apple carposphere microbial assembly.

Results

Influence of genotype and dynamics of carposphere microbial community assemblage at different fruit developmental stages and storage period

Differences in microbial composition among sample groups were assessed using permutational multivariate analysis of variance PERMANOVA and principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity. The R^2 values of PERMANOVA (Adonis) were used to explain the variance components of the different factors. A PERMANOVA model with "Stages" representing the fruit stage, "Cultivar" representing the fruit genotype and their interaction "Cultivar × Stages" as explanatory factors was used to examine the factors shaping overall variation in microbial community composition. We found that overall, "Stages" explained most of the microbial community variation (Adonis, bacteria: $R^2 = 0.44$, $p = 0.001$; fungi: $R^2 = 0.50$ $p = 0.001$), while "Cultivar" explained a lesser degree of the variation (Adonis, bacteria: $R^2 = 0.12$, $p = 0.001$; fungi: $R^2 = 0.19$, $p = 0.001$). The interaction between the two factors ("Cultivar × Stages") also exhibited significant impact on the variation between communities (Adonis, bacteria: $R^2 = 0.34$, $p = 0.001$; fungi: $R^2 = 0.18$, $p = 0.001$; Figure 2A).

The PCoA plot illustrates this effect with the samples largely separated by these two factors along principal coordinate axes 1 and 2 for both bacterial and fungal communities. When similar analyses were performed to check for differences in the community composition among stages separately for each cultivar (Supplementary Figure 3) or among cultivars separately for each stage (Supplementary Figure 4), significant differences (Adonis, $p \leq 0.01$) in both bacterial and fungal communities in each of the groups analyzed were observed except for the fungal community at maturity stage among cultivars ($p = 0.1689$). Pairwise comparisons between cultivars during each stage revealed that in both bacterial and fungal communities, significant differences (Pairwise.adonis, P adjusted < 0.05) between cultivar pairs were observed only at the

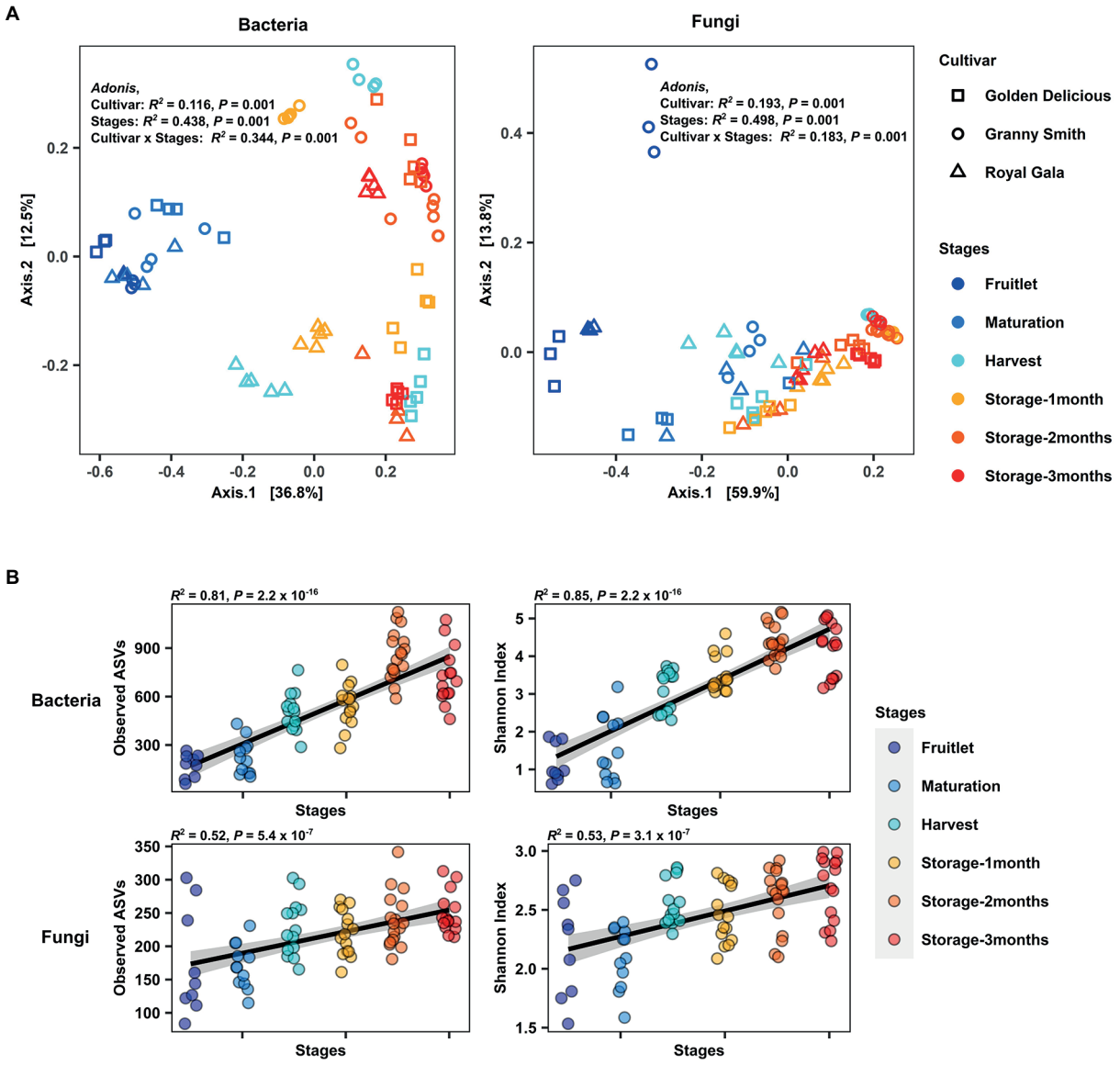

FIGURE 2
Overview of the apple carposphere microbiome. **(A)** Principal coordinate (PCoA) analysis of bacterial and fungal community based on Bray–Curtis dissimilarity with permutational analysis of variance (PERMANOVA) showed significant association of bacterial and fungal community compositions with fruit stages (developmental stages and storage period) and cultivar type (Adonis, $p < 0.001$). **(B)** Strong positive correlation (Spearman, $p < 0.0001$) between the fruit developmental stages and storage periods (plotted at the x-axis) and bacterial and fungal richness (Observed ASVs) as well as Shannon diversity (plotted at the y-axis) in the apple carposphere microbiome.

latter stage of fruit development (harvest) and during storage (Supplementary Table 3). Similar pairwise comparisons between individual stages revealed that the bacterial community differed in composition between most of the stage pairs (Pairwise.adonis, P adjusted < 0.05), although the R^2 values explaining the variance components were lesser for storage period pairs as compared to pairs from early developmental stages (Supplementary Table 4). In the case of fungal community composition, significant differences were seen in pairwise comparisons between early developmental stage pairs (Pairwise.adonis, P adjusted < 0.05) except between maturation and harvest stages (Pairwise.adonis, P adjusted $= 0.06$), but no differences were seen for storage period pairs (Pairwise.adonis, P adjusted > 0.05). Conducting pairwise comparisons among each individual stages of each cultivar revealed significant differences between many stage pairs in the fungal community but little to no significant differences between stages in the bacterial community after corrections for multiple testing (Supplementary Table 5).

A multivariate homogeneity of group dispersions test (PERMDISP2) based on Bray–Curtis dissimilarity among the samples grouped by stage and cultivar was used to determine if

there were differences in variance (beta-dispersion) between sample groups. Our results for bacterial communities revealed significant differences in variances among sample groups at different stages (Betadisper, bacteria: $p = 0.003$; fungi: $p = 0.008$), with lower variance (distance to centroid) at the early fruitlet stage compared to later stages of fruit development and during storage periods (Supplementary Figure 5A). Statistically significant differences in the bacterial community were only observed between the fruitlet and one-month of storage (Tukey HSD, $p = 0.008$) while in the fungal community, differences were seen between the fruitlet and 1-month of storage (Tukey HSD, $p = 0.003$), and between the fruitlet and 2-months of storage (Tukey HSD, $p = 0.007$). No significant differences in variances were observed between different cultivar groups (Betadisper, bacteria: $p = 0.933$; fungi: $p = 0.491$; Supplementary Figure 5B).

We then investigated α-diversity of the apple carposphere microbiome in samples by estimating within-sample richness (Observed amplicon sequence variants: ASVs) and Shannon diversity and using Spearman's correlation to determine their association with fruit stages. We found a significant increase and strong positive correlation in richness and Shannon diversity with fruit stages in both the bacterial (richness: $R^2 = 0.81$, $p < 2.2 \times 10^{-16}$; Shannon index: $R^2 = 0.85$, $p < 2.2 \times 10^{-16}$) and fungal community (richness: $R^2 = 0.52$, $p = 5.4 \times 10^{-7}$; Shannon index: $R^2 = 0.53$, $p = 5.3 \times 10^{-7}$; Figure 2B). Interestingly, when analyses were conducted separately for the three cultivars, there were significant increases and positive correlations in both bacterial and fungal richness and Shannon diversity in all the three cultivars, except in "Royal Gala," which did not show a positive correlation between fungal richness and diversity with fruit stage (Supplementary Figure 6). No significant differences were detected in overall α-diversity among the three cultivars (Kruskal–Wallis test) in the bacterial community (richness: $p = 0.65$; Shannon index: $p < 0.56$). Significant differences were observed, however, for fungal communities across cultivars in richness ($p = 0.00018$) but not in the Shannon Index ($p = 0.073$). Pairwise Wilcoxon tests among the three cultivars indicated that "Royal Gala" samples had a lower fungal richness and Shannon Index, relative to "Golden Delicious" and "Granny Smith" (Supplementary Figure 7). Collectively, these results confirm that the composition of microbial communities in the apple fruit is influenced by fruit genotype and display significant and dynamic assemblages at different fruit developmental stages and storage period.

Community succession in apple carposphere microbial assemblages

After determining the effect of the host (fruit development stage, storage period, and genotype) on the microbial assemblage of the apple carposphere, we next investigated the dynamics of these microbial community assemblages over time. To do this, we performed a Mantel correlation analysis between temporal distances (based on Euclidean dissimilarity as periods between the six fruit sampling stages) and microbial community distances (based on Bray–Curtis community pairwise dissimilarity) from all sample pairs. Strong evidence of community succession was evident in both the bacterial (Mantel: $R^2 = 0.60$, $p = 0.001$) and fungal communities (Mantel: $R^2 = 0.57$, $p = 0.001$) in the carposphere microbiome over time (Figure 3A). Similar strengths of community succession was also seen when the sample pairs were analyzed separately for each of the three cultivars (Supplementary Figure 8). Confirmation of the results of these temporal dynamics in community composition was observed in the trends of relative abundance of the dominant bacterial and fungal ASVs (>1%) across fruit stages and cultivars (Figure 3B). Prominent declines in the relative abundance were observed of initially dominant ASVs belonging to the genera *Pseudomonas*, *Pantoea, Aureobasidium,* and *Vishniacozyma,* while increases in *Cladosporium, Stemphylium,* and *Alternaria* were evident, relative to the abundance of other genera (Figure 3C).

The dynamic nature of the apple carposphere microbiome structure over time raises questions regarding the role and fate of the core successional microbes and their persistence across different fruit stages. We identified 15 bacterial ASVs and 35 fungal ASVs, across all the sampling stages and cultivars, as core successional microbes based on 95% occupancy and no limit on the percentage of their contribution to relative abundance. Tracking these core microbes across the different fruit developmental stages and the storage period revealed contrasting dynamics in bacterial and fungal communities (Figure 4A). The collective contribution of the bacterial core microbes to the total abundance decreased dramatically over the course of the successive sampling times (from 84.2% during the fruitlet stage to 26.5% at the end of the storage period; Figure 4A). This trend was mainly due to dominance of two core members (ASV1_*Pseudomonas* and ASV3_*Pantoea*) during the early stages of fruit development followed by a dramatic decrease at the later fruit development stages, while another core member ASV2_*Cellulosimicrobium* became predominant at harvest and during the period of storage (Figure 3C). In contrast, the 35 ASVs that made up the fungal core microbiome consistently predominated in their collective abundance (contributing >85% of fungal abundance) throughout all of the fruit developmental stages and storage period (Figure 4A). Linear Discriminant Analysis Effect Size (LEfSe) analysis was used to determine taxa that most likely explained differences between the fruit stages based on their abundance. The analysis identified 20 bacterial and 11 fungal ASVs at different fruit stages that were differentially abundant (LDA score > 4.0, $p \leq 0.01$; Figure 4B). Notably, all of the 11 fungal and some of the bacterial (8 out of 20) ASVs that differentiated the fruit stages were core taxa members. These results indicate that the core fungal and bacterial microbiomes are highly persistent and remain stable (especially in the fungal community) during community succession, potentially due to higher abundance of these microbes in the environment, leading to higher immigration and adaptation to the apple carposphere than the other members of the microbiome.

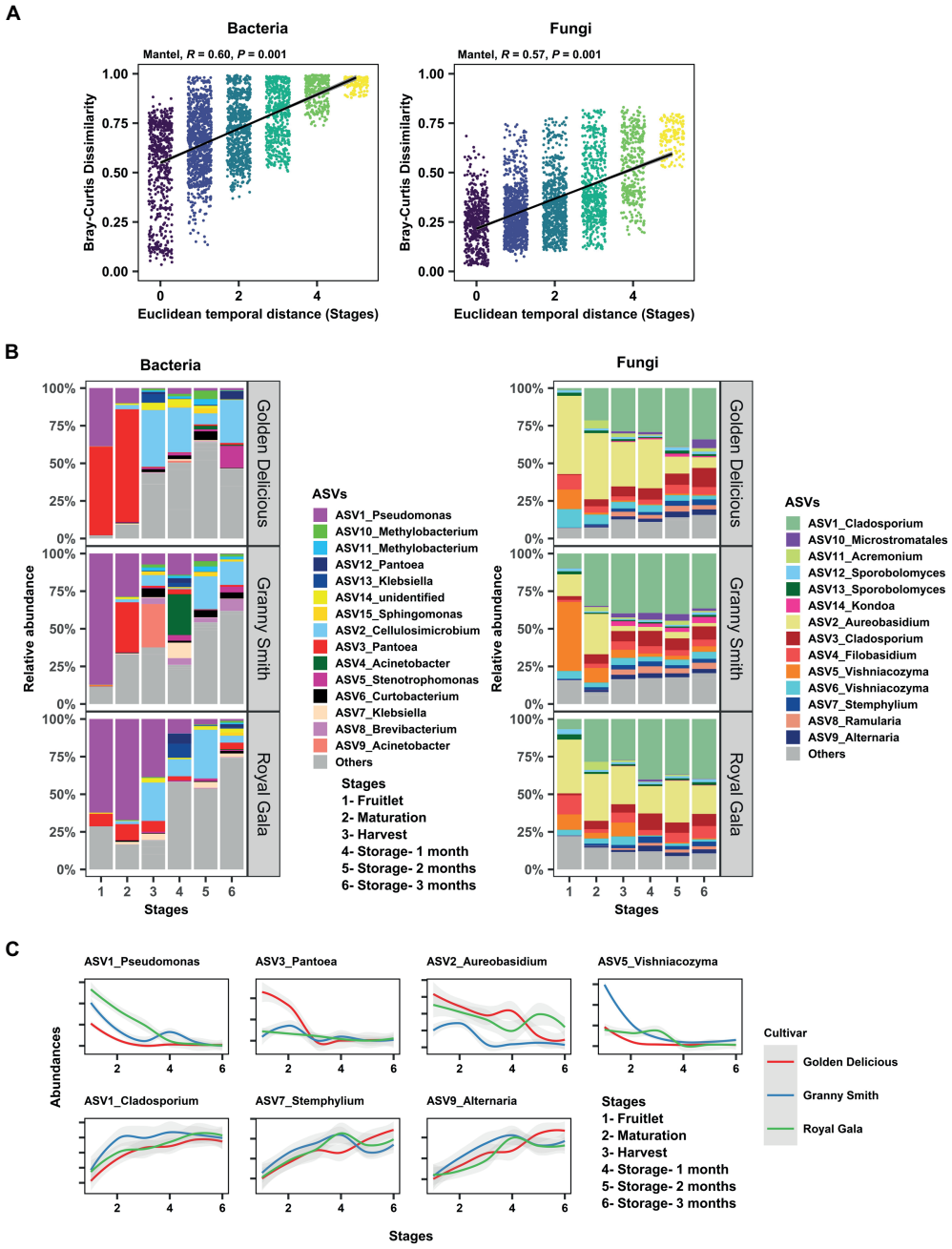

FIGURE 3
Community succession of the apple carposphere microbiome. **(A)** Mantel correlation between Euclidean temporal distance (fruit stages) and Bray–Curtis community dissimilarity showed strong succession in both bacterial and fungal communities. **(B)** Temporal change of the relative abundances of dominant bacterial and fungal amplicon sequence variants (ASVs) at each sampling stages in the carposphere microbiome of three apple cultivars as visualized using barplots. **(C)** Temporal change in the abundances of some ASVs corresponding to the genera *Pseudomonas*, *Pantoea*, *Aureobasidium*, and *Vishniacozyma* showed characteristic increasing trends in abundances while ASVs corresponding to genera *Cladosporium*, *Stemphylium*, and *Alternaria* showed decreasing trends in abundances in the carposphere of three apple cultivars.

Turnover drives community succession in the carposphere microbiome of apple

With the finding of strong community succession, despite the dominance and persistence of a few core ASVs, we explored the ecological processes underlying the strong community changes that impacted the rest of the microbial community (non-core members) over the course of fruit development and storage periods. To exclude the influence of abundance of the dominant core ASVs, we used Sorenson pairwise dissimilarity (β_{SOR}), a β-diversity metric that is independent of species abundance, as well as richness variance (unlike the Bray–Curtis dissimilarity

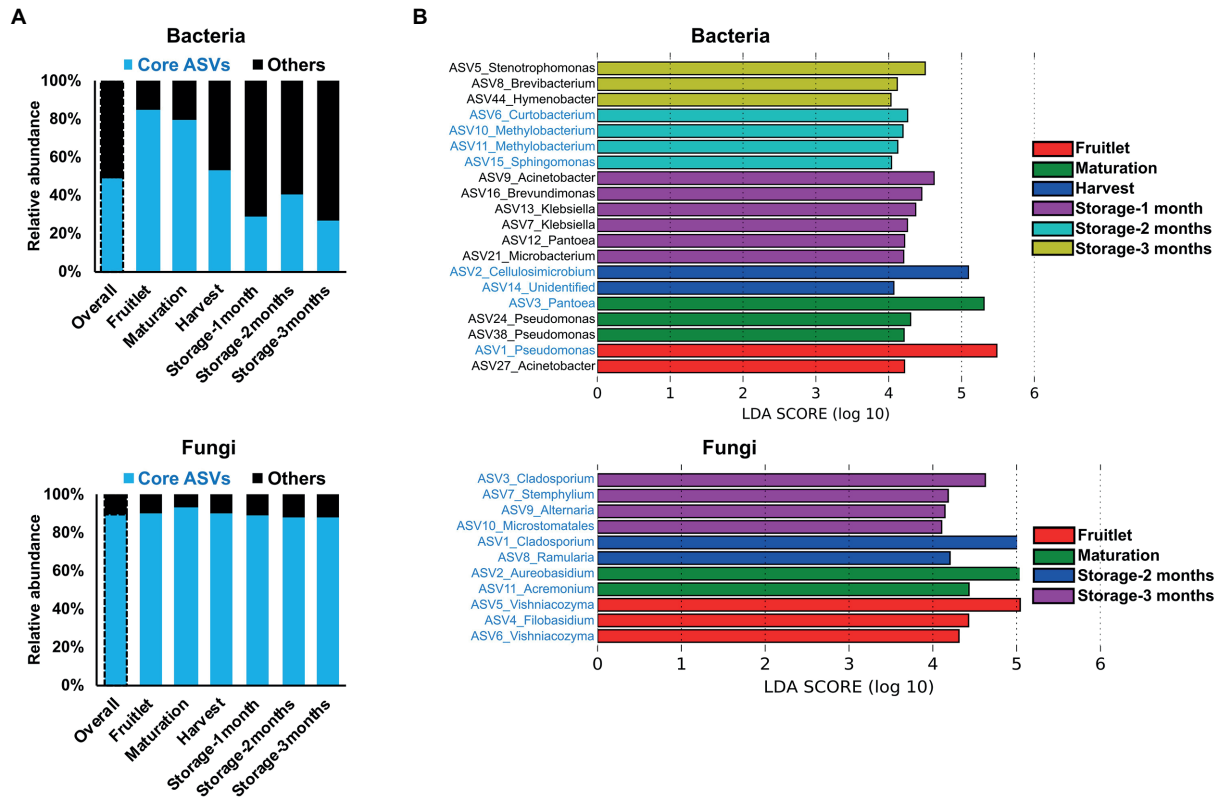

FIGURE 4
Core microbiome dynamics and distribution of the apple carposphere microbiome. **(A)** Distribution of core microbiome in the apple carposphere showed decreasing collective contribution of bacterial core amplicon sequence variants (ASVs) to the total bacterial abundance and a contrasting dominant and consistent contribution of fungal core ASVs to the total fungal abundance. **(B)** Identified biomarker ASVs (LDA score>4.0, $p \leq 0.01$) during different fruit stages using Linear Discriminant Analysis Effect Size (LEfSe) analysis comprised of both non-core and core ASVs in bacteria while all fungal biomarker ASVs comprised of only core ASVs. Biomarker ASV names in blue are core-members.

previously used to describe community succession), to explain the variation in microbial composition over time. We first calculated β_{SOR}, and then partitioned it to discriminate turnover (Simpson dissimilarity; β_{SIM}) from nestedness (β_{SNE}) – the two antithetic processes that reflect species replacement and species loss, respectively. A mantel test revealed that the temporal distance (fruit developmental stages and storage periods) exhibited a significant association with both the turnover and nestedness components of bacterial (β_{SOR}: $R^2 = 0.59$, $p = 0.001$; β_{SIM}: $R^2 = 0.36$, $p = 0.001$; β_{SNE}: $R^2 = 0.40$, $p = 0.001$) and fungal community variations (β_{SOR}: $R^2 = 0.57$, $p = 0.001$; β_{SIM}: $R^2 = 0.35$, $p = 0.001$; β_{SNE}: $R^2 = 0.31$, $p = 0.001$; Figure 5A). The overall total Sorenson's β diversity value (β_{total} value = bacteria: 0.975; fungi: 0.962) was almost entirely contributed by the turnover component measured as the Simpson dissimilarity value ($\beta_{turnover}$ value-bacteria: 0.959; fungi: 0.949), as compared to the nestedness component measured as nestedness, the resultant fraction of the Sorenson dissimilarity value ($\beta_{nestedness}$ value-bacteria: 0.015; fungi: 0.013; Figure 5B). These data indicate that changes in the identities of the microbial taxa that are present through succession is driven mostly by turnover. In contrast, nestedness plays only a minor role in carposphere microbiome assembly and dynamics. Since turnover was the dominant component underlying β-diversity during succession in both bacteria and fungi, we performed a distance-based test for homogeneity in multivariate dispersions (PERMDISP) to gauge the strength and timing of turnover using the β_{SIM} across the developmental stages and storage periods and visualized the results in PCoA plots. Results revealed strong turnover in species identities among the samples from the three developmental stages (fruitlet, maturation, and harvest) and little or no turnover in samples after harvest during the three time points sampled during storage (Figure 5C).

Pattern of universality in the carposphere microbiome

In our study of the apple carposhpere microbiome we further addressed the question of whether the carposphere microbiome assemblage follows an underlying universal ecological dynamics model. In microbial systems where universality exist, the

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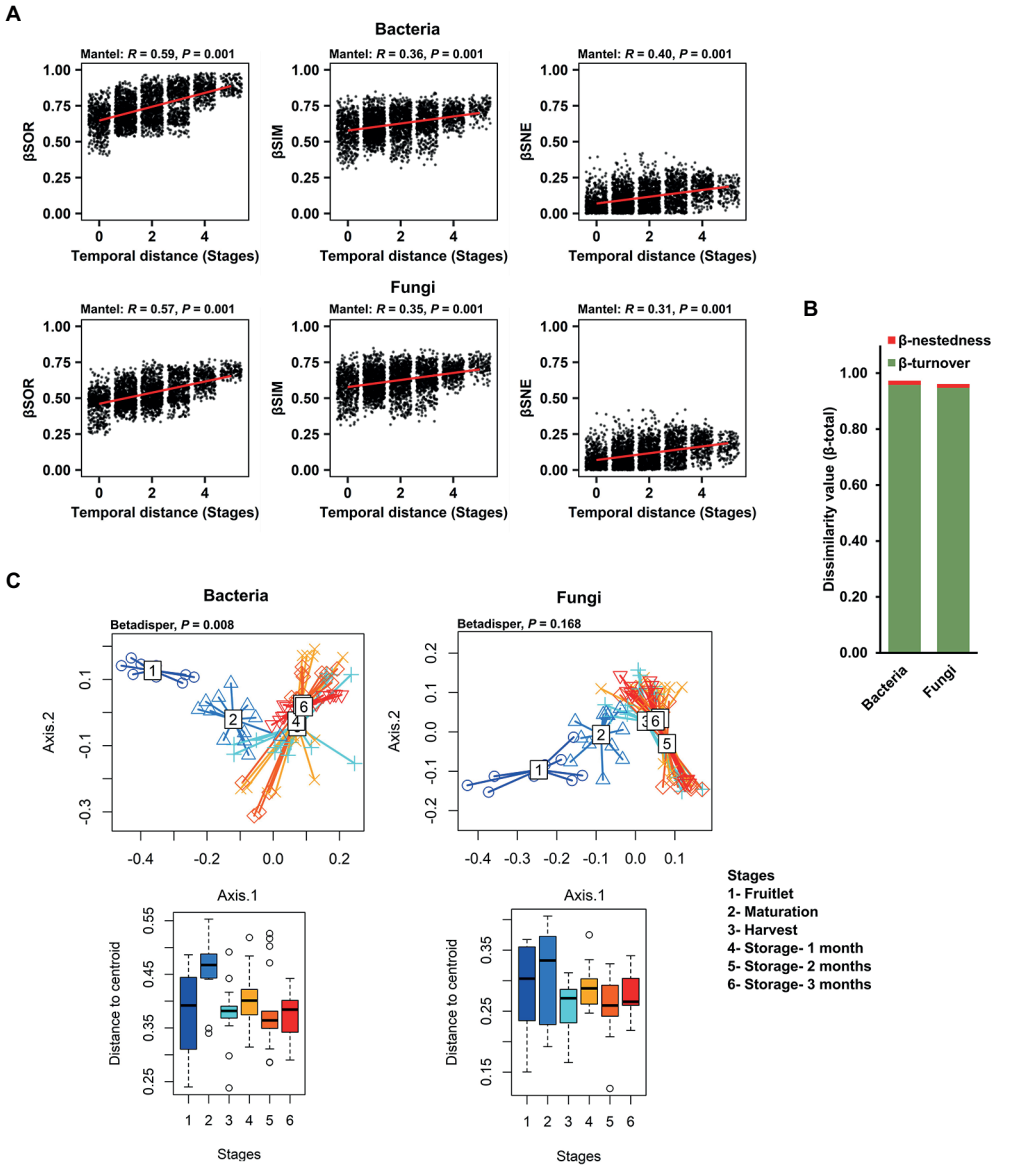

FIGURE 5
Role of two patterns, turnover and nestedness in the change in carposphere microbiome community composition over time. **(A)** Mantel tests to explore correlation of temporal distance (stages) with the compositional variance of bacterial and fungal community showed significant and biologically meaningful associations with the overall compositional variance (measured by Sorenson pairwise dissimilarity; β_{SOR}) and also with both the turnover (Simpson pairwise dissimilarity; β_{SIM}) and nestedness (Sorenson pairwise dissimilarity minus Simpson pairwise dissimilarity; β_{SNE}) components after partitioning. The Sorenson and Simpson metrics differ from the Bray–Curtis metric in that they do not take into account relative abundances. **(B)** The overall compositional variance (β_{-total}) was almost entirely contributed by the turnover component ($\beta_{-turnover}$) rather than nestedness ($\beta_{-nestedness}$) in the apple carposphere microbiome. **(C)** Turnover of both bacterial and fungal community composition through stages, demonstrated by PCoA plots using Simpson dissimilarity showed strong bacterial as well as fungal compositional turnover among stages 1–3 (developmental stages) followed by significantly less or no turnover for stages 4–6 (storage periods) but none were significantly different from other stages in case of the fungal community.

compositional variation between sample groups mainly originates from differences in the sets of colonizing species, as opposed to individual dynamics where the samples exhibit a high degree of variability in both community assemblages and abundance profiles. We re-normalized dissimilarity (root Jensen–Shannon divergence) between all our samples for just the shared ASVs and plotted it against the overlap of the community assemblages, obtained from the relative abundances of the shared ASVs. A nonparametric regression and bootstrap sampling was performed to calculate the dissimilarity–overlap curve (DOC) and its confidence interval. A flat DOC is expected in case of individual dynamics. When a DOC displays a characteristic negative slope in the high-overlap region, universality is supported with inter-taxa interactions and the level of support is determined by the fraction of the negative slope (f_{NS}),

which is the fraction of pairwise comparisons found where the DOC slope is negative. The DOCs from the present data exhibited significant negative slopes (bacteria: $p = 0.009$; fungi: $p = 0.009$) with f_{NS} values of 15.4% for the bacterial community comparisons and 62.6% for the fungal community comparisons (Figure 6). To validate the universality on a larger scale, we applied the DOC analysis to a data set of from our previous study on apple fruit microbiomes from multiple geographical locations and fruit tissue types (Abdelfattah et al., 2021). We observed significant negative slopes in overall fungal community comparisons ($f_{NS} = 48.7\%$, $p = 0.009$) but not significant in bacterial community comparisons ($f_{NS} = 11.5\%$, $p = 1.0$; Supplementary Figure 9A). Interestingly, the level of support for universality in the fruit fungal community comparisons was highest in peel tissues ($f_{NS} = 36.7\%$), followed by tissues from the calyx end ($f_{NS} = 23.1\%$), and tissues from the stem end ($f_{NS} = 18.5\%$; Supplementary Figure 9B).

Cross-domain and microbe-microbe interactions in the apple carposphere

Strong cross-domain interactions were observed, based on Mantel correlations between the bacterial and fungal community, using distance matrices based on both Bray–Curtis dissimilarity ($R = 0.56$, $p = 0.001$), where taxa abundance and richness are considered, and Simpson dissimilarity ($R = 0.56$, $p = 0.001$), where the variance due to abundance and richness was removed (Figure 7A). This cross-domain association was also supported by the detection of significant positive correlations (Spearman's) in richness (Observed ASVs, $R = 0.55$, $p = 9.2 \times 10^{-8}$) and within sample diversity (Shannon Index, $R = 0.55$, $p = 1.1 \times 10^{-7}$) between the bacterial and fungal communities (Figure 7B). Furthermore, we generated a correlation network (27 nodes, 79 edges, network density: 0.279; characteristic path length: 2.181) to check for potential associations at the genera level, and the resulting network revealed significant co-occurrences among dominant bacterial and fungal ASVs (Figure 7C). The taxa involved comprised 15 bacterial and 12 fungal ASVs connected by 56 co-presence and 23 co-exclusion associations, and included plant pathogen genera, such as *Ramularia*, *Alternaria*, and *Stemphylium*, and biocontrol agents, such as *Aureobasidium*, *Vishniacozyma*, and *Filobasidium*. The genera *Kondoa*, *Ramularia*, and *Pantoea* were identified as putative hub taxa, based on both a high node degree and betweennesss centrality (Figure 7D).

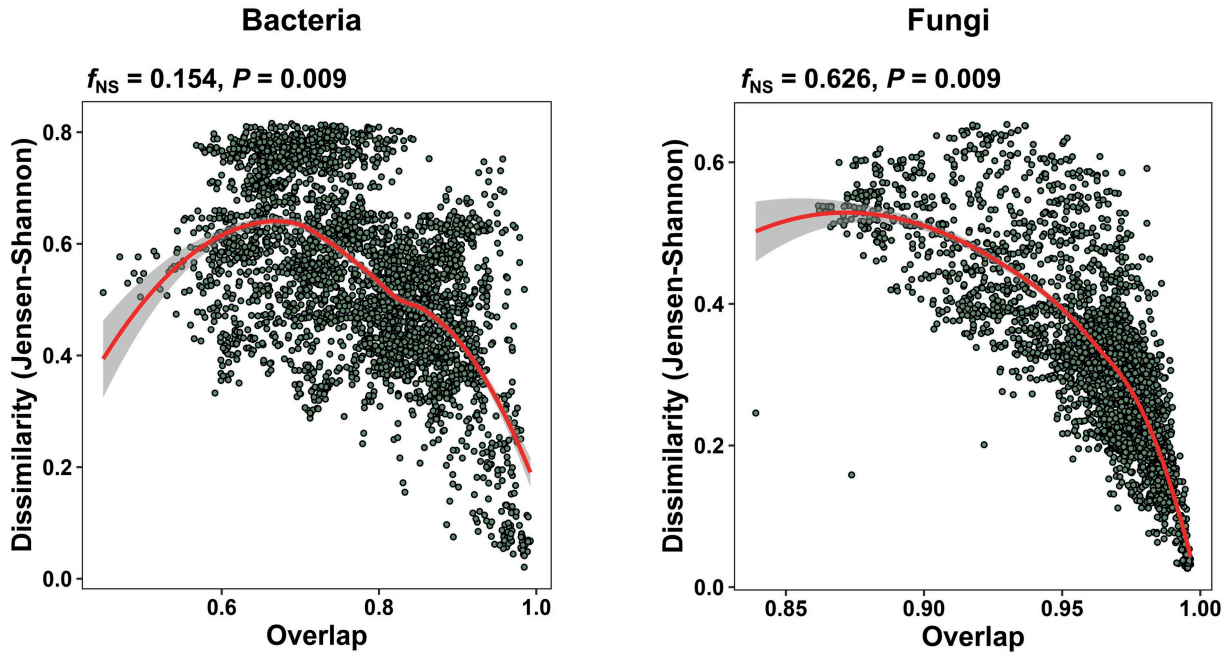

FIGURE 6
Universal ecological dynamics of the apple carposphere microbiome. Dissimilarity-overlap curves (DOC) for all bacterial and fungal samples of the apple carposphere microbiome showing significant negative slopes ($p = 0.009$). For DOCs, the overlap and dissimilarity based on root Jensen–Shannon divergence of sample pairs were calculated and each sample pair was represented as a point in the dissimilarity–overlap plane. A nonparametric regression and bootstrap sampling was performed to calculate the DOC and its confidence interval. The DOCs are indicated in red. The fraction of negative slope (f_{NS}) is the fraction of data points in the interval where the DOC shows a negative slope and supports the level of universality. Higher f_{NS} value was observed in the fungal community than the bacterial community suggesting stronger level of universality in the former than the later although both were significant.

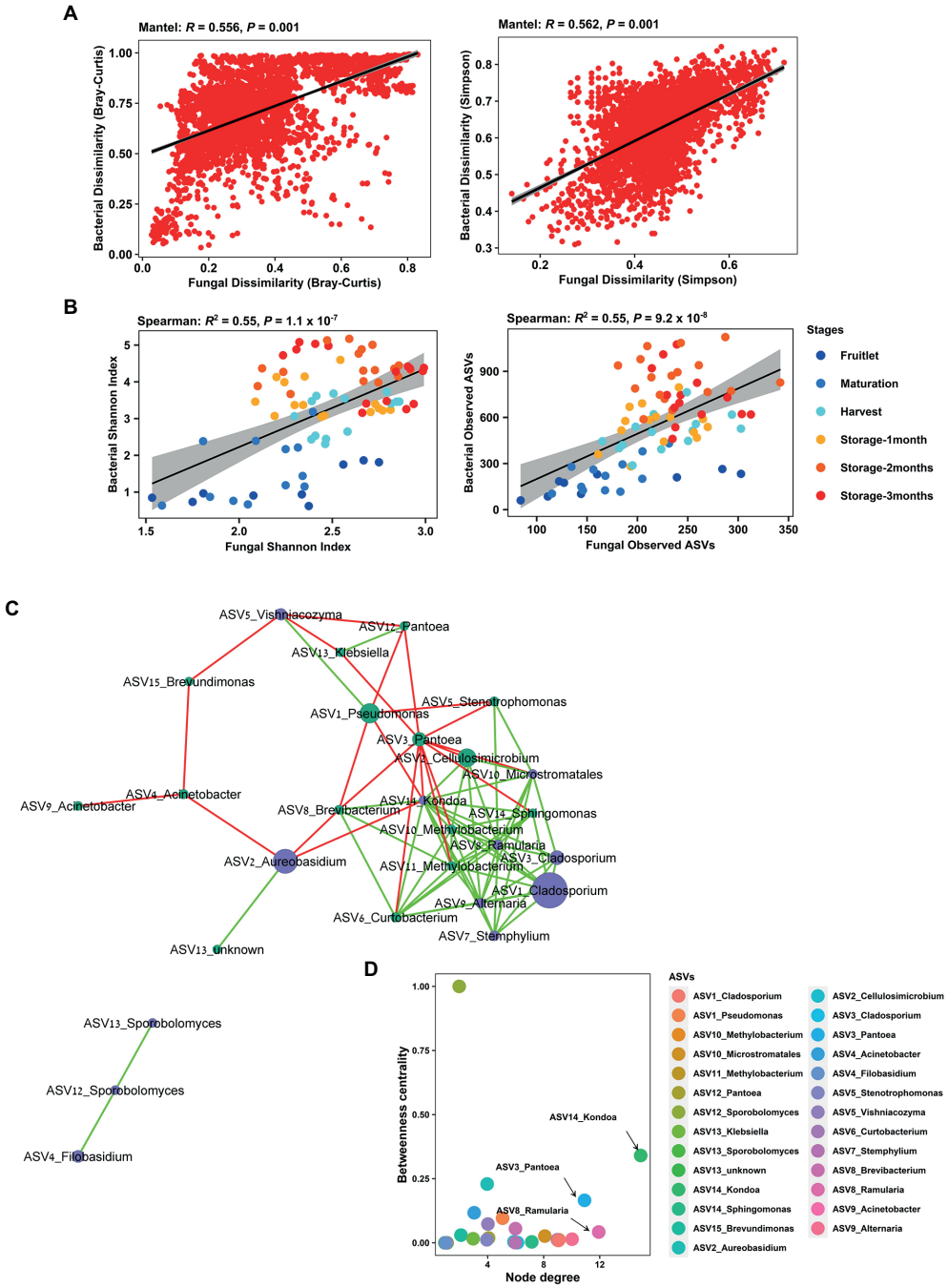

FIGURE 7
Cross-kingdom correlations between bacterial and fungal communities. **(A)** Mantel tests showing strong correlation of bacterial and fungal Bray–Curtis dissimilarity and Simpson pairwise dissimilarity comparisons across all the samples in the apple carposphere microbiome. **(B)** Significant correlation of bacterial and fungal community richness (Observed ASVs) and Shannon diversity indices across all the samples in the apple carposphere microbiome. **(C)** Co-occurrence network generated in CoNet and visualized in Cytoscape displayed significant strong positive and negative co-occurring relationships between dominant fungal and bacterial ASVs. Node colors represent ASVs from bacterial (green) and fungal kingdoms (blue). Green edges represent copresence (positive correlation) and red edges represent coexclusion (negative correlation) between relative abundance profiles indicated by the size of the nodes. Significance was estimated from multiple metrics including Spearman correlation, Pearson correlation, Bray–Curtis dissimilarity and Kullback–Leiber divergence. **(D)** Keystone taxa analysis-Betweenness centrality vs. node degree of all ASVs in the cross-domain network of the apple carposphere microbiome. Nodes with high degree represent putative hub taxa in the network and nodes with high betweenness centrality represent potential key connector species. Both these measures are indicators for potential keystone species. The taxa *Kondoa*, *Ramularia*, and *Pantoea* may act as potential keystone species in the apple carposphere microbiome.

Discussion

Epiphytic and endophytic microbiota are integral components of the carposphere in horticultural crops and may play a contributing role in determining produce quality and shelf life. The drivers involved in the assemblage of fruit-associated microbial community, however, are just beginning to be investigated. Our principal objective in the present study was to elucidate the patterns of apple epiphytic carposphere microbiome assembly and dynamics during fruit development in the orchard and in storage utilizing three commercial apple cultivars. Our findings revealed that the structure and assembly of the apple carposphere microbiome is strongly influenced by the stage of fruit development and that the fruit genotype also exerts a significant influence (Figure 2). A significant effect of host genotype was previously reported for apple fruit endophytic microbiota (Liu et al., 2018). This may be attributed to the fact that endophytic microbes are sheltered from external factors and environmental fluctuations, and because the plant host is able to exert more control over their colonization and dynamics, in a genotype-specific manner. Our finding that the host genotype can also influence, albeit to a lesser extent, the fruit epiphytic community in this study is remarkable, considering that epiphytes are exposed to numerous external factors. The genotype effect observed on the fruit epiphytic community could possibly be attributed to differences in the degrees of host facilitative priority effects. Indeed, evidences of host genotype modulated facilitative priority effects on microbial assemblies has been reported in plant–microbe interactions (Halliday et al., 2020; Leopold and Busby, 2020).

Despite seeing strong microbial community succession across fruit stages, we observed that a fraction of the total observed ASVs comprised a core successional microbiome (15 out of 17,655 bacterial ASVs and 35 out of 3,224 fungal ASVs) and dominated in their abundance relative to other taxa (non-core members) and persisted throughout the stages of fruit development and storage (Figures 3, 4). Indeed, most of the core bacterial and genera identified by this approach like *Pseudomonas*, *Pantoea*, *Methylobacterium*, *Cladosporium*, *Aureobasidium*, *Filobasidium*, *Vishniacozyma*, and *Alternaria* among others were also frequently isolated from the fruit washings using traditional culture methods (data not shown). We suggest that this core successional microbiome could be prioritized in future studies, due to the fact that they were shared in all three cultivars and persisted throughout the different stages of fruit development and after harvest when the apples were placed in storage. Notably, these taxa included all the core groups identified in our previous global (geographically) study of the apple microbiome (Abdelfattah et al., 2021). The core taxa belonging to *Pseudomonas*, *Pantoea*, *Vishniacozyma*, *Filobasidium,* and *Aureobasidium* were differentially abundant during early fruit stages (fruitlet and maturation). These taxa contain species with demonstrated biocontrol activity against fungal pathogens that infect apples (Filonow et al., 1996; Ippolito et al., 2000; Nunes et al., 2001; Lutz et al., 2013; Wallace et al., 2017), while other core taxa, such as *Cladosporium*, *Ramularia*, *Stemphylium,* and *Alternaria* were differentially abundant during sampled storage time points and include potential postharvest pathogens of apple fruit (Videira et al., 2015; Lutz et al., 2017). Core microbiomes across different plant species, such as *Arabidopsis thaliana,* rice, sugarcane, barley, soybean, and fruit crops, such as grapes, apple, and citrus, share common members, including *Pseudomonas*, *Methylobacterium*, *Sphingomonas,* and *Cladsporium*. The fact that a set of core taxa persist in the apple carposphere starting from early fruit development through harvest and during cold storage strongly suggest that these taxa of are well-adapted to the fruit peel of apples, regardless of environmental conditions, cultural management practices, and genotype.

Microbial assembly in plant-associated microbiomes is an ecological process that involves complex interactions among diverse groups of microbes, their host plants, and the environment. Successional patterns have been reported in a few host plants, including sorghum (Gao et al., 2019, 2020), grasses and forbs (Hannula et al., 2019), and in *Baccharis linearis* (Gazitúa et al., 2021). Those studies revealed varying contributions of turnover and nestedness in shaping the dynamics of the successional assemblages. Our study on the apple carposphere indicated that the successional events involve both turnover and nestedness processes across the different stages of fruit development and storage (Figure 5A). The contribution of turnover in the community dynamics, however, far outweighed the contribution of nestedness in both bacterial and fungal communities (Figure 5B). Our results also revealed that microbial community turnover was maximum during the fruit developmental stages with little to no turnover occurring during storage (Figure 5C). The strong turnover driving community succession in the apple carposphere may perhaps be attributed to the significant niche differences present in the orchard during fruit development and the stable and regulated conditions that occur in storage. As previously stated, available niches on the apple fruit surface are subject to constant fluctuations as fruit develops and matures, with niches expanding as the surface area in growing fruit increases, which presumably continues until harvest. During these stages, the expanding carposphere is anticipated to facilitate continuous microbial immigration (hence more richness and turnover) and allow for coexistence of taxa that can adapt to the same niche environment (HilleRisLambers et al., 2012). After harvest and during storage, the overall dispersion of the communities responsible for variances in the composition over time do not exhibit any clear and predictable patterns. Developing, ripening, and senescing apple fruit undergo a series of biochemical events, including soluble sugar accumulation, change in pH, a decline in host defense response, ethylene production, increased respiration, flesh softening, etc. (Toivonen and Brummell, 2008), and all these events potentially influence the dynamics of the fruit microbiome. Sustained low temperatures during storage can also exert a profound effect on fruit microbiome dynamics, with previous

studies reporting a general decrease in diversity and fluctuations in the relative abundance of microbial taxa in stored apples (Bösch et al., 2021). As such, incorporating information of these fruit physiological parameters and their influence on the fruit microbiome will provide a more comprehensive picture of the factors shaping the ecology and dynamics of fruit-associated microbiome, and we acknowledge the lack of this information as a limitation in the present study.

The existence of an underlying universal ecological dynamic in fruit microbiomes was found (Figure 6), similar to those reported for microbiomes in humans and some agricultural-associated microbial ecosystems (Bashan et al., 2016; Van Geel et al., 2017; Verbruggen et al., 2018). Our result is supported by further analysis of fruit microbiome datasets from our previous study characterizing the apple fruit microbiomes from multiple geographical locations (Supplementary Figure 9). Notably, the level of universality was higher in fungal communities than in bacterial communities in all the datasets examined. A more stable pattern of community dynamics in fungi relative to bacteria is also supported by our result showing more stable abundance distribution patterns in core successional microbes (Figure 4A). Importantly, in regards to the practical use of biocontrol technologies is that the existence of universal ecological dynamics suggests the carposphere may respond consistently across systems (cultivars, geographic locations) to microbiome manipulations (e.g., management practices, biocontrol applications, etc.).

We observed a strong correlation between bacterial and fungal microbiomes (a cross-domain association) as indicated by their significant correlation in their community composition and diversity (Figures 7A,B). In some cases, bacteria and fungi may co-occur in syntrophic guilds where, besides other interactions, the members interact mutually by producing metabolites for others to use (Hoffmann et al., 2013). We also generated a co-occurrence network from across all samples to investigate microbe-microbe interaction dynamics and revealed several individual bacterial and fungal taxa, including putative plant pathogens as well as biocontrol agents, with significant positive or negative co-occurrences among them (Figure 7C). This network approach may be insufficient for interpreting species interactions and does not indicate causal relationships (Röttjers and Faust, 2018). Identifying these correlations and hub microorganisms through co-occurrence networks, however, provides a useful starting point for experimental studies aimed at testing relationships and constructing synthetic communities to manipulate fruit–microbiomes.

In summary, we demonstrated that fruit developmental stage and the length of storage significantly shape assemblages of microbial communities on fruit surface, while fruit genotype also plays a role in the overall assembly and dynamics. We observed strong succession in microbial communities of the apple carposphere that is strongly driven by turnover events during the different fruit developmental stages. The underlying ecological dynamics of the apple carposphere largely follow a universal model. We found that a set of core taxa persist throughout the stages of fruit development and after harvest and identified specific taxa, including known biocontrol agents of plant pathogens, that were differentially abundant during the different stages of fruit development. Further studies should investigate core microbial members for direct functional interactions with fruit pathogens and their interactions with the resident microbiome.

Materials and methods

Experimental set-up and sampling

In order to minimize variations due to effects of environmental conditions, we selected three apple cultivars—"Royal Gala," "Granny Smith" and "Golden Delicious," that were planted at the Matityahu Experimental Farm, Agricultural Research Organization (ARO), Northern Israel (3380400400 N, 3582700400 E, altitude 667 m). The three cultivars were planted in the same plot of the orchard where several other apple cultivars in separate rows were also planted with 3-m row gaps and each row consisted of 35–40 individual trees. Three rows, one each of the three cultivars were tagged for use in this study. The cultivars "Granny Smith" and "Golden Delicious" have different genetic lineages arising from chance seedlings while "Royal Gala" is a product of traditional breeding between "Kidds Orange Red" and "Golden Delicious." All trees of the three cultivars were the same age (5 years) and under the same maintenance program. The apple trees received fungicidal and insecticidal sprays against apple scab, powdery mildew, codling moth, fruit fly and mite during the previous seasons (Supplementary Table 1) but their application was avoided during the duration of this experiment (2019 season).

Sampling of fruit was done from all the selected trees during three fruit developmental stages *viz.* at fruitlet stage (50–60 days after anthesis; DAA), at maturation stage (110–120 DAA) and at harvest stage (150–170 DAA), followed by another three times at monthly intervals from the harvested fruit lot during cold-storage periods (1°C; Figure 1). Details of dates of sampling, number of replicates for each sampling time and average fruit sizes during different sampling times are summarized in Supplementary Table 2. These stages were selected to encompass the major physiological stages of fruit during development in the field and storage after harvest. The onset of anthesis of the three cultivars occurred around the same time (between March 20 and 30, 2019). The fruits from all the three cultivars were collected at the same date for fruitlet stage on 29th May 2019 (50–60 days after anthesis; DAA) and for maturation stage on 28th July 2019 (110–120 DAA). Due to differences in ripening times of the three cultivars, harvesting was done on different dates ("Royal Gala" on 27th August 2019, "Golden Delicious" on 3rd September 2019 and "Granny Smith" on 16th September 2019). Briefly, 3–5 fruits from each tree were picked manually using

sterile gloves during fruitlet and maturation stages and 20–30 fruits from each tree during harvest stage. At each picking time, fruits from all the trees for each cultivar were pooled, collected in sterile corrugated fiberboard boxes, marked and transported immediately to the laboratory for sampling. All the fruits picked at fruitlet and maturation stages were immediately used for sampling while enough fruits were picked at harvest stage to be sampled immediately and also after different storage times. For this, harvested fruits of the three cultivars were all stored together in the same cold-storage room (1°C, 85% relative humidity), in separate containers. Prior to sampling, these stored fruits were removed from storage and placed at room temperature for 5 h and allowed to warm. Weather information (temperature and humidity) during the course of the experiment (from March 2019 to October 2019) is shown in Supplementary Figure 1.

DNA extraction and sequencing of 16S rRNA and ITS genes

Epiphytic microorganisms were obtained by swabbing the entire fruit surface using sterile cotton tips moistened with phosphate buffered saline (PBS). Swabbing was done on 20 fruits (using a swab for each fruit and later pooled) to represent one sample replicate for all sampling stages and cultivars. A fixed number of 20 fruits each per replicate was used to obtain the samples from each cultivar and sampling points in order to account for variation arising from the expanding fruit surface area as the fruit developed. This way, using different set of 20 fruits for each sampling replicate, a total of 1,640 fruits were used to obtain a total of 82 samples with ≥3 replicates per sample depending on the availability of fruits. The cotton swabs were collected in 50 ml falcon tubes containing 20 ml of PBS, gently shaken (150 rpm, 10-min) in a rotary shaker, sonicated for 5-min in a water bath sonication followed by a 30-s vortex. After aseptically removing the cotton tips, the microbial suspension obtained was used to extract DNA by using the Promega DNA Purification kit according to the manufacturer's instructions. To check for contamination introduced during extraction, elution buffer and specimen-free DNA isolations were performed and used as negative controls.

Extracted DNA was used for amplicon PCR reactions to amplify the bacterial 16S rRNA region using the 515F/806R primer set, and the fungal internal transcribed spacer (ITS) region using the ITS3/KYO2 primer set along with peptide nucleic acid (PNA) clamps to block the PCR amplification of apple plastid and mitochondrial sequences (Caporaso et al., 2011; Toju et al., 2012). PCR conditions were performed as described in our previous studies (Abdelfattah et al., 2021). Library preparation following amplification was done as specified in the Illumina 16S Metagenomic Sequencing Library Preparation guide precisely as outlined. Sterile water and empty wells were sequenced as negative controls. Subsequent library size, quality, and confirmation of the absence of adapter dimers were done on an Agilent 2100 Bioanalyzer (Agilent). Paired-end sequencing of amplicons was conducted on an Illumina MiSeq (Illumina) sequencer with a V3 600-cycle Reagent Kit (Illumina).

Data processing and analysis

Demultiplexed forward and reverse reads of both 16S rRNA and 1TS gene sequences were trimmed, merged, denoised, and chimeras removed for quality control using default parameters in DADA2 (Callahan et al., 2016) as integrated in QIIME2 (Bolyen et al., 2019). The taxonomies of the high-resolution amplicon sequence variants (ASVs) obtained were performed using the GreenGenes and UNITE databases for 16S rRNA reads (DeSantis et al., 2006) and ITS reads (Abarenkov et al., 2010) respectively. Negative controls were used to identify the contaminant sequences and removed before any analyses were performed. A total 17,655 bacterial ASVs (11,540,568 reads) and 3,224 fungal ASVs (13,110,514 reads) were obtained. To account for differences in sequencing depth, the ASV feature tables were rarified to the depth of the smallest sample (31,899 for 16S rRNA and 84,687 for ITS) and all downstream analyses were performed on this rarefied ASV tables.

Rarefaction curves for both 16S rRNA and ITS reads were constructed and confirmed the minimum depths used were sufficient to reach saturations of diversity in both bacterial and fungal communities (Supplementary Figure 2). Bray–Curtis dissimilarities were calculated and subjected to principal coordinate analysis (PCoA) using the ordination function in the phyloseq package in R v3.5.1 to visualize the variations in microbial community compositions between groups. In addition, permutational analysis of variance (PERMANOVA) was carried out to assess the effect of fruit stage and genotype on the microbial community variation detected by Bray–Curtis dissimilarities using the adonis function in the vegan package. To test for homogeneity among microbial communities, β-dispersions using Bray–Curtis and Simpson dissimilarities were explored by the betadisper function in the vegan package in R.

The α-diversity indices (Observed ASVs and Shannon Index) were calculated using the estimate richness function in the phyloseq package. Statistically significant differences in diversity metrics were identified using the nonparametric Kruskal–Wallis for comparisons between all the groups, and pair wise comparisons between samples were made using the Wilcoxon's test.

To visualize the relative abundances of ASVs, bar plots and line graphs were constructed using the ggplot2 package. The core microbiome was calculated based on ASVs present in 95% of the investigated samples using core function in the microbiome package. Linear discriminant analysis (LDA) effect size (LEfSe; Segata et al., 2011) that utilizes a combination of Kruskal–Wallis test and pairwise Wilcoxon rank-sum test with linear discriminant analysis (LDA > 4), was used to determine a ASVs that best characterize each fruit developmental stage and storage period.

Bray–Curtis dissimilarities between sample pairs and Euclidean dissimilarities were used to construct distance matrices of temporal distances (fruit stages) and Mantel tests were performed to explore the correlations between these matrices. Sorensen pair wise dissimilarity (β_{SOR}) distances between sample pairs were measured based on presence/absence data (to remove the richness variation), and then partitioned into those contributed from turnover (Simpson pair wise dissimilarity; β_{SIM}) and nestedness-resultant dissimilarity (β_{NES}) using the betapart package, followed by Mantel tests to explore the correlations between these matrices with the temporal distance (fruit stages).

To assess the universality in ecological dynamics across fruit microbial communities, we used the dissimilarity overlap curve (DOC) approach using the DOC package. Briefly, the DOC was constructed by plotting, for each possible sample pair of microbial communities, the dissimilarity on the *y*-axis (root Jensen–Shannon divergence, calculated from only the ASVs shared by the two communities) against the fraction of ASVs that overlap on the *x*-axis. Universality is supported where the DOC dips as the overlap grows, and this level of support is proportional to the fraction of pairwise comparisons where the DOC slope is negative (termed the fraction of negative slope, f_{NS}). For the smoothed DOC curve, the initiation of negative slope signifies the median of initiation of negative slopes obtained from DOCs of 1,000 bootstrapped data sets.

To explore microbe-microbe correlations, we selected ASVs with relative abundances >1% identified across all the samples, merged the bacterial and fungal ASVs and constructed a co-occurrence network using the CoNet (Faust and Raes, 2016) application (v.1.1.1.beta) implemented in Cytoscape (v.3.7.2; Shannon et al., 2003). Briefly, Pearson's correlations, Spearman's correlations, Bray–Curtis dissimilarity and Kullback–Leibler divergence, were performed and used to create an initial network. The edgeScores randomization function was then employed to perform 100 row-wise permutations with 1,000 highest and lowest scoring edges retained. The reboot renormalization function was then used to check for compositional bias to construct a merged final network based on a score distribution of 100 bootstrap iterations. Significance of the correlations was calculated with Brown's method and Benjamini–Hochberg FDR method was used for correcting multiple comparisons. The network was finally visualized using an organic layout in Cytoscape (v3.7.2).

Data availability statement

The sequencing data generated from 16S rRNA and ITS genes in this study has been deposited to the NCBI SRA database under the accession number Bioproject ID: PRJNA770498.

Author contributions

VZ, AK, AB, and SD designed the study. VZ, AK, AB, AA, VS, SS, OF, RB, SF, SW, MW, and SD contributed in conducting the experiment. VZ, AK, and AB performed the analyses. VZ and SD wrote the first draft. All authors contributed to the article and approved the submitted version.

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Supplementary material

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Deciphering the microbial diversity associated with healthy and wilted *Paeonia suffruticosa* rhizosphere soil

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Plant health is closely related to the soil, where microorganisms play a critical and unique role. For instance, *Paeonia suffruticosa* is an emerging woody oil crop in China with attractive development and utilization prospects. However, black root rot causes wilting of the aboveground plant parts, which significantly affected its seed yield and quality. Studies found that soil microorganisms are critical in maintaining plant health, but how changes in the soil microbial communities affect the healthy and diseased oil peony is unclear. Therefore, our present study used high throughput sequencing and BIOLOG to analyze the rhizosphere soil microbial communities of healthy and diseased oil peonies. Our results revealed that the physical and chemical properties of the soil of the diseased plants had changed, with the ability to metabolize the carbon source being enhanced. Moreover, our research highlighted that the oil peony-infecting fungal pathogenic genus (*Fusarium*, *Cylindrocarpon*, and *Neocosmospora*) was closely associated with oil peony yield reduction and disease aggravation. Further network analysis demonstrated that the bacterial and fungal networks of the diseased plants were more complex than those of the healthy plants. Finally, the inter-kingdom network among the diseased plants further indicated that the lesions destroyed the network and increased the intraspecific correlation between the fungal groups.

KEYWORDS

***Paeonia suffruticosa*, root rot, microbial network, plant pathogen, rhizosphere microbial community**

Introduction

Oil peony, a unique new woody oil crop in China, is among the world's top woody oil tree species, which includes olive, *Camellia oleifera*, and oil palm. The peony seed oil contains up to 92.26% unsaturated fatty acids, with over 42% α-linolenic acid, also known as "blood nutrients" (Cheffi et al., 2019). The *Fusarium* species complex often causes root rot, a classical soil-borne disease that attacks many economically important crops, including potato and camellia (Kendrick, 2003; Dugan et al., 2010; Hernandez et al., 2021). Root rot in oil peony is caused by *Fusarium solani*. At the disease onset, the pathogen enters the plant through the roots, then expands to the medulla, followed by the scattered fleshy roots, and even infects the entire root, thereby finally causing severe production losses annually.

Various soil biochemical factors affect the growth and survival of soil pathogens and the plant's nutrient availability. Among them, soil microorganisms are crucial factors for plants and pathogens, since they are critical in regulating soil fertility, nutrient cycling, promoting plant health, and protecting plants from diseases (Lauber et al., 2008; Faoro et al., 2010; Rousk et al., 2010). Microbiota associated with the host plant, especially their roots, determines the soil-borne pathogen's infection capability. Thus, assembling a self-serving rhizosphere microbiota is vital for both plants and pathogens. Plants recruit beneficial microbes to stimulate plant growth, elicit plant systemic defense, and attack pathogens. When pathogens invade plants and cause diseases, the original rhizosphere microecological balance is disturbed, subsequently changing the soil properties, microbial community structure, and metabolic functions (Li et al., 2010; Jaffuel et al., 2016; Mueller et al., 2016). Microbial community composition is primarily influenced by the cooperative and competitive interactions among the numerous microbial members that help maintain plant health (van der Heijden et al., 2016; Duran et al., 2018). Currently, the co-occurrence networks are widely used in inferring potential microbial interactions (De et al., 2018; Hernandez et al., 2021). The uniformity and complexity of the networks have been shown to be critical for stable host–microbial interactions (Wang et al., 2021).

Such an analysis is vital, as a balanced microbiome is crucial for the health of humans, plants, and the environment, while diseases are often associated with microbial dysbiosis (Hooks et al., 2017). Compared to healthy individuals, communities with microbial imbalances have more significant differences in their composition. Additionally, high microbial diversity and stable community composition have significant effects on the prevention of pathogen invasion and establishment (Ning et al., 2011), counteract the pathogen growth (Yuan et al., 2012), competition with pathogens for nutrients (Weller et al., 2002), direct promotion of plant growth (Langfelder and Horvath, 2007), and modulation of host immunity. Therefore, understanding the soil microbial composition of the rhizosphere is the key to understanding the spread of soil-borne diseases. Nevertheless, our current understanding of the potential interactions within the complex plant-associated microbiomes and their response to pathogen invasion still remains limited.

Therefore, this study evaluated the characteristics and differences between the diseased and healthy plants, including the soil's physicochemical properties and the microbial community. In this study, we hypothesized: (1) soil properties are correlated with the soil microbial community and root rot, and (2) soil microbial community is altered in the root rot infected soils. For this purpose, we employed the BIOLOG and Illumina Miseq high-throughput sequencing technologies to compare the rhizosphere soil microbial communities of the healthy and diseased oil peony plants in different regions, thus providing theoretical support for the biological control of the oil peony root rot disease.

Materials and methods

Sample selection and physiochemical soil properties

All samples were collected from the main oil peony plant bases in Shanxi Province, China, at Wuxiang (112°51′56″ E, 36°50′40″ N), Xiangyuan (11258′15″ E, 36°27′42″ N), and Huguan (113°11′56″ E, 36°7′24″ N) (**Supplementary Figure 1**). The three oil peony planting bases were empty farmland before the planting of oil peony began in the last five years. Fertilization, weeding, and other daily management measures remained the same. The oil peony cultivar used was *P. suffruticosa*, and mature peonies were sampled at all sites. At each site, healthy plants not only referred to the above-ground plant growth being good and consistent without leaf wilting but also having an underground fleshy root surface without black spots. Accordingly, the diseased plants referred to the noticeable wilting and death of the aboveground parts, i.e., yellowing and wilting of the leaves, along with the blackening of the underground fleshy roots. Three replicates of each of the healthy and diseased plants were collected from three different plots (20 m × 20 m) at each site. From each plot, soil samples were randomly collected using the five-point sampling method. Soils of the same type collected from the same plot were thoroughly mixed into one sample. Rhizosphere soil (defined as the soil that adheres to the root) was collected from the roots through manual shaking. Soil samples were put into labeled self-sealing bags and transported to the laboratory to determine their physical and chemical properties. Each soil sample was ground, sieved through a 2-mm sieve, and divided into three portions for further processing, i.e., stored in a 4°C refrigerator for BIOLOG, air-dried for chemical property analysis, and stored at −80°C for DNA extraction. Samples were marked with letters and numbers as follows: H1: healthy plants in Wuxiang; D1: diseased plants

in Wuxiang; H2: healthy plants in Xiangyuan; D2: diseased plants in Xiangyuan; H3: healthy plants in Huguan; D3: diseased plants in Huguan.

Physical and chemical properties of soil

The soil's physicochemical parameters were measured according to the oven dry-weight method, which helped estimate the soil moisture content. Then soil pH was determined using a glass electrode meter (Sartorius PB-10) in a suspension of 1 g of soil in 5mL of distilled water. Available P (AP) was extracted using sodium bicarbonate and subsequently measured by the molybdenum blue method. The available K (AK) was determined by flame photometry (Wu et al., 2016; Xia et al., 2016), while the available N (AN) was determined using potassium persulfate oxidation. The organic matter (OM) content was determined as described previously (Inclán et al., 2015; Restrepo et al., 2015).

DNA extraction, polymerase chain reaction amplification, sequencing analysis

Total genomic DNA was extracted directly from these samples using the FastDNA® spin kit according to the manufacturer's protocol (MP Bio, Santa Ana, United States). DNA concentrations were then determined using a NanoDrop ND-2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, United States). The DNA's integrity was assessed *via* 1% agarose gel electrophoresis. The bacterial 16S rRNA gene's V3-V4 hypervariable regions were amplified using the 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) primers. To amplify the fungal ITS sequences, the primers ITS1-F (5′-GGAAGTAAAAGT CGTAACAAGG-3′) and ITS1-R (5′-GCTGCGTTCTTCATCGATGC-3′) were used. Polymerase chain reaction (PCR) reaction system was as follows: Phusion Master Mix (2×) 15 μl; Primer (2 μm) 3 μL; 10 μl template DNA; H_2O 2 μL, total 30 μl. Reaction procedure: pre-denaturation at 98 °C for 1 min; The 30 cycles included (98 °C, 10 s; 50 °C, 30 s; 72 °C, 30 s); It was then extended for 5 min at 72 °C. 16S rRNA and ITS rRNA tag-encoded high-throughput sequencing were carried out on the Illumina MiSeq platform (Novogene, Beijing, China). Pairs of reads from the original DNA fragments were merged based on the previously described method, with sequencing reads being assigned to each sample according to the unique barcode. Sequences were analyzed through the QIIME software package (Quantitative Insights into Microbial Ecology) and the UPARSE pipeline. The reads were first filtered using the QIIME quality filters while using the default settings for Illumina processing in QIIME. Then the UPARSE pipeline was utilized to detect the operational taxonomic units (OTUs) at 97% similarity. For each OTU, a representative sequence was selected and used to assign taxonomic composition employing the RDP classifier.

Microbial metabolic function

The functional diversity of the soil microbial communities was estimated using the BIOLOG method. EcoPlates were used for the BIOLOG assay to determine the microbial carbon source utilization profile. One gram of fresh rhizosphere soil from around diseased or healthy plants was mixed with 99 ml of 0.85% sterile NaCl solution, then shaken for 30 min on a reciprocal shaker. Then, 150 μL of the solution was added into each well of the EcoPlate with 31 carbon sources and incubated in darkness at 25°C. Plates were read every 24 h at 590 nm with a total time of up to 168 h. Average chromogenic development (AWCD) was used to evaluate the carbon source utilization capacity of microbial communities in the rhizosphere soil samples of healthy and diseased plants. The utilization of 31 carbon sources by the microbes in each sample was measured as described previously, and a principal coordinate analysis (PCoA) was used to detect the time-course of substrate utilization in healthy and diseased rhizosphere samples.

Statistical analyses

The original data obtained by sequencing were spliced and filtered to obtain valuable clustered data. The operational taxonomic unit (OTU) was clustered under 97% similarity to calculate the number, annotate species, and obtain taxonomic information. The Alpha diversity indices (Chao1 index, Shannon index, and Simpson index) and the Goods-coverage index were calculated using Qiime software, while the taxonomic differences between the groups were compared utilizing the least-significant-difference (LSD) test with *t*-test adjustment. The statistical significance was $p = 0.05$ and heat maps were generated using custom R (version 3.2.5). Redundancy analysis (RDA) using the vegan package R (version 3.2.5) analyzed the relationships between microbial community structure, microbial species, and environmental variables.

Network analysis

A network of OTU levels between the fungi and bacteria was constructed using rhizosphere microorganisms from both healthy and diseased plants to evaluate the complexity of the interactions between microbial taxa. Using the Spearman correlation analysis (SparCC), a tool for estimating correlation values from component data, the SparCC correlation with statistical significance ($P > 0.05$) was included in the network

analysis. Nodes in the reconstructed network represent gate-level classification units, while edges represent significant positive or negative correlations between the nodes. The corresponding network graph relies on node number, edge number, modularity, community number, average node connectivity, average path length, diameter, and cumulative degree distribution. The data co-occurrence analysis used the psych package in R 3.5.2, while visualization and attribute measurement employed the Gephi computing network. The hub taxa per network were identified as within and among module connectivity, while the network stability was measured by the proportion of negative or positive correlations and modularity.

Results

Physical and chemical properties of soil

The physical and chemical properties of the soil are presented in **Table 1**. The ANOVA analysis revealed a difference between the healthy and diseased soils, where the soil's AK, AP, and OM decreased significantly in the diseased soils as compared to the healthy soils. AN also showed a decreasing trend but with no significant difference. Compared with the healthy soil, pH and water content did not show any trend changes. Finally, the correlation analysis highlighted that AK was significantly positively correlated with OM (0.82) and AP (0.66) (**Supplementary Figure 2**).

Soil microbial diversity

We assigned all raw sequence data to each sample based on their barcode sequence. We obtained 1184901 bacterial 16S rRNA and 1270956 fungal ITS high-quality reads from 18 samples, sorted into 3904 and 2872 bacterial fungal OTUs. The rarefaction curve indicated that most microorganisms were detected in the samples. We used the Shannon, Simpson, and Chao 1 indices to evaluate and compare the diversity and richness of bacterial communities among different soil samples (**Figure 1**). The results revealed no significant difference between the healthy and diseased soil. However, the differences in the soil's microbial diversity at different sampling points were evident, with the overall effect of the diseased soil on microbial diversity and richness being non-significant.

Soil microbial community composition and structure analysis

When grouping the OTUs at the phylum level of bacteria, we found that the diseased plants had more bacterial phyla, whereas healthy plants had a greater number of fungal phyla, with no significant differences between the diseased and healthy soils (**Supplementary Figure 3**). Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Chloroflexi, and Firmicutes were the dominant phyla existing in all the samples, with Proteobacteria being the most dominant phylum accounting for 45.36% (H1), 43.12% (H2), and 28.49% (H3) in the healthy soils and 40.73% (D1), 44.13% (D2), and 51.38% (D3) in the diseased soils. Similarly, we observed no significant difference among the fungi at the phylum level between the healthy and diseased soils. For the fungi, Ascomycetes, Balloonomycetes, Basidiomycetes, and Mortierella, were the dominant phyla across all samples, with Ascomycota being the most dominant phylum accounting for 24.80% (H1), 25.82% (H2), and 41.45% (H3) in healthy soils and 38.49% (D1), 32.34% (D2), and 46.31% (D3) in diseased soils.

We further explored the microbial community at the genus level and found differences in the microbial community structure in the healthy and diseased soils from different regions through the *t*-test (**Figure 2**). The abundances of thirty bacterial genera and six fungal genera were significantly different between the healthy and diseased soils ($p < 0.05$), as indicated by the *t*-test. Twenty-six bacterial genera were more abundant in the diseased soils, with bacterial genera like *Marmoricola, Polycyclovorans, Pseudoduganella,* and *Geodermatophilus* being more abundant in the healthy soils. Moreover, fungal genera like *Chaetomium, Geomyces,* and *Stephanonectria* were abundant in the healthy soil, while *Fusarium, Cylindrocarpon, Neocosmospora* were abundant in the diseased soil. Among them, *Fusarium* showed significant differences between diseased and healthy plant soils ($p < 0.01$).

Relationship between microbial community and environmental variables

Redundancy analysis showed that the microbial community structure was affected by the plants' health and soil properties (**Figure 3**). The microbial community structure in H1, H2, and H3 was correlated with AK, AP, and OM, while the microbial community structure in D1, D2, and D3 was correlated with pH. The redundancy analysis highlighted that the first and second components explained 35.74% and 26.85% of the total bacterial and total fungal variations, respectively. In the bacterial communities, the soil OM, AP, and AK were positively correlated with *Pseudarthrobacter*, *Marmoricola,* and *Pseudomonas* and were negatively correlated with *Dongia, Sphingomonas Acidobacteria*, *Gaiella*, *Nocardioides*, *Lysobacter,* and *Nocardioides.* With respect to the fungal communities, the soil OM, AP, and AK were positively correlated with *Trichocladium*, *Mortierella*, *Aspergillus, Solicoccozyma,* and *Preussia,* while being negatively correlated

TABLE 1 Physicochemical parameters of the soil samples.

Samples	Available N/ mg·kg^{-1}	Available P/ mg·kg^{-1}	Available K/ mg·kg^{-1}	Organic matter/ g·kg^{-1}	pH	Water content/ %
H1	33.95 ± 1.98a	17.84 ± 2.41b	392.59 ± 11.75a	21.80 ± 0.90a	7.74 ± 0.02bc	13.34 ± 0.28b
D1	31.07 ± 1.44ab	11.01 ± 0.42c	335.61 ± 8.12b	19.13 ± 0.58b	7.68 ± 0.03c	14.05 ± 0.13a
H2	27.6 ± 0.91b	26.00 ± 1.22a	335.6 ± 23.46b	18.98 ± 0.77b	7.83 ± 0.05ab	12.47 ± 0.12c
D2	31.01 ± 1.54ab	11.04 ± 0.75c	243.87 ± 9.43c	15.68 ± 0.47c	7.9 ± 0.02a	11.81 ± 0.13d
H3	28.95 ± 1.36b	23.74 ± 0.93a	372.24 ± 7.29ab	16.63 ± 0.76c	7.8 ± 0.02b	10.85 ± 0.07f
D3	29.34 ± 1.44b	9.33 ± 0.45c	224.48 ± 21.25c	13.34 ± 0.65d	7.9 ± 0.05a	11.37 ± 0.15e

All data are presented as the mean ± SE. Different lowercase letters in the same column indicate significant ($p < 0.05$) differences between the healthy and diseased soils. H1, healthy plants in Wuxiang; D1, diseased plants in Wuxiang; H2, healthy plants in Xiangyuan; D2, diseased plants in Xiangyuan; H3, healthy plants in Huguan; D3, diseased plants in Huguan.

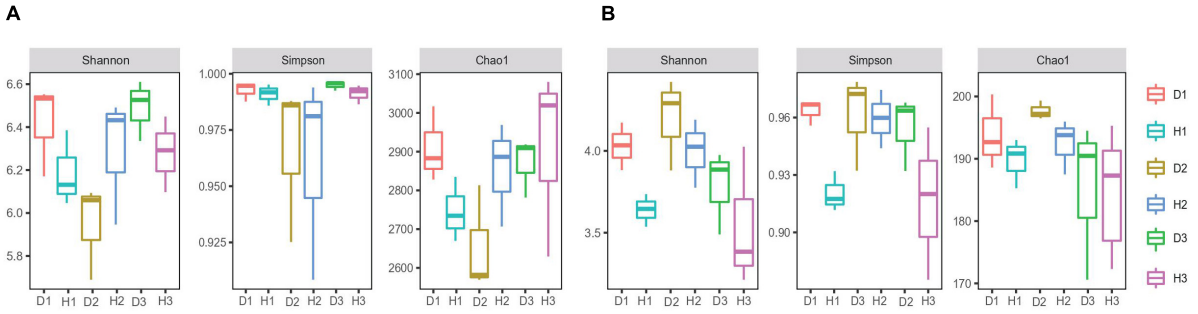

FIGURE 1
Microbial alpha diversity. The α-diversity indices of soil bacterial **(A)** and fungal **(B)** communities in the soils of healthy and diseased oil peony, Chao1 index for calculating bacterial abundance, Shannon index, and Simpson index for calculating bacterial diversity, respectively.

with *Fusarium*, *Gibberella*, *Dactylonectria*, *Neonectria*, and *Neocosmospora*. Thus, the soil properties, especially the available nutrients, highly influence the community structure of soil microbes.

Microbial metabolic function

We also analyzed the average absorbance of soil microorganisms and found that the carbon source utilization rate in the samples increased with time, with the carbon source targeted metabolic ability of diseased plants being higher than that of the healthy plants. After principal component analysis (PCA) of the AWCD value of the soil samples, the microbial flora of healthy and diseased soils showed a separated state of carbon substrate utilization, thus indicating the differences in microbial communities between the healthy and diseased soil samples. Upon comparing the utilization rates of six different functional groups (carbohydrates, amino acids, carboxylic acids, multipolymer, amines/amides, and phenolic compounds) by microorganisms (Figure 4A), we found that the utilization rate of carbohydrates by microbial communities in diseased soil was significantly higher than those in healthy soil. Additionally, although the amino acid utilization rate in soil microbial communities increased, there was no significant difference in the utilization rate of the other carbon sources. Furthermore, the thermal diagram (Figure 4B) indicates that in all samples, the substrate D-Xylose, i-Erythritol, L-Arginine, L-Asparagine, L-Phenylalanine, L-Serine, Tween 40, Tween 80, Phenylethyl-amine, and Putrescine were metabolized rapidly in diseased soil. The substrates, including D-Cellobiose, Glucose-1-Phosphate, D-Glucosaminic, D-Galacturonic, D-Malic Acid, and α−Cyclodextrin presented high utilization rates in healthy soils. The significant differences in the carbon substrate utilization patterns between the healthy and diseased soil communities involved L-Asparagine and phenylethyl-amine, with high and low utilization in the diseased and healthy samples, respectively.

Co-occurrence network analysis

To investigate how the disease affected the community structure of the peony microbiome, we performed a co-occurrence network analysis to explore the connection complexity within the rhizosphere microbiomes of both healthy and diseased soils (Figure 5). We also analyzed the bacteria–bacteria (BB), fungi–fungi (FF) intra-kingdom networks, along with the bacteria–fungi (BF) inter-kingdom networks. Based on the intra-kingdom network analysis (Figure 5A), we observed

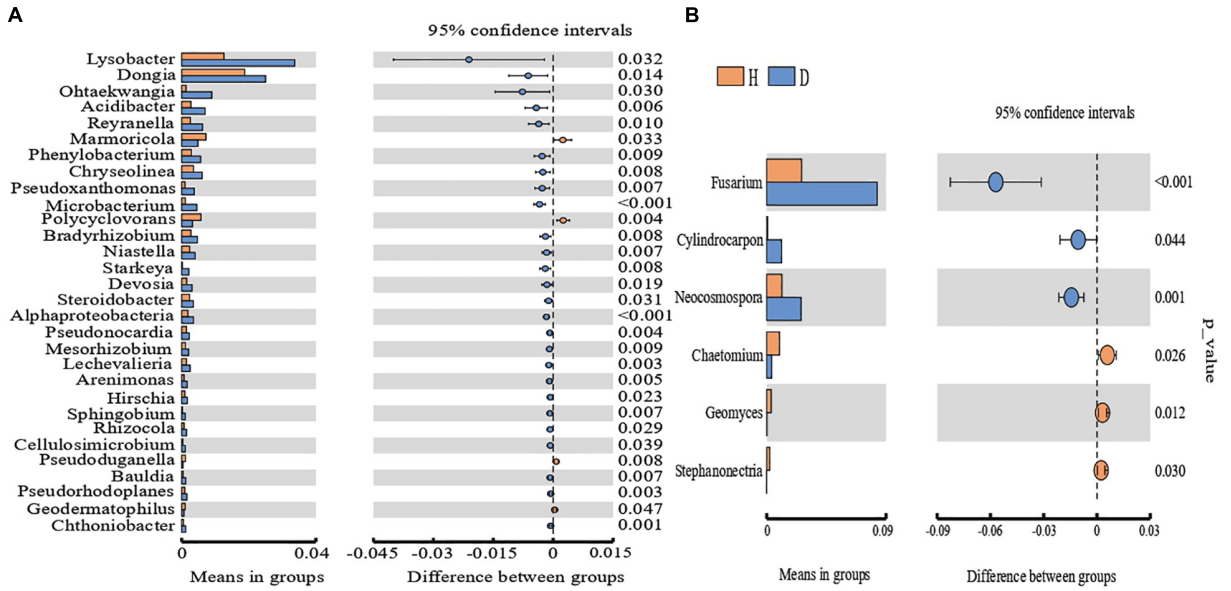

FIGURE 2
The difference in the relative abundance of bacteria **(A)** and fungi **(B)** between healthy and diseased plants was calculated, and the *t*-test was used to calculate the *P*-value ($P < 0.05$). H and D refer to healthy and diseased plant soil.

a higher proportion of negative edges and modularity in the fungal networks (proportion of negative edges/modularity: 27.2%/0.49 and 31.4%/0.41 in the healthy and diseased soils, respectively) than in the bacterial networks (proportion of negative edges/ modularity: 0.67%/0.486 and 16.6%/0.495 in healthy and diseased soils, respectively). Furthermore, based on the number of nodes and edges, the network in diseased soil was more complex than in the healthy soil. The inter-kingdom co-occurrence networks further indicated that diseases destabilized the network and increased the intra-kingdom correlations among the fungal taxa (**Figure 5C**). The proportion of negative edges and modularity were higher in the diseased networks (proportion of negative edges/modularity: 38.2%/0.431) than in the healthy networks (proportion of negative edges/modularity: 21.1%/0.524) (**Figure 5B**). The BF inter-kingdom correlations were primarily negative (58.0% and 57.3% in the healthy and diseased networks, respectively), while negative correlations dominated the intra-kingdom correlations (93.0% BB and 72.8% FF in the healthy plants network, and 60.0% BB and 68.6% FF in the diseased network) (**Figure 5D**). The top-10 hub taxa were six bacteria and four fungi, and they were the same in both the healthy and diseased networks.

The topological roles of the nodes were defined by the within-module connectivity (Zi) and among-module connectivity (Pi). In all networks (**Supplementary Figure 4**), we found that 4.6% of the nodes were connectors (37 nodes), while 0.3% were module hubs (three nodes). Although we found no network hub in the four networks, the diseased network had more hubs (12 connectors in the bacterial network and nine connectors and one module hub in the fungal network) than the healthy network (eight connectors in the bacterial network and eight connectors and two module hubs in the fungal network). For the bacterial communities, the eight connectors in the healthy network belonged to *Lechevalieria, Stenotrophomonas, Rhodoplanes*, and *Nocardioides,* respectively. However, the 12 connectors in the healthy network belonged to *Reyranella, Sphingomonas,* and *Bradyrhizobium,* respectively. Regarding the fungal communities, *Solicoccozyma* and *Acremonium* were present in the diseased networks, while *Chaetomium* was in both the healthy and diseased networks.

Discussion

Effects of plant diseases on physical and chemical factors of soils

Microbes affect the growth of crops by affecting the soil's physical and chemical properties, and consequently the vital transportation of nutrients needed for their cultivation (Jiang et al., 2017). The reduction in AK, AP, and OM decreased the soil quality and weakened plant resistance to pathogens (Janvier et al., 2007).

We analyzed the physicochemical properties of the rhizosphere soil of diseased and healthy plants in three areas. Compared to the healthy soils, the microorganisms in the root rot soil had higher carbon utilization ability, thereby

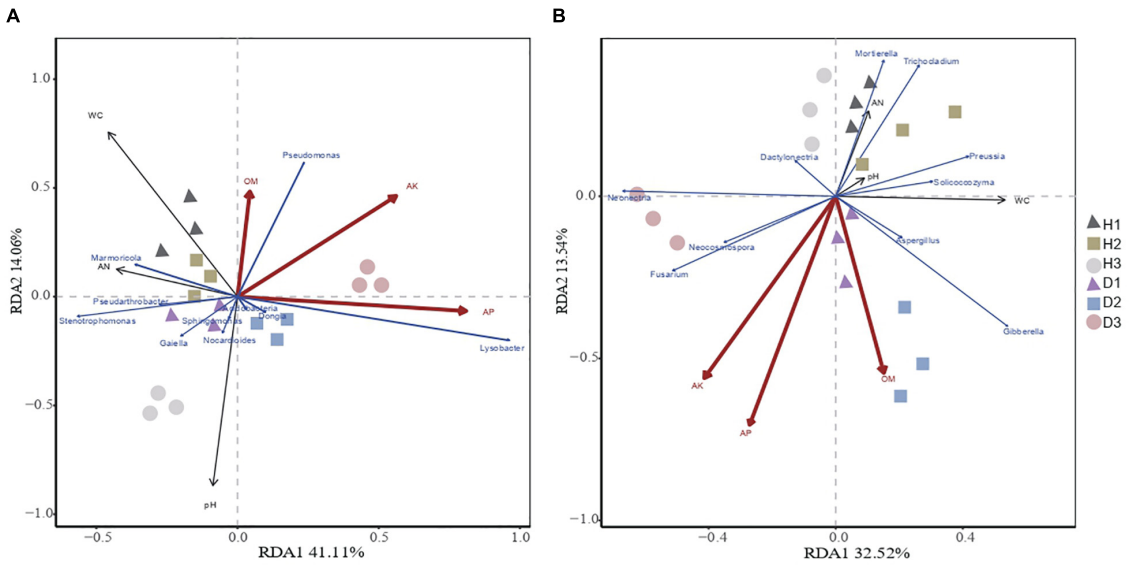

FIGURE 3
Redundancy analysis of abundant bacterial **(A)** and fungal **(B)** genus and soil properties for healthy and diseased samples from three sampling areas. The"H1," "H2," and "H3" refer to the three healthy soils. The "D1," "D2," and "D3" refer to the three diseased soils, respectively.

potentially decreasing the OM content. The OM content and available nutrients were significantly decreased in the diseased soils, thus affecting the plant and soil microorganisms on soil nutrient utilization. Root rot actually increased the carbon source utilization capacity of plants, and the reduction of soil OM accelerated the occurrence of root rot, thus resulting in an inevitable vicious cycle that deepens the plants' susceptibility.

Changes in the microbial composition of diseased plants

This study presented no significant difference in bacteria and fungi between the diseased and healthy soils according to the phylum level's diversity, richness index of the rhizosphere microorganisms, and OTUs. This result follows the results of previous studies (Jumpponen and Jones, 2010; Xu et al., 2012). We conducted further studies at the genus level, and upon comparing the diseased and healthy soils, we observed differences in the rhizosphere's resident bacterial and fungal genera. These microbial genera may be the key genera to inhibit or aggravate the occurrence of root rot. In this study, we found abundant fungal OTUs belonging to *Fusarium*. The proportion of *Fusarium* in the diseased soils of D1, D2, and D3 increased by 3.38%, 1.15%, and 9.76%, respectively, as compared to healthy soils. Following our previous study results, *Fusarium* is the oil peony's root rot pathogen (Liushuang, 2020). Moreover, sequencing results also showed that the number of *Fusarium* in the diseased soil was significantly higher than in the healthy soil. This study showed that the relative abundance of *Cylindrocarpon* and *Neocosmospora* in the fungal groups in diseased soil was significantly higher than in the healthy soil (**Figure 3B**). Furthermore, *Cylindrocarpon* and *Neocosmospora* were also the pathogenic genera in the soil of the root rot-infected plants. For example, *Cylindrocarpon* can cause peanut root rot (Caputo et al., 2015; Chojak-Koniewska et al., 2017). The relative abundance of *Chaetomium* is high in the healthy soil, and *Chaetomium* has been reported to produce antibiotics like chitin to inhibit the growth of pathogens, thus acting as an antagonistic fungus in the root rot soil (Pangesti et al., 2017). The significantly increased *Mycobacterium* and *Promicromonospora* in the soil of the root rot plants were bacteria related to OM degradation (Jin et al., 2018; Baranowski et al., 2019). *Dongia*, *Ohtaekwangia*, *Reyranella,* and other nutrient cycling-related bacteria also showed an increasing trend in the diseased plant soil (Piubeli et al., 2019; Sun et al., 2020; Zhang et al., 2020). The increase of these bacteria accelerated the degradation of soil nutrients, thus decreasing the soil nutrient content.

Relationship between the available nutrient content and plant diseases

Soil microorganisms not only mutually affect each other but also interact with the surrounding environment. Increasing evidence indicated that root exudates initiate and modulate the dialog between the plants and soil microbes comprising both pathogenic and beneficial microbes (Zhao et al., 2016). Plants attract beneficial microbes by emitting volatile organic

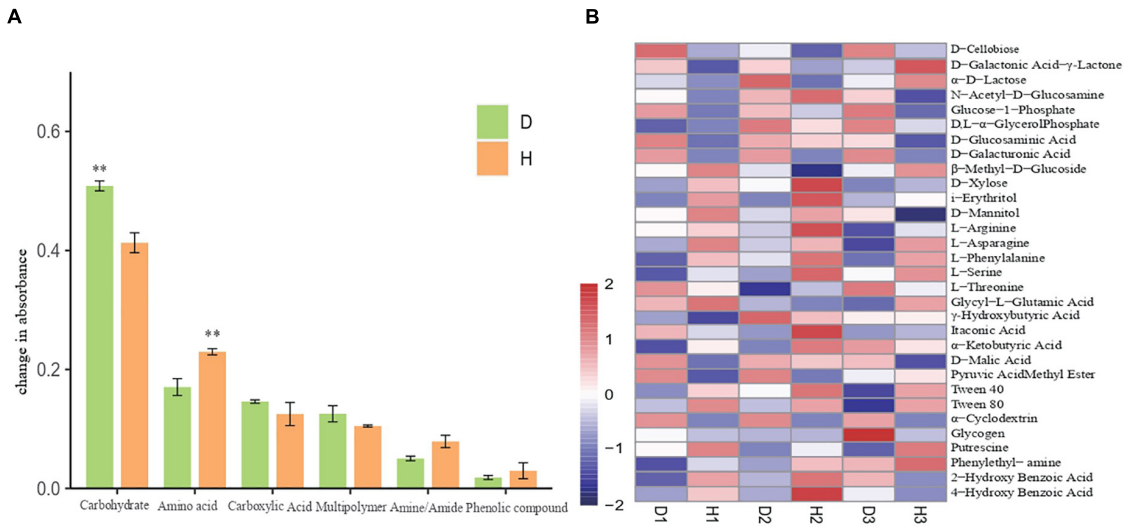

FIGURE 4
Six groups carbon source utilization rates of soil microorganisms of healthy and diseased oil peony **(A)**. Differences in utilization of 31 carbon sources by soil microorganisms **(B)**. The "D1," "D2," and "D3" refer to the three diseased soils, respectively.

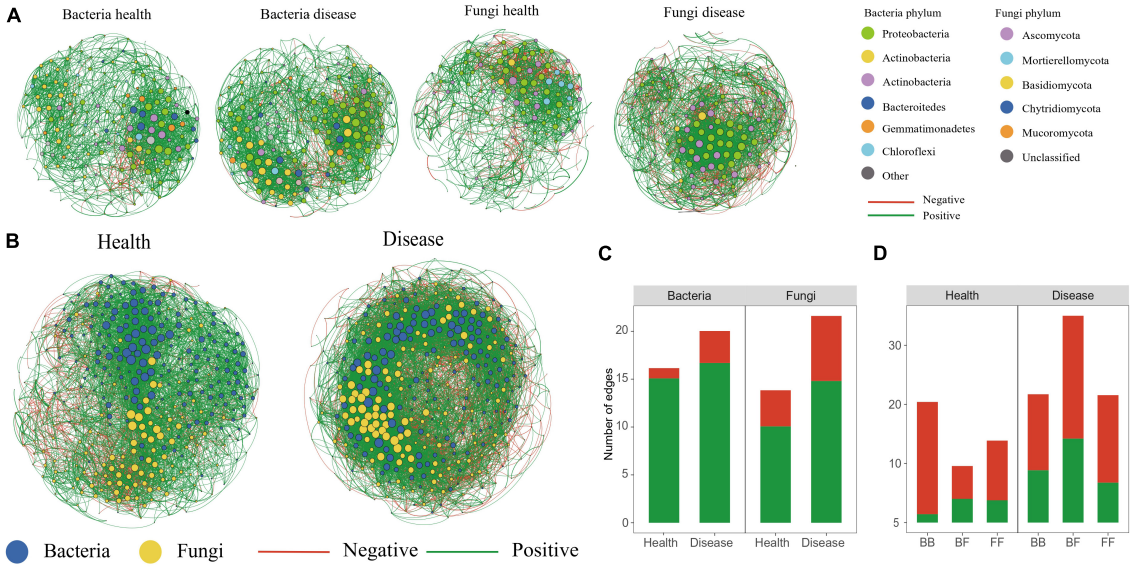

FIGURE 5
Co-occurrence networks. Intra-kingdom co-occurrence networks **(A)**. The nodes are colored according to bacterial phylum and fungal phylum. Node size indicates the degree of connection. Edge color represents positive (green) and negative (red) correlations. Number of positive and negative edges of bacterial and fungal groups in health and disease networks **(C)**. Interkingdom co-occurrence networks **(B)** contained both bacterial and fungal taxa. Number of bacterial–bacterial (BB), bacterial–fungal (BF), and fungal–fungal (FF) correlations in the healthy and diseased networks. Green and red colors of the edges and column indicate positive and negative correlations, respectively **(D)**.

compounds or modifying the synthesis and secretion of particular root exudates (Berendsen et al., 2018; Jun et al., 2018; Liu et al., 2019; Gao et al., 2021). After planting oil peony for 5 years, the characteristics of the rhizosphere soil changes due to the shifting of the root exudates' features because of the oil peony physiological change. This may systemically affect interactions between the plants and microbes *via* alteration of the microbial community structure. Therefore, our study aims to further understand the microbial activity mechanism and, through redundancy analysis, show a significant correlation between the soil properties and microbial community structure, which was affected by the OM and available nutrients in

the diseased soil. Since the abundance of *Stenotrophomonas*, *Lysobacter*, *Nocardioides*, *Gaiella*, and *Sphingomonas* were negatively correlated with OM and the available nutrients, and root rot reduced the soil OM and the available nutrient content, we inferred that these bacteria might accelerate the soil's carbon cycle. The abundance of *Marmoricolam* was positively correlated with both OM and the available nutrient content, and *Marmoricola* was also believed to improve the antifungal activity of the soil. Regarding fungi, redundancy analysis results indicated that the abundance of the *Fusarium* was negatively correlated with both OM and the available nutrients. Therefore, future studies can focus on controlling the content of the pathogenic *Fusarium* by manipulating the content of the organic compounds and available nutrients.

The carbon metabolism of diseased plants increased

The BIOLOG ECO microplate technology can competently describe the metabolic functions of the microbial communities, especially for the environmental microorganisms (Choi and Dobbs, 1999). AWCD is related to the number and species of microbiota that utilize the single carbon source in a soil microbial community, thus reflecting their overall capacity to utilize the carbon source (Choi and Dobbs, 1999; De Gens et al., 2000). In this experiment, the AWCD of the soil microbes from the healthy and diseased plants exhibited the conventional microbial growth curve (from the adaptation to the stable phase), thereby presenting an increase during the culture time and being consistent with the previous studies (Kong et al., 2006; Wang et al., 2018). The growth curve showed that the higher the AWCD value, the higher the soil microbial metabolic activity (Wang et al., 2011). The diseased plants had higher AWCD values than the healthy plants. Thus, both the soil microbial activity and metabolic ability of the diseased plants were higher than the healthy plants. The pathogenic invasion of these plants altered the microbial community structure, which consequently increased their carbon source utilization, and this reduced the bioavailability of nutrients in plants, thereby aggravating the disease (Lan et al., 2019). In this study, the soil microbial metabolism enhancement may be related to the increase in specific microbial groups that can utilize carbohydrates and carboxylic acids. Additionally, plants release phenolic acids into the soil *via* aboveground leaching, root secretion, and plant residue decomposition. This directly affects the soil's nutrient status, while indirectly affecting the plant growth by regulating soil microbial activity. In this study, the increase in metabolic capacity may also be due to the release of substances from the root post root rot infection and plant wilting, which causes more leaves to enter the soil, thus providing more carbon sources for soil microorganisms. The utilization rate analysis of the six carbon sources highlighted that the carbohydrate utilization ability of the soil microorganisms to in the root rot-infected oil peony root zone was significantly improved. This may be related to the increased abundance of related microbial taxa, e.g., *Dongia*, *Ohtaekwangia*, and *Reyranella*, in the root zone soil, which have been previously reported to degrade carbohydrates. Furthermore, this may be due to the root necrosis caused by root rot, which causes root organic components like sugars, organic acids, and amino acids to enter the soil, which may selectively increase the related microbial groups.

Changes in microbial community structure of diseased plants

Cooperative and competitive interactions among the microbial species and network modularity can affect the community stability (Faust K; Coyte et al., 2015). In this study, fungal networks and their central taxa in healthy and diseased plants have more negative correlations than the bacterial networks. Mutual negative interactions indicate that ecological competition can reduce the stability of the microbial communities by inhibiting cooperation (Coyte et al., 2015). Therefore, the host may reduce its resistance to external stress due to microbial competition (Wagg et al., 2019). In stark contrast to bacterial communities, pathogen invasion affected the fungal communities more, probably due to enhanced positive intra-kingdom correlations we observed among the fungal taxa in diseased networks as compared to the healthy ones. Furthermore, lower modularity in the diseased fungal network may exacerbate the destabilizing effect due to the higher prevalence of cross-module correlations among the taxa (Grilli et al., 2016; Hernandez et al., 2021).

Many positive and negative correlations in the fungal network showed that the fungal community was more sensitive to diseased plants than the bacterial community.

Our results indicated that the diseased plants decreased the bacterial network complexity, but increased the fungal network complexity. The contrasting pattern of these networks parallels recent observation based on the macroecological soil patterns of *Fusarium* wilt (Yuan et al., 2020). Previous studies demonstrated the importance of the network complexity (Wagg et al., 2019) and hub taxa (Toju et al., 2018; Yu et al., 2020) in supporting the ecosystem functions. The fungal connectivity, mainly belonging to the intra-kingdom cooperative interactions, increased in the diseased soil, thus inducing the ecological importance of the fungal taxa. Additionally, we found dominating cooperative correlations within each microbial kingdom, but the competitive correlations dominated between the bacteria and fungi, since both the bacteria and fungi typically compete for similar plant-derived substrates (De et al., 2010).

Network hubs are the essential microorganisms in the microbial network (Qi et al., 2019). More network hubs, like keystone taxa, possibly made more frequent exchanges of

materials and information among the microbial species in the diseased network than in the healthy network. Deciphering the network hub is critical for harnessing the plant microbiome to enhance the plant's growth and health (Toju et al., 2018; Trivedi et al., 2020). Indeed, we found that several potential beneficial bacteria, like *Reyranella*, *Sphingomonas,* and *Bradyrhizobium,* were enriched in the diseased plants. We also identified them as the core taxa, i.e., present in all samples in the plant microbiomes. Nevertheless, previous studies showed that *Reyranella*, *Sphingomonas,* and *Bradyrhizobium* colonized plant rhizosphere and were vital for modulating the host performance, especially in suppressing plant pathogen (Zhang et al., 2020; Matsumoto et al., 2021). Therefore, our results indicated that the host plant might selectively regulate the community abundance of some core taxa under pathogen stress. Furthermore, we found that several fungal taxa, like *Solicoccozyma* and *Chaetomium*, were enriched in the diseased plants and identified as hub taxa in the co-occurrence networks, thereby having an antagonistic effect on the pathogens (Pangesti et al., 2017; Stosiek et al., 2019).

Conclusion

This study highlighted the significant differences in the rhizosphere microbial community structures between the healthy and diseased oil peony plants. After pathogen invasion, the increase of bacteria related to nutrient cycling in soil, such as *Dongia, Ohtaekwangia, Reyranella*, etc., the decrease of antagonistic bacteria and fungi, like *Chaetomium, Marmoricola*, etc., and the increase of pathogenic fungi, like *Cylindrocarpon* and *Neocosmospora*, caused the imbalance of soil microbial community structure and eventually led to plant root rot. The soil's physical and chemical properties changes and the modified carbon source utilization also indirectly indicated the changes in the rhizosphere microbial communities of the root rot plants. Moreover, the network's diagram complexity and some parameters indicated that the rhizosphere microbial community had indeed changed. Given that oil peony is economically important and root rot has always been a serious concern, future studies can use our study results to screen antagonistic bacteria against root rot through the study of rhizosphere microorganisms of root rot plants. They can then use its interaction with other microorganisms in the network diagram to synthesize bacterial agents that can treat oil peony root rot, and also provide methods for biological control of oil peony root rot.

Data availability statement

The data presented in this study are deposited in the NCBI (https://www.ncbi.nlm.nih.gov/) repository, accession numbers PRJNA850114 (16S) and PRJNA850456 (ITS).

Author contributions

MJ, MC, SL, and JZ contributed to the conception and design of the study. SL and MJ performed a lot of experiments. MJ conducted statistical analysis and wrote the first draft of the manuscript. MC, XS, SL, and JZ wrote part of the manuscript. All authors contributed to the revision of the manuscript, read, 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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A review on effective soil health bio-indicators for ecosystem restoration and sustainability

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Preventing degradation, facilitating restoration, and maintaining soil health is fundamental for achieving ecosystem stability and resilience. A healthy soil ecosystem is supported by favorable components in the soil that promote biological productivity and provide ecosystem services. Bio-indicators of soil health are measurable properties that define the biotic components in soil and could potentially be used as a metric in determining soil functionality over a wide range of ecological conditions. However, it has been a challenge to determine effective bio-indicators of soil health due to its temporal and spatial resolutions at ecosystem levels. The objective of this review is to compile a set of effective bio-indicators for developing a better understanding of ecosystem restoration capabilities. It addresses a set of potential bio-indicators including microbial biomass, respiration, enzymatic activity, molecular gene markers, microbial metabolic substances, and microbial community analysis that have been responsive to a wide range of ecosystem functions in agricultural soils, mine deposited soil, heavy metal contaminated soil, desert soil, radioactive polluted soil, pesticide polluted soil, and wetland soils. The importance of ecosystem restoration in the United Nations Sustainable Development Goals was also discussed. This review identifies key management strategies that can help in ecosystem restoration and maintain ecosystem stability.

KEYWORDS

ecosystem restoration, ecosystem stability, resilience and resistance, soil health, bio-indicators, molecular bio-indicators

Highlights

- A total of 250 reported studies (including original research, review papers, opinion papers, etc.) on ecosystem restoration and soil sustainability were reviewed.
- Forms of soil degradation were discussed along with important soil bio-indicators.
- A connection was established between soil health, soil sustainability, and ecosystem restoration.
- Management strategies were proposed to deal with altered soil scenarios.
- Advanced molecular techniques for assessing sensitive soil bio-indicators were briefed.

Introduction

With the current advent of the changing climate, the state of environmental degradation and destruction of different ecosystems is considerably taking place on a "catastrophically short timescale" (Novacek and Cleland, 2001), which is estimated to accelerate from 1,000 to 10,000 times than the normal rate (Wilson, 1988). Biological diversity or biodiversity is of pivotal importance to sustain a better future. Humans have a great responsibility toward maintaining and enhancing global biodiversity at an ecosystem level. On a more anthropocentric level, natural ecosystems provide human society with food, fuel, and timber. Habitat loss is the leading cause of species extinction (Wilson, 1988) and ecosystem service decline (Daily, 1997). The two ways to reverse this trend of habitat loss are conservation of currently viable habitats and restoration of degraded ecosystems.

To understand the importance of ecosystem restoration, attention must be drawn to the cause of ecosystem degradation and its consequences. Ecosystem degradation is an outcome of long-term environmental degradation. By definition, environmental degradation is any change or disturbance to the environment perceived to be deleterious or undesirable. The deterioration of the environment is manifested in different ways through the depletion of resources, such as air, water, and soil resulting in habitat destruction, the extinction of wildlife, and pollution leading to the complete destruction of ecosystems. The degradation of the environment mainly occurs due to overexploitation of resources for short-term economic goals *viz*. deforestation for mining, building roads, or exploitation of flora and fauna. Some natural phenomena like active volcanoes, hurricanes, droughts, and earthquakes may also be responsible for causing damage to the ecosystem. Many of the world's ecosystems have undergone significant degradation with negative impacts on biological diversity. Fundamentally, ecosystem degradation is an environmental problem that diminishes the capacity of species to survive. Ecosystem restoration can be the only solution to the existing crisis.

Ecosystem restoration is essentially the "process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed" (SER, 2004). The practice of ecosystem restoration includes a wide scope, such as erosion control, reforestation, usage of genetically local native species, removal of non-native species and weeds, revegetation of disturbed areas, daylighting streams, and reintroduction of native species, as well as habitat and range improvement for targeted species. The nutrient cycles and energy fluxes are the most basic and essential components of ecosystems (Ochoa-Hueso et al., 2021). An understanding of the full complexity and intricacies of these cycles is necessary to address any ecological processes that may be degraded. A functional ecosystem, which is completely self-perpetuating or natural, is the ultimate goal of restorative efforts (Farrell et al., 2021). Since these ecosystem functions are emergent properties of the system, monitoring and management are crucial for the long-term stability of an ecosystem.

The management of the ecosystem for its sustainability and stability calls for appropriate measures, such as monitoring of soil health particularly in degraded agricultural soils. It is already established that excessive use of inputs has degraded many fertile lands in the recent past. Therefore, it has now become more pertinent to identify and quantify the soil properties to arrest further degradation. To facilitate sound recommendations (for fertility restoration) to the farmers, robust information on soil health is required.

Over the years, ecosystem restoration has proliferated with several collaborative and multidisciplinary global projects called the following: 1. The EcoHealth Network (relies on the mutual benefits of ecological and human health), 2. The UN System of Environmental-Economic Accounting (SEEA EA); a standardized ecosystem accounting framework merging with economic frameworks, 3. The Natural Capital Project (NatCap), where global partnership existed between the wellbeing of people was aimed *via* ecosystem restoration and nature-based solutions, and 4. The INCASE project, to test the natural capital collaboration with scientists and stakeholders at a river catchment scale, was developed in Ireland (Farrell et al., 2021). Realizing the importance of the subject and growing interest in research, several conferences have been organized in the last few years; to name a few, "The 9th World Conference on Ecological Restoration" by the Society for Ecological Restoration (CIFOR, CGIAR, June 2021) and the National Conference on Ecosystem Restoration, by the University of Florida (July–August 2021) were also conducted recently.

Restoration ecologists and other conservation biologists agree that habitat is the most important locus of biodiversity protection. There has been a growing realization and appreciation in restoration ecology for the role of soil ecology in restoring and maintaining diverse biological communities both above- and belowground (Farrell et al., 2020; Cavender-Bares et al., 2021). Therefore, soil health restoration is important to consider while designing ecological restoration strategies. Soil dynamic properties can be used to monitor and assess the consequences of restoration activities on ecosystem functioning and services. In any case, finding suitable soil health indicators to monitor ecological restoration activities at different scales requires a full understanding of soil-plant–ecosystem relationships (Raiesi and Salek-Gilani, 2020). Current methods for assessing soil health fail to provide a complete picture of the status of the functioning soil system. Although knowing these aids management decisions, they lack any indication of the dynamic ways, in which soils need to respond to anthropogenic stresses and disturbances.

One of the ways to address this is to comprehensively determine soil microbial community characteristics and the biogeochemical properties they influence. To date, the exploration and application of soil ecological knowledge to

restoration questions have been dominated by soil microbial ecology. Since micro-organisms could act directly or indirectly on organic matter decomposition and the promotion and maintenance of several soil properties, some characteristics of soil microbial communities have been used as ecological indicators of ecosystem disturbances and plant cover restoration (Gama-Rodrigues et al., 2008; Muñoz-Rojas et al., 2016); e.g., the microbial biomass is considered one of the most sensitive and effective indicators because it is directly influenced by biotic and abiotic factors (Karlen et al., 2019; Nunes et al., 2020). Soil microbial and biochemical properties, such as the metabolic quotient (qCO_2) (ratio basal respiration: microbial carbon C) and microbial quotient (qMIC) (ratio microbial C: organic C), are also cited in the literature as efficient properties to evaluate the soil health (Maini et al., 2020; Simfukwe et al., 2021). On the other hand, soil biochemical properties are already established indicators of soil health, but there is still no consensus as to how they should be used. Generally, biochemical properties related to the biological cycling of the elements (carbon, nitrogen, phosphorus, and sulfur) are used to diagnose soil health. These properties include both general biochemical parameters (i.e., microbial biomass C, dehydrogenase activity, and nitrogen mineralization potential) and specific biochemical parameters (i.e., the activity of hydrolytic enzymes, such as phosphatase, urease, and β-glucosidase). Biochemical properties can be used both individually, as simple indices, or in combination using complex equations derived from mathematical combinations or the application of statistical programs (Lehmann et al., 2020). The results described in the literature for both are contradictory and question the validity of the use of biochemical properties as health indicators. Complex expressions, in which different properties are combined, are thought to be highly suitable for estimating soil health, although their use is limited to the area and situation, in which they have been described (Rinot et al., 2019; Thiele-Bruhn et al., 2020). Currently, there is a knowledge gap in the literature regarding the systematic compilation of the studies meant for the understanding of ecosystem restoration under various ecological conditions over the decades. The objective of this review article is to summarize some of the many attempts that have been made for the quantitative evaluation of soil health under various ecological constraints, including microbial and biogeochemical indicators, and proposed management strategies that improve ecosystem restoration measures.

So far, several studies have been conducted that specifically determine the role and impact of soil microbial and biochemical indicators under several land uses, management practices followed in the recent past or from several years, and stressed soil (drought and salinity, which change the soil's physical and fertility attributes) conditions in different parts of the world. However, the authors of this work felt the urge for a systematic and informative compilation of studies meant for the understanding of ecosystem restoration in various situations that may be suitable for global readers.

We have categorically emphasized the studies, which are solely based on identifying the bio-indicators, that were either influenced by the natural or the altered management systems. Future research priorities can be set for those who have focused on environmental management and fulfilling sustainable development goals. Our sole purpose behind this compilation is not only to gather literature but to also make our best attempt to analyze what progression was made over the years to identify the microbial and biochemical indicators under different situations that comes in *via* both thought process and technologically wise. We, the team of authors, have searched for 250 scientific kinds of literature (including original research, review papers, opinion papers, etc.) over three decades from different corners of the world in this aspect, and have compiled and presented it suitably to the global readers. In the present scenario, it is more important to assess where we are, and in which direction we are leading for ecosystem restoration.

This review summarizes the many attempts that have been made at the quantitative evaluation of soil quality (based on the selected soil indicators) that eventually relate to ecosystem restoration of agricultural soil and other degraded soils and how important the role of microbial and biochemical indicators is in the whole process. Measuring the soil quality, restoration, and resilience are all realized as interconnected and point toward a sustainable soil system, concerning both agriculture and the environment. Over and above, a brief discussion on proposed management strategies and how they can uplift the ecosystem restoration status also took place in our compilation. The prominence of ecosystem restoration in Sustainable Development Goals as proposed by the United Nations was also highlighted.

Concept of microbial/biochemical indicators

There are many definitions of "Bio-indicators" if we search in the literature. Moreover, it has been slightly modified from time to time. Hodkinson and Jackson (2005) mentioned that "a bio-indicator is a species or a group of species that reflects biotic and/or abiotic levels of contamination of an environment," whereas Stankovic and Stankovic (2013) described, "a bio-indicator is an organism or a part of an organism or a community of organisms, which contains information on the quantitative aspects of the quality of the environment; exposure of organisms can be measured by either levels or effects." So far, it is established that soil microbial parameters, which provide information on the biomass, activity or functionality, and diversity of microbial communities have been widely proposed as bio-indicators of soil health (Gómez-Sagasti et al., 2012). Bio-indicators play a substantial role, together with common

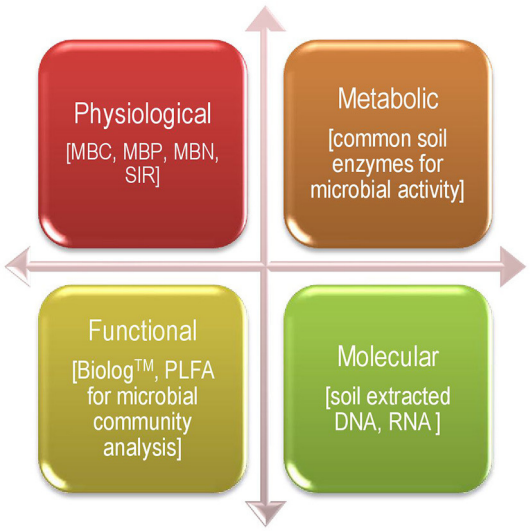

FIGURE 1
Soil bio-indicators as classified on the basis of techniques of measurement.

soil chemical indicators, in the reclamation of soil health. However, not much attention has been paid to identifying the set of ideal/sensitive bio-indicators for stressed or problematic soils, which demands equal attention for soil remediation or restoration, a globally important sustainability issue.

These days, an extensive number of techniques/approaches are frequently involved to estimate common soil microbial parameters (bio-indicators), and, hence, they can be broadly classified into four categories: 1. Physiological, 2. Metabolic, 3. Functional, and 4. Molecular (Figure 1). There are good numbers of bio-indicators that were investigated for agricultural systems and used for indexing soil health and sustainability under long-term agro-ecosystem (Masto et al., 2007; Bhaduri and Purakayastha, 2014; Bhowmik et al., 2016, 2017a; Bhaduri et al., 2017a; Fortuna et al., 2018a,b) at altered management practices in a wide range of agricultural soils (Koper and Piotrowska, 2003; Kang et al., 2005; Bhattacharjya et al., 2017), such as a climate-dominated natural soil (Bastida et al., 2006), pesticide-afflicted soils (Saha et al., 2015, 2016a,b), hydrocarbon-polluted soil, and a mine–reclaimed soil (Mukhopadhyay et al., 2016). All possible relations between soil health and plant-microbe interactions in the plant rhizosphere with a special mention of soil bio-indicators have been explicitly discussed in other literature too (Bhaduri et al., 2015, 2017b, 2018; Bhowmik et al., 2017b, 2019).

Methods

The idea of the review was conceived by the lead author who felt the need for such systematic compilation, and, subsequently, the team of authors was formed. After rounds of group meetings, the layout of topics was finalized, and the authors started to search for relevant information on different time scales. For searching relevant literature Google Scholar was trusted as a search engine. The authors used some common keywords, such as soil bio-indicators, ecosystem restoration, polluted soils, problem soils, land use intensification, agricultural sustainability, microbial community, soil and ecosystem responses, soil nutrient cycling, wetland pollution, and sustainable development goals. Systematic classification of published literature was grouped as per the major headings of the review. After the finalization of the first draft, the topics were redistributed for all possible checks among authors, and further refinement was done wherever possible. Two authors in the team dedicated their time to editing and the manuscript was finalized thereafter. At the time of the revision, a team of authors addressed all comments by anonymous reviewers.

Results and Discussion

The behavior of bio-indicators under distinct soil scenarios

Soil microbial assemblages and functions are robust indicators of environmental responses to disturbances in managed and natural ecosystems. However, the choice of keystone soil microbial indicator(s) can be calibrated with the type of scientific questions to be addressed (Siddig et al., 2016; Schloter et al., 2018). In this review article, we report potential soil microbial indicators that can serve as early warnings of ecological responses to a range of conditions, including land-use intensification and land management practices, pollution of heavy metal and organic wastes, ecosystem restoration projects, and environmental disturbances.

Land use intensifications and land management practices

Traditional microbial community analysis, along with soil biochemical and biogeochemical analyses, can be fruitful in investigating the soil and environmental consequences of long-term farming practices (García-Orenes et al., 2016). Kumar et al. (2018) recently suggested the use of 16S rRNA amplicon sequencing-based operational taxonomic units (OTUs) to demonstrate that the community structure of beneficial soil bacteria like diazotrophs, which are known to increase in fields, receive balanced fertilization as compared to the fields receiving single nutrient, such as nitrogen. Changes in soil microbial community composition and structure can be indicative of land-use intensification, such as the conversion of native rangeland to silvopasture or sown pasture and subsequent alteration of

plant cover and nutrient inputs (Xu et al., 2017). The occurrence of keystone taxa, however, can also inform about the influence of land management practices on the structure of soil (and root) microbial network, thus, soil and ecosystem functions (Bhowmik et al., 2016; Banerjee et al., 2018, 2019).

The sensitivity of microbial structural diversity as a function of land-use intensification can vary based on the ecosystem (or, dominant vegetation) type. For instance, intensive soil management practices in an agricultural system in Brazil greatly reduced the value of indices for soil microbial diversity (Siqueira et al., 2014), but those indices were not effective to explain the ecological consequences of land use intensifications in tropical rainforests of Amazonia (de Carvalho et al., 2016). The functional potential of the soil biota represented by the active fraction of the microbial community in the Biolog assay can serve as an alternative indicator to assess the influence of land use intensifications (Gomez et al., 2004). Other than vegetation types, changes in soil microbial diversity as a function of land-use intensification can also be contingent on biome types or climate and underlying edaphic properties (Houlbrooke et al., 2011; Trivedi et al., 2016).

A recent study indicated that agricultural intensifications could alter the expressions of soil biochemical indicators (e.g., enzyme activity) quicker than soil microbial diversity indicators (Pérez-Brandán et al., 2016). To that end, soil enzyme activities are often used as effective indicators for assessing the environmental consequences of applications of agricultural chemicals for soil nutrition (Gaind and Singh, 2016; Sihi et al., 2017) and xenobiotic pesticides (Sahoo et al., 2016; Mahapatra et al., 2017). One can also evaluate the ecological consequences (e.g., soil carbon loss) of land use management intensities, along with a wide spatial gradient of edaphic factors, using microbial ecophysiological traits, including growth efficiency, qCO_2, and indicator proteins (Malik et al., 2018). Hereby, we discuss the use of specific bio-indicators for a wide range of soil ecosystems:

Mine deposited soil and its reclamation

Mine-deposited or mine-reclaimed soil has a close association with heavy metal toxicity. Hence, only a few kinds of literature cited some work in a similar direction. Earthworm (*Eisenia foetida*), as a bio-indicator, has been tested to assess Hg-toxicity and bio-availability in mine tailings. In another study, acidophilic bacteria were identified after 16S-rRNA profiling of bacterial community from extremely and moderately acidic lead-zinc mine tailing samples. A study from an Indian mine located at Ranigunj coalfields, dealt with environmental soil quality index, screened the bio-indicators, and differentiated their behaviors at underground mine (better dehydrogenase and fluorescein activities) and open cast mine soils (peroxidase activity) (Masto et al., 2015).

Rehabilitated soils include soils under cultivation or any productive use of mankind after keeping barren/fallow for a few to several years. Hence, their structure and properties are altogether different as compared to other soils. An early study (Majer, 1983) discussed that ants can serve as good bio-indicators owing to numerical abundance, size, and species richness, especially in the case of mine-rehabilitated soils. Another study also supported this finding showing that a close association exists between aboveground ant activity and belowground decomposition processes in terms of soil microbial biomass carbon at mine-disturbed sites in Northern Territory, Australia. This provided evidence that ants could be used as indicators of restoration success, following disturbance (Andersen and Sparling, 1997). In other instances, for long-term heavy metal polluted (Zn, Pb, Cd toxic) soil, urease and invertase enzyme activities were identified as "progress indicators" for soil rehabilitation (Ciarkowska et al., 2014). Schimann et al. (2012) proposed the ratio of soil Denitrifying Enzyme Activity (DEA) and Substrate Induced Respiration (SIR) as ecosystem indicators after disturbances of gold mining in tropical rainforests of French Guiana.

Fatty acid methyl ester (FAME) biomarkers can be used effectively to evaluate the reclamation progress of surface mines. Within this context, Mummey et al. (2002) observed an increased ratio of bacterial: fungal FAME biomarkers that also co-occurred with an increment of SOM content, indicating improved soil health due to ecosystem recovery after surface-mine reclamation. Interestingly, Anderson et al. (2008) suggested using MBC instead of soil organic carbon (SOC) as an indicator of ecosystem stability from surface coal mining in a semiarid area, even after a decade-long reclamation management practice. However, MBC alone may not always be a sensitive indicator of soil restoration in heterogeneous mountain systems like Pa'ramo in the High Tropical Andes (Abreu et al., 2009). Hence, bio-indicators should be carefully chosen for restoration project monitoring based on the prior knowledge of their degree of similarities and dissimilarities between reclaimed and pristine sites of similar geographic territories (White and Walker, 1997).

Trace (heavy) and organic pollutant affected soils

Expressions of stress proteins (AKA heat shock proteins), especially hsp70 and hsp60, have long been used for biomonitoring of exposure of soils to trace (or, heavy) metals and organic pollutants (Huggett, 2018). Ghotbi and Morgan (2007) synthesized the applicability of stress proteins expressed by soil bacteria under stressed conditions, such as sudden fluctuations in soil pH after metal contamination. Heavy metal contaminations are also known to increase the abundance of low molecular weight proteins in soils (Zhang et al., 2013). Stimulation of metal-resistant microbes is another

widely observed response to heavy metal contamination in soil (Doleman, 1994; Shi et al., 2002). The effects of heavy metals on soil microbial communities can also be found to be reported in studies conducted by Zhao et al. (2014) and Chu (2018).

The abundance of functional genes related to fatty acid metabolism (e.g., *acc*, *fab*, and *fad* genes) can be used to evaluate contamination of organic biomolecules like hydrocarbon, especially in soils subjected to oil contamination from industrial effluents (El-Bestawy et al., 2005). Taxonomic profiling of soil microbiome informed by 16S rDNA analysis can also be used to evaluate the hydrocarbon exposure to soil, where the enrichment of Proteobacteria, Gamma Proteobacteria, and Bacteroidetes may indicate potential effects of the oil spill. A more traditional soil microbial diversity indicator like the Shannon diversity index also served as a useful indicator of soil contamination in a recent study conducted by Patel et al. (2016). Additionally, Galitskaya et al. (2015) indicated that metabolic quotient and cellulase activity are two very sensitive bio-indicators of radioactive oil waste.

There are several bio-indicators reported from heavy metal polluted soils. Various organisms, including microbes, fungi, plants, animals, and humans, are being used to identify and monitor the level of toxic metals in the ecosystem, encompassing air, water, sediment, soil, and the food chain Stankovic et al. (2014). Nematode diversity is a frequently used parameter, where maturity index (MI) is an established bio-indicator to assess the degree of heavy metal polluted soil (Coleman et al., 1998; Korthals et al., 1998). A recent report by Martin et al. (2018) mentioned *Pinus halepensis* trees as bio-indicators of heavy metal pollution, where tree rings and barks were used to evaluate environmental contamination. Another study reported that Cryptogamic biota, e.g., lichens and bryophytes, are useful to identify the degree of heavy metal toxicity in Zn-Pb polluted sites (Rola and Osyczka, 2018).

Desert soil/barren lands

Few researchers also attempted to identify promising bio-indicators for desert soils or barren lands. They have opined that rain frequency and continuity were determinants for desert soil under a xeric moisture regime that may cause changes in soil conditions and, the substrate utilization pattern of the microbial community as determined by the MicroResp^{TM} analysis (Saul-Tcherkas and Steinberger, 2009). Guan et al. (2015) concluded that soil nematode communities serve as bio-indicators under sandy ecosystems, whereas the plantation of *Caragana microphylla* improved nematode diversity. Another study stated that there was a beneficial impact of sand-stabilizing shrubs for various hydrolase and oxidase enzymes involving soil C-cycle (polyphenol oxidase, cellulose, and β-glucosidase) and N-cycle (nitrate reductase and urease) conducted at Tengger Desert, China (Hu et al., 2016).

Anthropogenic stressed soils

Soil invertebrate species are often reported as useful bio-indicator for monitoring qualitative and quantitative environmental changes in soil due to anthropogenic activities (Paoletti et al., 1996). Pollution creates a shift in the community structure of nematodes; for light to moderate pollution, the abundance of sensitive species is reduced while keeping the abundance of tolerant species unaffected; whereas the decrease in tolerant species is noticed under heavily polluted soils, due to toxic effects or a decline in microbial activity (Korthals et al., 1998).

Radioactive materials polluted soils

Radioactivity leaves a long-term footprint on soil ecological behavior. Although not many, few researchers revealed some interesting facts and findings. Zaitsev et al. (2014) reported after extensive radioecological research around the historic Chernobyl site (in and around Russia, Ukraine, Belarus, and Kazakhstan) that earthworms, millipedes, collembolans, and oribatid mites were the most appropriate bio-indicators of different radioactivity levels and types of radioactive pollution. A recent report indicated that radioactivity could induce biological effects in soil earthworms (family: Lumbricidae) *viz*. impairment of reproduction of individuals and reduction in population density; however, a higher survival rate of *Aporrectodea caliginosa* was observed after additional acute γ-irradiation with a dose of 2,270 Gy, which may be a sign of adaptation for higher doses of ionizing radiation and establish them as bio-indicator under soil contamination with radionuclides and heavy metals (Rybak et al., 2021). Another study reported that soil macro-invertebrates (e.g., ground beetle) served as important bio-indicators with 3–37 times lower abundance, and biodiversity at the contaminated sites enriched with uranium and arsenic over the control soil (Gongalsky, 2003). Gaso et al. (1995) discussed the role of land snails (*Helix aspersa* Müller) as bio-indicators in sites contaminated with radionuclides (^{226}Ra, ^{137}Cs, and ^{40}K) for two decades. Aquatic mosses were also identified as bio-indicators of radioactive contamination (Hongve et al., 2002).

Pesticide polluted soils

Pesticides help control agricultural pests but often affect non-target soil biota and their processes (Pimentel, 1995; Aktar et al., 2009). The fate of the pesticide in the soil system depends on the chemical composition of the pesticide along with the soil's biotic and abiotic conditions (Pal et al., 2006). Depending on the transformation, the products could interact and disrupt the biological processes in the soil, thereby, influencing bio-indicators of soil health. However, most of the studies have found discordant results because of numerous interacting factors and complex soil dynamics. Researchers have investigated the effect of different types of pesticides, including

insecticides, fungicides, and herbicides, and found that they have a positive or negative effect on soil microbial biomass (Chowdhury et al., 2008; Saha et al., 2015; Kumar et al., 2020). Repeated application of pesticides has been reported to significantly lower the soil microbial biomass, mainly the fungal populations, and has been reported to increase certain bacterial populations (Smith et al., 2000; Pal et al., 2006; Singh et al., 2015). Molecular techniques like DGGE were also to determine the effect of specific pesticides on the soil microbial community (Lo, 2010). They found that carbofuran stimulated the population of *Azospirillum* and anaerobic nitrogen fixers in flooded and non-flooded soil. However, specifically in non-flooded soils, butachlor reduced the population of *Azospirillum* and anaerobic nitrogen fixers. Soil respiration can serve as a bio-indicator to evaluate the effect of these chemicals on microbial CO_2 respiration. Many studies reported the favorable effect of pesticide application on microbial growth and activity, whereas few studies show the adverse effect of pesticides on microbial respiration (Jail et al., 2015). Microbial metabolic quotient was found to increase due to chemical pesticide application indicating the requirement for microbes to use more energy for maintenance. The literature is replete with the effect of pesticide application on soil enzymatic activity (Saha et al., 2016a,b; Sahoo et al., 2017). Enzymes involved in nutrient cycling, including dehydrogenase, were mostly inhibited, whereas cellulose activity was stimulated due to pesticide application (Tu, 1992; Haney and Senseman, 2000; Chowdhury et al., 2008; Riah et al., 2014). Most of the studies to date have focused on soil incubation experiments but not much on field studies.

Wetland assessment and restoration

Like many other natural and managed ecosystems, the use of microbial metabolic indicators, coupled with hydric soil indicators, has been suggested for designing assessment strategies in wetland ecosystems (Merkley et al., 2004). Mieczan and Tarkowska-Kukuryk (2017) recently demonstrated the efficacy of using microbial loop components (or, microbial food webs) as a monitoring tool for evaluating ecological disturbance in a restored carbonate-rich fen. On the other hand, Dziock et al. (2006) reported the usefulness of using microbial diversity and community comparison indices to evaluate the effect of wetland pollution. Ratios of oligotrophic:copiotrophic organisms, such as the ratio of ammonia-oxidizing archaea (AOA) to ammonia-oxidizing bacteria (AOB), can be used to assess the success of wetland restoration projects (Martens-Habbena et al., 2009). To that end, Sims et al. (2012) recently proposed the use of the ratio of ammonia monooxygenase (*amoA*) gene copies for AOA to AOB for evaluating the effect of the nutrient loading in oligotrophic wetlands, where an increased value of AOA: AOB gene copies may indicate healthy conditions in those wetlands.

Molecular technologies like nucleic acid fingerprinting, especially terminal restriction length fragment polymorphism (tRFLP) and fluorescence *in situ* hybridization (FISH), along with microbial community-level physiological profile (CLPP), are also generally used as indicators for wetland trophic status (Castro et al., 2002; Sizova et al., 2003; Costa et al., 2007). Lipid biomarkers based on phospholipid fatty acid (PLFA) analysis also hold promise for assessing the efficacy of wetland restoration and management projects (Sims et al., 2013). A greater abundance of fungal biomarkers and increased ratios of gram-negative: Gram-positive bacteria served as an indicator of the restoration status of a calcareous subtropical wetland of Florida Everglades (Inglett et al., 2011). In contrast, a higher prevalence of spore-forming and stress-tolerant gram-positive bacteria like *Actinomycetes* can indicate the onset of environmental stresses, including extreme oligotrophy, drought, or warming in wetlands (Saetre and Baath, 2000; Yao et al., 2000).

Impact of bio-indicators on soil nutrient cycling, soil quality, and ecosystem viability *vis-a-vis* sustainability

Soil is one of the most important natural resources, which determines the existence of plants and animals and governs human civilization. Soil conservation and maintenance are prerequisites for the environment and ecosystem stability. Soil degradation is a common phenomenon and is caused by different natural and manmade factors; hence, its restoration needs to be assured naturally or by human intervention. However, if we do not care for it properly, then soil degradation would reach an advanced stage, and its restoration would become practically difficult as there will be a need for site-specific techniques (conservation agriculture and integrated nutrient management) of restoring soil quality with a strategy to increase soil, water, and nutrient use efficiency (Lal, 2015). Quantification of soil degradation *vis-à-vis* restoration needs some measurable properties that can define the extent of the process. Nowadays, soil as a medium of plant growth is mainly defined in terms of its quality or health.

Soil is a heterogeneous, porous, living, and natural and dynamic system, which is crucial to maintaining the entire ecosystem, and its importance in crop production is judged by its inherent capacity to support crop growth and is reflected by soil quality and health, which is governed by different soil properties. There is always a debate in using the terms soil quality and soil health being defined differentially by different workers in several ways. The broad definition of soil quality as proposed by the Soil Science Society of America (SSSA) is "The ability of a specific type of soil to function within natural or managed ecosystem boundaries, to sustain plant and animal productivity, and maintain or improve air quality and water to support human health and livable" (Karlen et al., 1997). In other ways, soil quality can also be defined as the

"fitness for use," "capacity of a soil to function," "ability to sustain productivity and maintain environmental quality," etc. (Lal, 1993; Acton and Gregorich, 1995; Karlen et al., 1997). The quality of the soil depends on the climatic conditions of the region, soil characteristics, vegetation, anthropogenic influence, management strategy, etc., and complex interaction among them. Soil management and cropping practices are commonly known to alter the factors affecting almost all the biological processes in the soil, as well as soil quality (Bhaduri and Purakayastha, 2014). As a complex functional state, soil quality cannot be measured directly, but it may be inferred from management-induced changes in soil properties, better known as soil quality indicators (the measurable or quantifiable soil attributes). However, these indicators are neither well-defined, nor the set of accepted or approved parameters to characterize or define soil quality exist. The choice of soil attributes and its interpretation of measurements are not simple and straightforward because of their complexity and site-specificity (Bünemann et al., 2018). Moreover, it would be unrealistic or impossible to use all soil attributes as indicators, so a minimum set of soil attributes (better known as the minimum data set) encompassing chemical, physical, and biological soil properties are selected for soil quality assessment (Larson et al., 1994).

Biological indicators for soil nutrient cycling and soil quality

Apart from physical and chemical properties, soil quality, or soil health is also very closely linked to its biological properties, which consist of different attributes like microbial diversity, microbial biomass, enzyme quantities and activities, mineralizable carbon, nitrogen, phosphorus and sulfur, soil respiration, soil organic matter/carbon, and labile carbon pools, etc. It has been well-established that biological properties respond more rapidly to changes in agricultural management practices or environmental changes (Doran et al., 1996; Bhowmik et al., 2019; Kumar et al., 2019). In this context, biological indicators might be important and sensitive indicators to evaluate the effect of different management practices on soil health. The parameters commonly used for soil health assessment are mainly focused on soil properties, which change significantly with time after changes in management practices. It is well-understood that some of the soil's physical, as well as chemical, parameters will take significantly more time to show their changes as compared to biological parameters. So, soil biological parameters could be treated as early indicators for soil health changes. Soil organisms and microbial activity are assumed to be directly responsible for soil ecosystem processes, especially in the decomposition of soil organic matter and related nutrient cycling and transformations, which is further helpful to meet the plant mineral nutrition (Jacoby et al., 2017). All these components are regarded as major components in the global cycling of materials, energy, and nutrients.

Among the different soil biological properties, Bhaduri et al. (2017a) observed that soil respiratory quotient was a key indicator of soil biological processes, indicating the carbon balance in soil across contrasting input management in the rice-wheat cropping system. However, dehydrogenase activity occupied the second position to influence soil biological quality, as revealed from the principal component analysis. Therefore, there is growing evidence that soil microbiological and biological parameters may possess potential as early and sensitive indicators for soil ecological stress and soil health assessment (Schloter et al., 2018). Soil micro-organisms play a crucial role in carbon and nutrient cycling in ecosystems. Soil microbial biomass (living part of soil organic matter) is the total microbial composition and is considered an important indicator of the soil quality index of soil fertility, which depends primarily on the rate of nutrient transformation and its availability, as well as the quality and quantity of organic inputs (Bending et al., 2004). It acts both as a source(s) and sink(s) of available nutrients and plays a critical role in nutrient transformation in terrestrial ecosystems (Jacoby et al., 2017). Based on the overall discussion that comes under the wider arena of the topic, a conceptual diagram for highlighting the scopes of soil health as an indicator of ecosystem resilience and stability has been presented (Figure 2).

Soil bio-indicators related to nutrient cycling in soil ecosystems

Although it is well-established that soil ecosystem functions are likely to be promoted with increased soil microbial diversity, the linkage between microbial diversity and carbon processing in the soil is often criticized based on the concept of functional redundancy (Maron et al., 2018). To that end, the use of microbial physiological indicators (e.g., carbon use efficiency and microbial turnover rate) are recently being promoted to evaluate the warming response of soil microbial community and subsequent decomposition of soil organic matter, SOM (Frey et al., 2013; Hagerty et al., 2014; Sihi et al., 2018; and Walker et al., 2018). These microbial indicators are increasingly being incorporated into microbial and enzyme-based models for predicting SOM decomposition under warming conditions (Allison et al., 2010; Sihi et al., 2016).

The microbial biomass in the soil is generally represented in terms of microbial biomass carbon (MBC), nitrogen (MBN), and phosphorus (MBP). Microbial biomass carbon (MBC) is a relatively small (1–4% of the total SOC pool), labile fraction that quickly responds to carbon availability and is also strongly influenced by the crop management practices and system perturbations usually followed (Verma et al., 2010). It indicates the soil's ability to store and recycle nutrients and energy, and it also serves as a sensitive indicator of change and future trends in organic matter levels and equilibrium. Microbial biomass nitrogen constitutes a significant part of the potentially

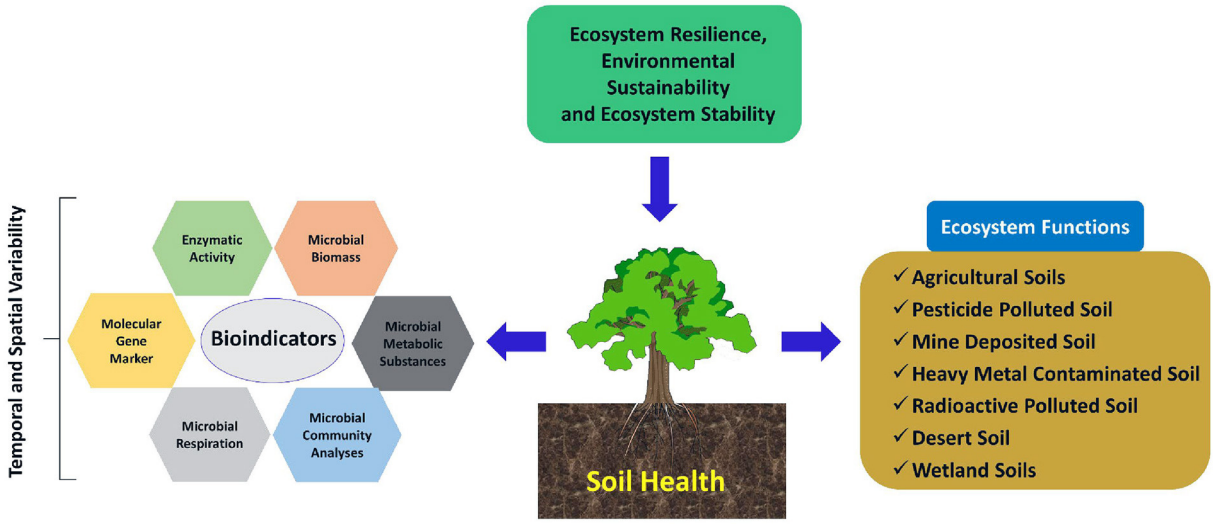

FIGURE 2
Schematic representation of soil health as an indicator of ecosystem resilience and stability. Soil health can be quantified and qualified in terms of various bio-indicators (e.g., Microbial biomass, respiration, metabolic substances, and community analyses as well as some enzymatic activity and molecular techniques) both at temporal and spatial scales. Multiple soil management system can strategize as a critical factor to determine the response mechanism of different bio-indicators.

mineralizable-N and serves both as the transformation agent and source sink of N (Li J. et al., 2019). Consequently, the MBN may have significant impacts on N availability to plants and overall soil N cycling (Singh et al., 2009). Micro-organisms are an integral part of the soil phosphorus (P) cycle and, as such, play an important role in mediating the availability of P to plants. The microbial biomass phosphorous (MBP) is estimated to be around 2–10% of total soil P; however, during different stages of soil development and within litter layers (soil surface), this may be as high as 50% (Achat et al., 2010). The rapid turnover of P in the microbial pool may contribute a major source to the available P pool, as P released from microbial biomass is highly available for plant uptake, as well as the microbial immobilization of inorganic-P protects the P from physico-chemical fixation (Oberson et al., 2001).

Soil with a relatively higher organic matter usually develops larger microbial biomass. Since microbial biomass affects soil fertility and, hence, ecosystem functioning, the measurement of microbial biomass, activity, and nutrient levels have attracted considerable attention to studying complex biogeochemical soil nutrient cycling for nutrient availability. Apart from this, polysaccharides secreted by microbes help in toil aggregation, thereby playing an important role in improving the soil structure as a binding agent. The role of soil microbial biomass in nutrient flow, organic matter turnover, and soil structural stability has led soil microbiologists to use it as a tool for soil management and perturbation studies (Csitári et al., 2021). Thus, microbial biomass could be considered an important parameter for the assessment of soil functional status and soil health as a whole.

Microbial biomass C and N mineralization capacity have primarily been used to estimate changes in soil health due to management and use, whilst dehydrogenase activity, which is a general measure of viable microorganisms, has also been employed in soils affected by heavy metals and pesticides, as well as for the diagnosis of the degree of recovery of degraded soils. The microbial biomass C increased with the intensity of grazing in meadow soils (Banerjee et al., 2000) and with the cereal-pasture rotation (Verma et al., 2010), decreased when the soil was cultivated (Caldwell et al., 1999), and the effect of zero-tillage was not clear (Dalal, 1998). Furthermore, it increased in soils supplied with organic fertilizers (Dalal, 1998), showed an erratic behavior for inorganic N fertilization (Singh and Singh, 1993; Ladd et al., 1994), and did not react coherently to the presence of herbicides (Voos and Groffman, 1997). Moreover, microbial biomass could not be considered a good indicator of heavy metal toxicity in soils (Dalal, 1998). In laboratory experiments, it has been observed that soil microbial biomass decreases with the addition of Cd or Cu. The effect of heavy metals on microbial biomass is not very clear and depends on the nature and concentration of heavy metals. In general, at low concentrations, the effect may not be very pronounced; however, at high concentrations, the heavy metals reduced the content of microbial biomass (Yuangen et al., 2004).

Other potential bio-indicators like kinetic analysis (V_{max} and K_m) of nutrient-degrading enzymes (carbon, nitrogen, and phosphorus) can shed light on the stoichiometric controls of SOM decomposition under warming conditions (Sihi et al., 2019). A systemic impact of nutrient limitation

on soil microbial functions can be revealed by more advanced multi-omics techniques such as community proteogenomics (Yao et al., 2018). A shift in soil microbial biomass carbon:nitrogen:phosphorus (C: N:P) ratio can also be used to evaluate the effect of nutrient limitation in a system, where nutrient limitation in the soil can influence microbial biomass stoichiometry plasticity (Griffiths et al., 2012; Hartman and Richardson, 2013; Fanin et al., 2017). Other important environmental disturbances like drought events can be captured by investigating the richness of soil fungal community. However, the presence of biocrust-forming mosses is known to mitigate this response in arid areas (Delgado-Baquerizo et al., 2018). Fire events, which are often associated with warming and drought events in arid lands, can modulate activities of enzymes released by soil fungi, especially N-Acetylglutamate synthase (NAG), with a more pronounced effect with increased intensity and/or severity of fire (Boerner and Brinkman, 2003; Turner et al., 2007; Liao et al., 2013). The size and diversity of specific functional microbial groups, such as arbuscular mycorrhizal fungi (AMF) and nitrifying bacteria communities, also have the potential to characterize the effects of management on the sustainability of soil (Helgason et al., 1998; Chang et al., 2001). Environmental stresses in coastal regions can be quantified using a systematic evaluation of microbial community structure and function, especially those related to salt tolerance (Chambers et al., 2016). For example, a recent study identified specific soil bacterial taxa (Gammaproteobacteria and Bacteroidetes), where the prevalence of those taxa positively correlated with soil salinity and served as ideal bio-indicators for soil and ecosystem responses to sea-level rise and associated salt-water intrusions in saline soils (Rath et al., 2019).

Soil bio-indicators as influenced by management practices for ecosystem viability/sustainability

In agricultural production systems, enhanced soil health should be accompanied by high productivity without detrimental effects on the environment (Govaerts et al., 2006; Griffiths et al., 2010). Researchers have evaluated the effect of agricultural management systems on soil health. Reduced disturbance systems, such as reduced tillage, generally resulted in improved soil health characteristics. Karlen et al. (1994) reported that surface soil in no-till systems had higher aggregate stability, total carbon, microbial activity, and earthworm populations compared to conventionally tilled soil. Estimated soil loss measured by simulated rainfall collection was two to four times greater in plowed soil compared to no-till. Deciphering the landscape variability along with the soil properties is indispensable to planning land use and a sustainable agricultural system.

It is well-known that there are microbial parameters that have the potential to be used as soil health indicators. The microbial properties (viz., diversity and distribution) are sensitive to agronomic management (Bending et al., 2000; Marx et al., 2001). Therefore, information related to microbial characteristics may be used to study soil health. Microbial characteristics may act as consistent soil health indicators influencing soil quality. Microbial parameters are more likely to be affected by agronomic management practices than biochemical parameters. Bending et al. (2004) highlighted that both biochemical and microbial analyses may be carried out to compare the impacts of management on soil health. Land-use changes have a significant effect on organic carbon, nitrogen, and C: N ratios (Knops and Tilman, 2000), as well as subsequent crop productivity. Some reports suggested that restoration processes had a tremendous impact on the quality of soil (McKinley, 2001). Managing soil ecosystems play a pivotal role in the revival of degraded soils. It is predicted that, if the ecosystem is resilient, there will be no disturbance to the microbial structures, and it will remain as such for some time. To date, many indicators have been considered and tested as potential soil health indicators, and many of these indicators have been successful in a variety of agricultural systems (Glover et al., 2000; Reganold et al., 2001; Bhowmik et al., 2016).

Predominance and succession of soil microbiota are invariably related to temporal and spatial differences, which can be appropriately used as an indicator of soil health. A lot of curiosity exists related to the study of microbial biodiversity and its activity on soil health and quality. Studying microbial diversity with culture-based early techniques had several limitations that can now be removed by using advanced techniques like the use of molecular BIOLOG and techniques, denaturing gradient gel electrophoresis (DGGE), metagenomic community-based approaches, analyzing lipids and phospholipids profile, BIOLOG, etc. Soil microbial activity is different from microbial biomass and diversity. Microbial activity indicates a wide range of physiological activities intimidated by soil micro-organisms that include microbial respiration or soil respiration, metabolic quotient, soil enzyme activities, etc. For example, soil respiration is a function of the total soil biological activity, including microbial activity. Some researchers feel that quantification of soil microbial biomass does not give complete information regarding soil health/quality. It is more useful when used in combination with the quantity of respiration to derive the metabolic quotient, which has been used to provide information on changes to the structure and functioning of soil microbial communities (Wardle and Ghan, 1995).

Many metabolic substances can also be used as soil health indicators such as sterols (Ergosterol), antibiotics, protein (Glomalin or Glomalin-related soil proteins), soil enzymes (phosphatase, urease, sulphatase, and dehydrogenase), etc. Soil enzymes are important attributes that are involved in the dynamics of soil nutrient transformation and make nutrients available to plants. It is proteinaceous and is released by

microbes during their growth and activity. Therefore, overall soil microbial activity can be derived from soil enzyme activity (Bhowmik et al., 2019; Kumar et al., 2019) and related to soil health.

The microbial redox system and oxidative activities can be measured through soil dehydrogenase activity using various methods. For example, dehydrogenase activity may increase or decrease with tillage (Bergstrom et al., 1998) depending on the soil management. The application of organic manures, green manures chemical fertilizers, landfill effluents, and industrial waste tends to increase dehydrogenase activity (Bardgett et al., 1996; Langer and Günther, 2001; Dhull et al., 2004). The advantage of using soil dehydrogenase activity as an indicator is that it is not usually affected by heavy metals if present in the sample at lower doses (Yuangen et al., 2004). It has the potential to indicate the extent of recovery of degraded soils. For example, soil contaminated by petroleum spillage can be easily assessed using dehydrogenase activity (Margesin et al., 2000). Likewise, the soil management-induced changes can be assessed through microbial biomass C.

β-glucosidase is the most important enzyme involved in the carbon cycle. So, it is generally used for soil health evaluation. Previous reports (Saviozzi et al., 2001; Mawlong et al., 2021) suggested that β-glucosidase activity decreases significantly in arable soils owing to agronomic management, compared to undisturbed forest or meadow soils. β-glucosidase has a negative relation with agricultural practices (agronomic management). However, some agricultural practices, such as the application of organic manures, may increase the β-glucosidase activity (Mawlong et al., 2021), which may lead to an erroneous interpretation of results. Thus, its value as an indicator of soil quality is reduced. Another soil enzyme, urease, which is responsible for the catalysis of urea (urea hydrolysis), is generally not considered for measurement of soil quality as its activity is highly subjective to soil amendments (Chakrabarti et al., 2000), as well as soil management (Saviozzi et al., 2001; Mawlong et al., 2021).

By considering individual indicators or groups of indicators, soil health can be assessed; however, some researchers also developed indices based on the combination of biological indicators to evaluate soil health. For example, the Biological Index of Fertility (BIF) involves the measurement of respiration and enzymatic activities for its calculation (Vittori Antisari et al., 2021). Microbial index of soil (Mi) involves the measurement of microbial biomass Carbon (C) and Nitrogen (N), potentially mineralizable N, soil respiration, bacterial population, mycorrhizal infection, dehydrogenase, and phosphatase activities, and it is derived by Combining all the data with a crop index and nutrient index (Kang et al., 2005). Similarly, the Enzyme Activity Number (EAN) involves dehydrogenase, catalase, alkaline phosphatase, amylase, and protease enzymes (Beck et al., 1984). Soil Biological Fertility Index was proposed for soil monitoring (Pompili et al., 2008; Renzi et al., 2017), and it is based on soil organic matter (SOM = SOC × 1.724), basal respiration (C_{bas}), cumulated respiration (C_{cum}), microbial biomass carbon (C_{mic}), and metabolic quotient (qCO_2). This indicator is more precise and sensitive compared to other microbial and enzymatic activity (Pompili et al., 2008; Renzi et al., 2017).

Besides the soil parameters related to microbial activity, soil organic carbon content and its different fractions/ pool can widely be used for soil health measurement alone; however, its response to actual management practices is often slower than biological activity, which is closely linked to microbial activity. Therefore, it was believed that the activity of microorganisms involved in the carbon cycle is a better indicator than the carbon content itself (Smith, 2004; Marinari et al., 2006). In the study of soil carbon, labile carbon pools are widely used for soil quality assessment and will be a better and more sensitive indicator for soil quality. More often, SOC and its lability are highly recommended as an indicator of soil sustainability as it is associated with short-term nutrient cycling (Mtambanengwe and Mapfum, 2008; Bhowmik et al., 2017a). Different methods are used to measure the labile pools of carbon, as well as carbon fractions for the soil quality determination. Labile carbon and its relative proportion to total organic carbon content are also widely used as the index called Carbon management index (CMI), which is determined based on the labile carbon content and its proportion over the total carbon (Blair et al., 1995; Verma et al., 2010). Labile carbon determination varies depending on the method used; its values also vary, however, and it can also be considered as the soil quality indicator for most of the studies.

Bio-indicators related to nitrogen cycling in soil ecosystems and aquatic environment

Soil health-promoting management practices enable synergy between multiple soil functions and the prospect of soil-based ecosystem services, in terms of crop production, nutrient recycling, water infiltration and purification, and climate moderation. Worldwide, only half of the nitrogen (N) fertilizers are taken up by crops, thus, a particularly critical soil health need is to improve N use efficiency by understanding the microbial N transformations that help synchronize N availability with plant uptake, and in turn, decrease N losses from soil. Nitrification is the most important component of the biogeochemical N cycle and involves the oxidation of ammonia to nitrite, and then, to nitrate by groups of micro-organisms known as nitrifiers. Nitrification increases the likelihood of N loss from soils. In ammonia oxidation, the first step of nitrification is catalyzed by the *amoA* gene encoding the α-subunit of the ammonia monooxygenase enzyme. Ammonia oxidation is completed in two steps: by two distinct groups of nitrifiers, namely, ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). These two groups have shown huge potential as molecular bio-indicators.

Like the agroecosystem, water bodies and the aquatic environment are also interrelated to each other and depend on the activities associated with our agricultural management practices. The use of fertilizers, pesticides, and other agrochemicals has an immense impact on the aquatic environment. To quantify the changes in the aquatic environment, we also need some sensitive indicators to evaluate them. The main consequences of human activities are the release and addition of different organic compounds, pollutants, pesticides, agrochemicals, antibiotics, etc. (Sauve and Desrosiers, 2014; Geissen et al., 2015). Besides the addition of different chemicals, other stresses were also developed in the aquatic environment (like raise in temperature, acidification, acid mine drainage, etc.). In earlier times, different indicators like fish, invertebrates, aquatic animals, and animals are used to monitor the change in the aquatic environment (Li J. et al., 2019). After the presence of particular bacteria, fungi are also used to monitor changes in the aquatic environment. However, these indicators have several limitations like being unable to culture in the laboratory condition, microscopic counting, etc. Besides, in recent days, analysis of the microbiome (microbial community structure, diversity, and patterns) is used to assess the aquatic environment (Michan et al., 2021). In the heavy metal contamination site, the proliferation of resistant bacteria, and bacterial genes was used to quantify the impact (Thomas IV et al., 2020). Microbial abundance, diversity, and activity are significantly affected by the surrounding environment and are highly sensitive to natural and anthropogenic activities, hence, perfect potential indicators of environmental disturbances (Khan et al., 2018; Milan et al., 2018).

However, in recent days, with the development of advanced technologies, DNA-based techniques are being used to detect and quantify the microbes and resistant genes in water samples. Different technology like metagenomic analysis, 16 S RNA, PCR, and qPCR-based analysis are used for evaluation. Microbes have an impact on environmental changes, hence, based on these facts, mass spectrometry imaging (MSI) has been considered a general tool to study micro-organisms (Maloof et al., 2020). PCR-based techniques are also being used to study the molecular viral as they are sensitive, as well as highly specific.

Computational tools and bioinformatics are also employed as environmental monitoring tools, such as pesticide bioremediation. It is executed through an online platform of biodegradative (open access) databases and necessary information provided on biodegradation pathways, as well as microbes-mediated biodegradation of xenobiotic (pesticide) molecules (Nolte et al., 2018). These databases include the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD), Biodegradation Network-Molecular Biology database (Bionemo), Pesticide Target interaction database (PTID), Microbial Genome Database (MBGD), Biodegradative Oxygenases Database (OxDBase), BioCyc, and MetaCyc for both windows, as well as Linux operating systems (Arora and Bae, 2014). Another new bioinformatics approach, BarcodingGO, was developed by a team of scientists from Brazil, aiming for environmental DNA and bioinformatics as an environmental monitoring tool under simulated conditions (Nunes et al., 2021). This was performed through a biodiversity survey (analyzing DNA released by organisms living in a specific environment) to measure the impact of an environmental disaster, identified by the unique quick response (QR) codes that represented pre- and post-scenarios of environmental disaster.

A comprehensive and easy-to-read information table, encompassing different soil types and their bio-indicators, is presented (Table 1).

Role of ammonia oxidizers as soil bio-indicators sensitive to soil management practices and environmental conditions

Assessments of the ammonia oxidizer community have revealed that the AOA and AOB not only respond differently to abiotic and biotic soil characteristics (niche specialization) but also possess different patterns of nutrient utilization (niche differentiation) (Zhalnina et al., 2012). Metagenomic studies have enabled us to realize that these two assemblages have different substrate (ammonia) affinities like ammonia, AOA has higher affinity and substrate toleration (Prosser and Nicol, 2012). Acidic soil pH has been reported to support more AOA as compared to AOB communities (Nicol et al., 2008); however, results might vary with geographical location and site differences (Jiang et al., 2014). Metabolically, ammonia oxidizers have always been considered to be mainly autotrophs (Hallam et al., 2006). Recent evidence from agricultural soils supports the fact that the potential to assimilate organic compounds (i.e., heterotrophic or mixotrophic metabolism) can be more prevalent in AOA than in AOB (Xue et al., 2016).

Quantitative PCR (qPCR) analysis and nitrification kinetic studies are used to measure AOA and AOB, and their relative contribution to nitrification in agricultural soils. The relative contribution of AOA and AOB communities responding to nitrification might differ in agricultural soils. AOA co-exists with AOB in agricultural soil but responds differently to climatic conditions and nitrogen management (Kowalchuk et al., 2000; Taylor et al., 2012, 2013; Habteselassie et al., 2013; Jiang et al., 2014; Banning et al., 2015; Giguere et al., 2015; Bhowmik et al., 2016; Ouyang et al., 2016). There has been evidence in the literature that AOB communities, as compared to AOA, are more responsive to the application of N fertilizer and have dominated nitrification in most agricultural soils except for acidic soils (Jia and Conrad, 2009; Di et al., 2010; Zhang et al., 2010; Xia et al., 2011; Ai et al., 2013; Giguere et al., 2015; Ouyang et al.,

TABLE 1 Important bio-indicators identified for different soil ecosystems.

SI. No.	Soil or management practices	Identified bioindicators	References
1.	Agricultural management practices or environmental changes	Microbial biomass C, functionality and diversity of microbial communities, metabolic quotient (qCO_2), microbial quotient (qMIC), enzyme activities, mineralizable carbon, nitrogen, phosphorus and sulfur, soil respiration, soil organic matter/carbon and labile carbon pools, Carbon management index (CMI), Biological Index of Fertility, Microbial index	Blair et al. (1995), Andersen and Sparling (1997); Kang et al. (2005), Pompili et al. (2008), Verma et al. (2010), Gómez-Sagasti et al. (2012), Bhaduri et al. (2017a,b); Renzi et al. (2017), Bhowmik et al. (2019), Kumar et al. (2019); Li L. et al. (2019), Vittori Antisari et al. (2021)
2.	Land use intensification, different fertilizer history, Pollution of agrochemicals	Soil microbial community (composition and structure), soil enzyme activity, 16S rRNA amplicon sequencing based operational taxonomic units (OTUs)	Gomez et al. (2004), Saha et al. (2015, 2016a,b); Gaind and Singh (2016); Pérez-Brandán et al. (2016), Sahoo et al. (2016), Mahapatra et al. (2017), Sihi et al. (2017), Xu et al. (2017), Kumar et al. (2018)
3.	Exposure of soils to trace (or, heavy) metals, organic pollutants, mine tailing, radioactive waste	Earthworm, acidophilic bacteria, stress proteins (hsp70 and hsp60), functional genes related to fatty acid metabolism (*acc*, *fab*, and *fad* genes), Shannon diversity index, metabolic quotient and cellulase activity, urease and invertase enzyme activities, nematode diversity, maturity index, cryptogamic biota (lichens and bryophytes)	Coleman et al. (1998); Korthals et al. (1998); El-Bestawy et al. (2005); Ciarkowska et al. (2014); Galitskaya et al. (2015), Patel et al. (2016), Huggett (2018); Rola and Osyczka (2018)
4.	Gold mining in tropical rainforests	Ratio of soil denitrifying enzyme activity and substrate induced respiration	Schimann et al. (2012)
5.	Radioactive pollution, contaminated with radionuclides	Earthworms, millipedes, collembolans and oribatid mites, land snails, aquatic mosses	Gaso et al. (1995), Hongve et al. (2002), Zaitsev et al. (2014), Rybak et al. (2021)
6.	Wetland pollution and restoration	Microbial diversity and community comparison, Ratios of oligotrophic:copiotrophic organisms such as the ratio of ammonia-oxidizing archaea (AOA) to ammonia-oxidizing bacteria (AOB)	Dziock et al. (2006); Martens-Habbena et al. (2009)
7.	Warming response of soil and effect of climate change	Microbial physiological indicators (carbon use efficiency, microbial turnover rate), kinetic analysis (V_{max} and K_m) of nutrient (carbon, nitrogen and phosphorus) degrading enzymes	Frey et al. (2013), Hagerty et al. (2014), Sihi et al. (2018, 2019), Walker et al. (2018)
8.	Sandy ecosystems	Soil nematode communities	Guan et al. (2015)
9.	Fire	N-Acetylglutamate synthase (NAG)	Boerner and Brinkman (2003), Turner et al. (2007), Liao et al. (2013)
10.	Sea level rise and salt-water intrusions	Soil bacterial taxa (Gammaproteobacteria and Bacteroidetes)	Rath et al. (2019)
11.	Aquatic environment	Fish, invertebrates, aquatic animals, microbiome (microbial community structure, diversity and patterns), metagenomic analysis,16s RNA, PCR and qPCR-based analysis, mass spectrometry imaging	Li J. et al. (2019), Maloof et al. (2020), Michan et al. (2021)

2016; Song et al., 2016). On contrary, a recent study by Orellana et al. (2018) on soil metagenomes from agricultural soil showed the increased response of AOA populations to synthetic N fertilization as compared to AOB. All these above-mentioned studies have vastly benefitted directly or indirectly from the sequence database generated with next-generation sequencing (NGS).

The literature is replete with studies that suggest the potential of the nitrifier and denitrifier gene copy numbers to be sensitive indicators of key management practices and are

influenced by the amount of available nitrogen (N) and carbon (C), and climatic factors, such as soil temperature and moisture. Long-term organic management systems (20 years) increased the microbial diversity resulting in enhanced N mineralization potential (Berthrong et al., 2013). In acidic soil in China, the community composition of AOA, and not AOB, was affected due to fertilization (He et al., 2007). This trend was reversed in alkaline soil under similar fertilization regimes (Shen et al., 2008). In a 44-year-old grassland fertilizer experiment, organic input (cattle slurry) increased *amoA* gene of ammonia-oxidizing archaea (AOA) significantly, whereas chemical N fertilization increased the ammonia-oxidizing bacteria (AOB) population (Zhou et al., 2015). Rudisill et al. (2016) also reported that organic fertility management practices stimulate nitrification, which is mainly reflected by increased AOB activity. In Zn contaminated soil with the recovery of nitrification activity, a shift in the community structure of AOB was observed without any observable difference in AOA (Mertens et al., 2009). Previous studies by Wessén et al. (2011) and Tsiknia et al. (2015) across a diverse range of management practices and climatic conditions suggested that *amoA* gene from functional microbial communities of AOA and AOB was an effective biological indicator. In biogeochemical cycling, however, some studies suggest that the direct effect of management regulates nutrient availability and not necessarily soil microbial activity or diversity (Wood et al., 2015).

Bio-indicators, e.g., nitrification potential, as well as *amoA* gene, copy numbers of AOA and AOB, also respond to interactions between soil abiotic and biotic properties (Fortuna et al., 2012). Several meta-analyses have concluded that both AOA and AOB populations responded to the application of N (Carey et al., 2016; Ouyang et al., 2018). Robinson et al. (2014) showed that soil pH regulated AOA and AOB in urine-treated soils. A study by Höfferle et al. (2010) showed that sewage-polluted soil increased the abundance of AOB over AOA. Mundepi et al. (2019) reported that in poultry-litter-amended soils, an abundance of AOB decreased, whereas AOA increased. However, functionally, AOB was contributing more to nitrification despite lower populations as compared to AOA. Response of AOA and AOB to N addition and soil moisture conditions in a typical temperate steppe suggested that AOA and AOB had distinct ecological niches, and AOB was more sensitive to N and precipitation and was the main driver of nitrification in such soils (Chen et al., 2013). A study was conducted by Ouyang et al. (2016) to measure the response of ammonia-oxidizing nitrifiers in agricultural soils treated with ammonium sulfate or steer waste compost. The AOB, as compared to AOA, contributed to nitrification potential in these N fertilized soils. A study by Giguere et al. (2015) revealed that changes in AOB populations were more sensitive to net nitrification rates in cropped soil, whereas AOA populations responded more to non-cropped soil. Banning et al. (2015) reported that in semi-arid agricultural soil, *amoA* AOB dominated nitrification activity and was four-fold higher than *amoA* AOA gene copies in the 0–10-cm soil layer. In a 2-year field experiment, Muema et al. (2016) observed that the AOB was more sensitive to organic inputs, whereas AOA to chemical fertilization. On the other hand, Shen et al. (2015) reported that the long-term application of organic manures with or without mineral NPK fertilizer increases the population of AOA in acidic red soil. However, other studies observed that long-term or short-term manure fertilization increased the populations of AOB rather than AOA in paddy soil (Wang et al., 2014). Ammonia oxidizers are also sensitive to substrate concentration in the soil. High ammonia substrate concentration favored AOB, whereas low ammonia substrate conditions favored AOA in grassland soils treated with animal waste (Di et al., 2010). Liu et al. (2016) found that ammonia-oxidizing populations of AOA and AOB also responded to the difference in the intensities of grazing in a semi-arid grassland. However, another study found that in N-limited natural grasslands, AOA is an important driver of nitrification, whereas in N-amended grassland soils, the contribution of AOB toward gross nitrification is more dominant (Sterngren et al., 2015). Radl et al. (2014) reported that management practices like cattle overwintering could also be a major factor that could alter the AOA vs. AOB populations. Their study concluded that the AOA populations dominated in no grazing, whereas the AOB outnumbered AOA in severely grazed sites.

Ammonia oxidizers are also effective indicators of the amount of oxygen present in the soil. Liu et al. (2015) observed that AOA rather than AOB had better adaptability in flooded soils where the oxygen concentration was lower. Another group of researchers also reported that AOB was more sensitive to oxygen availability as compared to AOA (Ke et al., 2015). Under simulated stress conditions like drought (drying-wetting cycle) in grassland soil that rarely experience drought, both the AOA and AOB populations showed poor resistance and resilience (Thion and Prosser, 2014). However, a study conducted by Yarwood et al. (2013) reported that AOA had the potential to persist in a 12-year soil ecosystem with no organic matter input. To determine the resilience of nitrification activity under increased acidity levels, Sher et al. (2013) found that in arid and semi-arid soils under increased acidity levels, AOA was predominant during water stress conditions and in higher temperatures, whereas the AOB was predominant in humid conditions with higher precipitation.

Impact of bio-indicators on crop productivity: A global scenario

Intensive agricultural management has contributed to economic and social development. However, it is also responsible for land degradation, biodiversity loss and ground water depletion, and contamination (Kirschenmann, 2010). The

United Nations Environment Programme (UNEP) sponsored project, the Global Assessment of Soil Degradation (GLASOD), estimated that agricultural land has undergone soil degradation to an extent of 38%. In a certain type of soil, it has been observed that crop productivity has declined 3–12 times in degraded soils compared to normal soils (Kyawt et al., 2015). Among the crops, rice and wheat show the steepest decline in crop productivity in degraded/erosion-prone soils (Panagos et al., 2018). Without major capital investments and engineering inputs, agriculture has become unsustainable. More recently, there have been concerns about the maintenance of crop productivity and environmental quality to maintain the human population in the future (Liu et al., 2006). Tools for assessing soil health are required to ensure sustainable agriculture for future generations by evaluating the effects of management practices on soil processes. Soil quality or soil health has been considered an indicator of crop productivity (Doran and Parkin, 1994; Acton and Gregorich, 1995; Karlen et al., 1997). Crop productivity has been traditionally linked to soil health, wherein it is assumed that improvements in soil health will alleviate growth-limiting factors and, hence, improve yields (Miner et al., 2012).

Evaluation of soil health should involve physical, chemical, and biological soil properties (Bhardwaj et al., 2011). Until now, changes in physical, chemical, or biological soil properties are monitored for assessing soil quality. Many researchers believe that combining several indicators into a single index would give a better perception of soil health rather than individual parameters (Sharma et al., 2005). Some others feel that only the linked parameters are important soil functions that should be combined for the calculation of indices to predict the sustainability or productivity of an agro-ecosystem (Herrick, 2000). It is difficult to develop a single universally accepted standard for each indicator as the soils, as well as management practices, vary widely. It is even more difficult to combine these indicators to derive an index that is applicable to all soil types or agroecosystems. Despite the emphasis on multi-parameter indexing (Doran and Parkin, 1994; Diack and Stott, 2001; Mandal et al., 2008; Sharma et al., 2008; Bhardwaj et al., 2011), many researchers like Visser and Parkinson (1992) believe that laboratory procedures for determination of decomposition rates, microbial biomass C, and soil enzymes are efficient and can be used independently for assessment of soil health.

To date, many quantifiable soil quality indices have been developed by many researchers. Larson et al. (1994) established the concept of a Minimum Data Set (MDS). They recommended the importance of identifying the set of indicators that are more sensitive to a particular soil management practice and only the selected indicators should be considered and used to assess the sustainability of the management practice in question. Doran and Parkin (1994) worked on the same line and expanded the minimum data set of Arshad and Coen (1992) by including biological properties that were not included earlier. However, DuPont et al. (2021) suggested that soil health indicator minimum data sets should be regional and management of goals that specifically identify and quantify the factors that affect crop productivity.

Soil biological properties are sensitive, essential, and give more variable results. Therefore, biological properties are indispensable when considering the characterizing of soil health (DuPont et al., 2021). Among the bio indicators, only microbial biomass C is being used as the only biological parameter for calculating the soil quality index. The terrestrial SOC is a major pool of soil organic matter. Emphasized that SOC plays a major role in determining the degree of soil erosion. Soil microbial respiration and nutrient cycle are directly dependent on SOC. Other soil health indicators directly dependent on SOC are available to water (Hudson, 1994), infiltration rate and capacity (MacRae and Mehuys, 1985; Pikul and Zuzel, 1994), and soil aggregate formation and stability (Oades, 1984; MacRae and Mehuys, 1985; Tisdall and Oades, 2012). Soil organic carbon or SOM is directly linked to many other soil health indicators, as well as crop productivity (National Research Council, 1993; Cannell and Hawes, 1994; Larson et al., 1994). SOM is one of the few indicators that relate to both soil health and crop productivity (de Lima et al., 2008; Van Eekeren et al., 2010; Hanse et al., 2011). Doran and Parkin (1994) emphasized the role of cropping systems, along with soil management systems and their impact on SOM. It is reported that even if SOM is not correlated to crop productivity goal, it must be included in the minimum data set as it influences multiple soil functions, such as microbial activity, nutrient cycling, soil carbon accumulation, and water relations (DuPont et al., 2021).

The invertebrates have also been used as indicators of soil health but, to date, no universally accepted criteria for its use have been found. In the same line, Linden et al. (1994) worked on earthworms, which are the most widely used invertebrate as soil indicators. They reported that the determination of earthworm activity in some soils may be relevant but not crucial. It was suggested that earthworms play vital roles in processes like water infiltration and crop root aeration and development, but earthworms are not obligatory for these processes, and high-quality soils may exist even in absence of earthworms.

An account of "resistance and resilience" of bio-indicators

Resistance is the property of an ecosystem to remain "essentially unchanged" when subjected to disturbance/stress. However, resilience refers to the capacity to recover from disturbance or withstand ongoing pressures/stress. It is a measure of how well an ecosystem can tolerate disturbance(s) without collapsing. Resistance can be measured just after the disturbance, whereas resilience can be assessed progressively with time after the disturbance, i.e., can only be determined after

the disturbance has ceased. A major goal in restoration is to restore both resistance and resilience so that the restored entity is self-perpetuating and does not require ongoing interventions to be sustainable (Lake, 2013). Ecological restoration is widely practiced for rehabilitating ecosystems and habitats that have been degraded or impaired through human use or other causes. In other terms, resilience can also be defined as the capacity of a system to absorb disturbance and re-organize in ways that retain essentially the same functions, structures, identities, and feedback (Walker et al., 2004). This includes two important mechanisms of resilience, namely, resistance to change and recovery from change.

Studying soil resistance and resilience is another new and emerging facet of assessing soil health. In a study conducted by Griffiths et al. (2005), both physical and biological stability and resilience were studied for soils treated with Cd, Cu, and Zn, digested or undigested sewage sludge. They found that the rate of mineralization of DOC released by alternate drying/wetting cycle was reduced by Zn contamination, while biological resilience was increased in the Zn-contaminated soil and reduced by Cd contamination. The effects of metals (Cd and Cu-contaminated) on physical resilience, in terms of expansion indices (indicating soil aggregation), were greater than the effects on soil C. In another study after imposing heat stress at 42°C for 24 h, the abundance, structure, and activity of two specialized soil bacterial functional groups (denitrifiers and nitrite oxidizers) were studied periodically to assess resistance and resilience (Wertz et al., 2007). The behavior showed differential results while the nitrite oxidizers were more affected, but reducing the diversity of both groups did not impair either their resistance or their resilience following the disturbance. Kumar et al. (2014), in another study, found that the combined application of NPK (balanced) and FYM (from a long-term experimental soil under maize crop) was most effective in enhancing resistance and resilience of soil microbial activity in terms of substrate-induced respiration and dehydrogenase activity against heat stress imposed at 48°C for 24 h. Here, an attempt was made to accumulate the important information generated from soil resilience models for uplifting the overall crop productivity and environmental quality (Table 2).

Ecosystem restoration *vis-à-vis* sustainable development goal

The 2030 Agenda for Sustainable Development, adopted by all United Nations Member States, prepared a blueprint in 2015 for peace and prosperity for all people and the planet earth, for present and future scenarios. There are a total of 17 Sustainable Development Goals (SDGs), which are recognized for ending poverty and other deprivations with strategies that improve health and education, reduce inequality, and spur economic growth– simultaneously while tackling climate change and working to preserve our oceans and forests. Addressing the SDG has been an urgent call for action by all countries, both developed and developing, and in a global partnership.

It has been noticed and reported by several researchers around the world that key ecosystem renders numerous essential services to food and agriculture, including supply of freshwater, protection against hazards, and provision of habitat for species, such as fish and pollinators, which are diminishing rapidly. Moreover, the degradation of land and marine ecosystems has taken a toll on the wellbeing of 3.2 billion people and charges about 10 percent of the annual global GDP due to the loss of species and ecosystem services. Among the 17 goals, the SDG 15 of the 2030 Agenda for Sustainable Development is devoted to "*protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and halt biodiversity loss*" (source: United Nations, 2022).

Considering the importance, UN General Assembly declared "The UN Decade on Ecosystem Restoration 2021–2030," which aims to upscale the restoration strategies for degraded and destroyed ecosystems as an established measure to combat climate change and enhance food security, water supply, and biodiversity. Further, the successful restoration process of 350 million hectares of degraded land by 2030 could be able to generate 9 trillion US$ in ecosystem services and take an additional 13–26 GT of GHG out of the atmosphere (source: UNEP, 2019).

Ecosystem restoration is considered one of the fundamentals to achieving the SDGs, where several other globally challenging factors like climate change, poverty eradication, food security, water, and biodiversity conservation are closely linked.

The UN Environment, as well as the Food and Agriculture Organization (FAO) of the United Nations, has taken responsibility for the implementation of the UN Decade on Ecosystem Restoration and has prioritized the following areas in this regard (source: UNEP, 2019):

i. Innovations on biodiversity and land degradation
ii. Protection of the marine environment from land-based activities
iii. Protecting the ecological balance of food chains by conserving and sustainably using mangrove ecosystems
iv. Sustainable coral reefs management
v. Deforestation and agricultural commodity supply chains
vi. Sustainable nitrogen management
vii. Rangelands and pastoralism
viii. Sustainable blue economy
ix. Sustainable peatland management for tackling climate change

TABLE 2 Soil resilience model for improving agricultural productivity and environmental quality.

Study site	Targeted stress environment	Crop/cropping system	Implications	References
1. N-E Nile Delta, Egypt	Soil degradation by waterlogging salinization, and alkalinization	Irrigated and rainfed croplands	Soil degradation processes dominated over the soil resilience causing a decline soil productivity index by 45.82% of the total area over a span of 35 years	Kawy and Ali (2012)
2. South-central region of the State of Parana', Brazil	Long-term tillage impacts vs. continuous no-till	Soybean-maize system	Increasing labile C fractions under continuous no-till has been reflected for highest resilience index and productivity	de Moraes Sa et al. (2014)
3. Gongzhuling, Jilin province, China	Long-Term fertilization trials	Maize	Organic matter (FYM and straw) amendments mitigated the climate change effects on crop production by enhancing soil resilience and showing better SOC, nutrients and soil water storage	Song et al. (2015)
4. Southern England, Rothamsted research station and nearby areas	Physical stress (uniaxial compaction) and biological stress (transient heat or persistent Cu stress)	Arable and grasslands	OM and clay content critically determined the soil resilience; grassland soils were more resilient to both physical and biological stresses than the arable soils	Gregory et al. (2009)
5. IARI, New Delhi, India	Short-term heat stress imposed at soils of long-term fertilization trials	Maize	NPK + FYM was most resilient against heat stress in terms of soil microbial activity (substrate-induced respiration and dehydrogenase activity)	Kumar et al. (2014)
6. Scottish Agricultural College, Auchincruive Estate, Scotland	Heavy metal (Cd, Cu, Zn)-contaminated sewage sludge	–	Medium-Term (9 years after application) effects of metal-contaminated sludges on the stability and resilience of a sandy clay loam soil. The most obvious effect of sludge addition was an increase in soil C content, but there were no significant effects of metals on soil C. Medium-Term (9 years after application) effects of metal-contaminated sludges on the stability andresilience of a sandy clay loam soil. The most obvious effect ofsludge addition was an increase in soil C content, but therewere no significant effects of metals on soil C 9 years of metal-contaminated sludges reflected on the resilience, increase in soil C content was prominent by applying sludge while metal contamination disturbed the C- metabolism and physical resilience	Griffiths et al. (2005)
7. Inner Mongolian Grassland Ecosystem Research Station, Chinese Academy of Sciences	Mechanical (physical) stresses imposed under arable systems, including vehicle traffic	Ungrazed and undisturbed soils	Mechanical resilience of fine-textured sandy loam soil was improved by adding woodchip biochar, with further impact on stability, compressive behavior and cohesion. Better proportion of medium to fine pores and improved water retention was noticed	Ajayi and Horn (2017)
8. Rubite, Granada, Spain	Organic waste (olive-mill solid waste) and its vermicompost was applied to a degraded soil	Marginal agricultural lands	Soil resilience factors of the disturbed soil like the amplitude (*period of recovery*) and the elasticity *(speed of recovery*) to the initial state after disturbance, were successfully monitored by o-diphenol oxidase, β-glucosidase and dehydrogenase activities. Vermicomposting promoted microbial activities in the degraded soil without any toxicity effects	Benitez et al. (2004)

Conclusion and way forward

The ecosystem of the earth can be disturbed naturally over time or through anthropogenic intervention. In the changing era of climatic aberrations, chances of land degradation and loss of biodiversity (both above-ground and underground) are unprecedented. Hence, it is crucial to develop a sound understanding of how the recovery of soil ecosystems is possible. The existing and upcoming concepts of function-based soil quality, resistance, and resilience are interlinked processes that may help in ecosystem restoration. Sustainable soil processes as regulated by effective soil indicators can influence both crop productivity and environmental protection. Of late, environmental issues after anthropogenic activities like mining and agrochemical pollution have created much concern and can be monitored from time-to-time using soil bio-indicators. In this review, the latest techniques for measuring function-based soil bio-indicators for both arable soil and aquatic wetlands were discussed in detail, which shows the possibilities for future research and policy intervention. Apart from being the medium of the food production system, the soil is rather considered a crucial substance for protecting the valuable environment, hence, its sustenance is immensely important. Thus, an understanding of resistance and resilience after short- and long-term soil manipulation is equally relevant. Here, three interconnected aspects of soil quality, soil sustainability, and ecosystem restoration were discussed, fulfilling the aim of sustainable development goals. Climate change in different forms and degrees has been a real challenge over the years to save the soil and the precious ecosystems surrounding it. So, the chain of soil's ecosystem restoration capabilities is an important global issue where the functionality, behavior, and achieving sustainability goals are immensely important to remark and apply for maintaining a healthy soil for the generations to come.

Author contributions

DB conceptualized the idea. DB and DS outlined the review. DB, DS, and AB took the lead in drafting the manuscript with the help of other co-authors. BV, SM, and BD supplemented in manuscript preparation. AB, BD, and BV did further editing and technical checking in the manuscript. DB and BD produced visualization. All authors contributed to literature collection and compilation of information, and contributed to the article and approved the submitted version.

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Conflict of interest

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

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Improvement in the physiological and biochemical performance of strawberries under drought stress through symbiosis with Antarctic fungal endophytes

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Strawberry is one of the most widely consumed fruit, but this crop is highly susceptible to drought, a condition strongly associated with climate change, causing economic losses due to the lower product quality. In this context, plant root-associated fungi emerge as a new and novel strategy to improve crop performance under water-deficiency stress. This study aimed to investigate the supplementation of two Antarctic vascular plant-associated fungal endophytes, *Penicillium brevicompactum* and *Penicillium chrysogenum*, in strawberry plants to develop an efficient, effective, and ecologically sustainable approach for the improvement of plant performance under drought stress. The symbiotic association of fungal endophytes with strawberry roots resulted in a greater shoot and root biomass production, higher fruit number, and an enhanced plant survival rate under water-limiting conditions. Inoculation with fungal endophytes provokes higher photosynthetic efficiency, lower lipid peroxidation, a modulation in antioxidant enzymatic activity, and increased proline content in strawberry plants under drought stress. In conclusion, promoting beneficial symbiosis

between plants and endophytes can be an eco-friendly strategy to cope with drought and help to mitigate the impact of diverse negative effects of climate change on crop production.

KEYWORDS

root fungal endophytes, drought, Antarctic microorganisms, functional symbiosis, strawberry

Introduction

Climate change is affecting food security, and global warming is challenging agricultural production all around the world. The occurrence of intense and longer periods of drought, together with high temperatures (Grossiord et al., 2020), reduce crop yield and fruit quality. This is particularly important in countries like Chile with an export-based economy. The scarcity of water resources is the main cause of crop loss worldwide (Munns and Tester, 2008; Ksoury et al., 2015), which will soon become even more severe as desertification progressively affects more and more regions of the world (Vinocur and Altman, 2005). All this affects agriculture worldwide, mainly because crop yields are reduced (Leisner, 2020).

Drought stress is a global problem; however, it is a particularly important case in developing countries, where these abiotic stresses severely limit crops production (Gleick, 2000; Zhou et al., 2007). Thus, drought stress is produced when the water availability in the soil is reduced below critical levels that impairs normal plants' growth and development (Ramakrishna and Ravishankar, 2011). Plants display a suite of mechanisms to mitigate the negative impact of stress in general, including water deficit (Taìtrai et al., 2016). Different authors have described that the mechanisms can be boosted by symbiosis with microorganisms (Wani et al., 2015; Barrera et al., 2020; Hereme et al., 2020; Morales-Quintana et al., 2021). In this context, one of the most extreme environmental conditions for life is in Antarctica, including low water availability, high UV-B radiation, extremely low temperatures, and saline soils (Convey et al., 2014), and despite this, two vascular plants inhabit this hostile environment, namely, *Deschampsia antarctica* and *Colobanthus quitensis* (Parnikoza et al., 2007). Additionally, several studies have demonstrated that endophytic microorganisms, such as fungi and bacteria, isolated from Antarctic plants allow other hosting plants tolerate environmental stresses (Molina-Montenegro et al., 2016; Acuña-Rodríguez et al., 2019, 2022). Two species of fungi, *Penicillium chrysogenum* and *Penicillium brevicompactum,* were identified in Antarctic plants, and their functional role was investigated in their native host plant as well as in other plant species, such as tomato and lettuce (Molina-Montenegro et al., 2020). Recently, the effect of two Antarctic root endophytes, *Penicillium rubens* and *Penicillium bialowienzense*, was evaluated in the response of blueberry plants under cold and drought conditions (Acuña-Rodríguez et al., 2022).

On the other hand, strawberry (*Fragaria* × *ananassa* Duch.) plants have shallow roots, which make it a very sensitive crop to soil water deficit, specifically during flowering and fruit ripening. Strawberry is among the most widely consumed fruit in the world. The allo-octoploid cultivated strawberry is a financially important fruit. In 2012, the world strawberry harvested area was 241,000 ha with 4,516,810 tons of fruit production.[1] The coast of Maule and the metropolitan are the two main strawberry growing regions in Chile with 80% of the Chilean production.[2] In soils with low water availability, strawberry crops show a significant decrease in physiological parameters (Mozafari et al., 2019; Cai et al., 2020), which causes a significant reduction in yield, because the plant has a shallow root system, a large leaf surface, and a high water content in the fruit (Liu et al., 2007; Nezhadahmadi et al., 2015).

Previous studies reported the symbiosis of the *Fragaria* genus with various types of fungi (Rivera-Chaìvez et al., 2012; Salgado-Barreiro et al., 2012). Yokoya et al. (2017) isolated 61 different endophytic fungi from samples obtained from *Fragaria vesca* plants collected from different locations, demonstrating the plant's ability to associate with a wide range of endophytic fungal groups. In this sense, Watanabe and Inoue (1980) reported that the most predominant genus of fungi in strawberry plant roots is *Penicillium* spp.

In this article, we report the ability of two Antarctic fungal endophytes, *P. chrysogenum* and *P. brevicompactum,* to improve the performance of strawberry plants exposed to drought stress. Biochemical and physiological parameters suggest that fungal endophytes can support the plant to deal with the water deficit. Moreover, this symbiosis avoids detrimental effects on the yield and quality of fruits.

1 http://www.fao.org/home/en/

2 http://www.odepa.gob.cl/

Materials and methods

Plants and fungal material

Eighty-four plants of *Fragaria* × *ananassa* (Strawberry) cultivars 'Aromas' were obtained from a commercial company and grown in 1-L pots with a standard peat–perlite (1:1) mixture in greenhouse at the Universidad Católica del Maule, Maule Region, Chile. For the obtention of plants free of microbial endophytes (E−), half of them were treated with 2 g L^{-1} of the commercial fungicide Benlate (DuPont, Wilmington, DE, United States), based on previous reports (Ramos et al., 2018; Barrera et al., 2020; Hereme et al., 2020). In addition, all plants were treated with systemic broad-spectrum antibiotic rifampicin (50 μg mL^{-1}) to remove any bacteria (Ramos et al., 2018; Barrera et al., 2020; Hereme et al., 2020). Water was used as the control for the fungicide treatment in E + plants. After 2 weeks, five plants, each of E + and E−, were randomly inspected to verify the presence and/or absence of endophytes based on Barrera et al. (2020).

For the inoculation, two Antarctic fungal endophytes, previously identified by Molina-Montenegro et al. (2020), were isolated from *C. quitensis* and *D. antarctica* plants, each randomly collected from different locations near Henryk Arctowski Polish station in Admiralty Bay on King George Island, Antarctic Peninsula (Rabert et al., 2020). Sequencing of ITS from DNA isolated from axenic fungal strains was performed to verify the identity of *P. chrysogenum* (Genbank accession number: KJ881371) and *P. brevicompactum* (Genbank accession number: KJ881370)[3] based on Molina-Montenegro et al., 2016. The fungal inoculum was prepared as a 1:1 mix of the two strains isolated and identified as C1 and B2 (**Figure 1**) according to Molina-Montenegro et al. (2020). The rhizosphere of each individual plant was inoculated with 3 mL of concentrated solution with the mix of spores (1×10^6–1×10^7 spores mL^{-1}) from each fungal endophyte. The inoculation was repeated 15 days later to ensure an effective association of fungi with the plants. The symbiosis was verified as previously described (Barrera et al., 2020). Two plants of each treatment group were randomly selected to verify the presence or absence of endophytes in roots, staining a section of the root with trypan blue in an acid glycerol solution and observing it under a light microscope (iScope, Euromex, Netherland). Additionally, strawberry roots from the inoculated group were re-checked for the presence of the fungal endophyte at the end of the treatments (after 60 days) (See **Supplementary Figure 1**).

Experimental design

One hundred strawberry plants with (E+) and without (E−) endophytic fungi were subjected to 60 days of contrasting water irrigation regimens. Plants were divided into four treatments: (1) 100% of the water irrigation [water-holding capacity (WHC)] (400 ml) (W + E−), (2) 50% of WHC (200 ml) (W−E−), (3) 100% of WHC plus root-endophytes inoculation (W + E +), and (4) 50% of WHC plus root-endophytes inoculation (W−E +). The amount of water to drought condition was established based on a gravimetric methodology (Erdogan et al., 2016). Briefly, the pots were watered until saturation and freely drained until there was no change in the weight. The difference between this weight and soil dry weight was used to calculate 100% of WHC. Then, 100 and 50% of WHC for plant cultivation were periodically determined gravimetrically and maintained by daily irrigation.

Skin fruit color determination

Fruits without external damage were examined for their skin color. Six strawberries per treatment were chosen randomly for color measurement at the equator of fruit. The surface color of the fruits from different treatments was characterized using a colorimeter model CR-400 (Konica Minolta, Tokyo, Japan) and expressed in the Hunter scale (L*, a*, and b*). The CIELAB color representation was generated by a Nix Color converter.[4]

Physiological and biochemical determinations

Photosynthetic performance, chlorophylls and carotenoids content, free proline concentration, and membrane damage

The photochemical efficiency of PSII (Fv/Fm) was estimated in accordance with Maxwell and Johnson (2000). For this, plants were assessed for each condition (time of exposure and presence/absence of endophyte) using a pulse-modulated amplitude fluorimeter (FMS 2, Hansatech, Instrument Ltd., Norfolk, United Kingdom).

Chlorophyll (Chl) and carotenoids (Car) were extracted from shoot tissue (100 mg) with 80% acetone and determined at the following wavelengths: 663 nm (Chl *a*), 646 nm (Chl *b*), and 470 nm (Car) and calculated by the spectrophotometric method described by Lichtenthaler and Wellburn (1983).

The free proline concentration evaluation was determined following the protocol of Bates et al. (1973). In brief, shoot

3 https://www.ncbi.nlm.nih.gov/genbank/

4 https://www.nixsensor.com/free-color-converter/

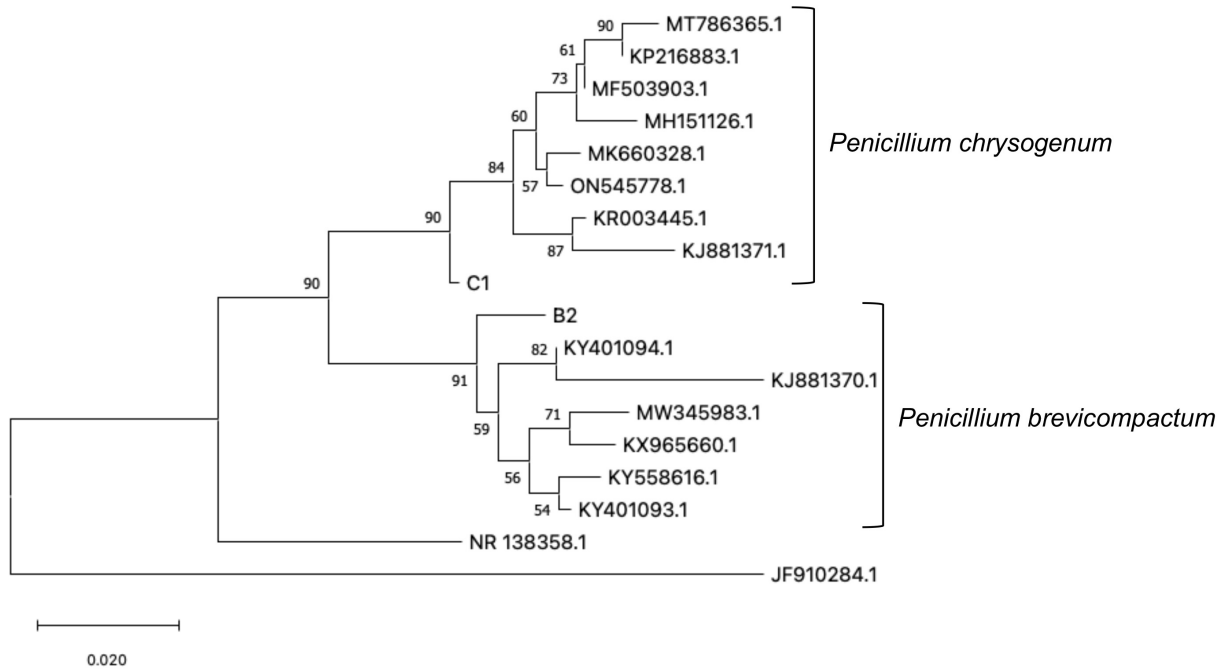

FIGURE 1
Phylogenetic relationship tree between 45 sequences of endophytic fungi based on the ITS rDNA sequences NCBI sequences: KR003445.1 *Penicillium chrysogenum*; MK660328.1 *Penicillium chrysogenum*; ON545778.1 *Penicillium chrysogenum*; MH151126.1 *Penicillium chrysogenum*; MF503903.1 *Penicillium chrysogenum*; MT786365.1 *Penicillium chrysogenum*; KP216883.1 *Penicillium chrysogenum*; MW345983.1 *Penicillium brevicompactum*; KY401094.1 *Penicillium brevicompactum*; KY558616.1 *Penicillium brevicompactum*; KY401093.1 *Penicillium brevicompactum*; KX965660.1 *Penicillium brevicompactum*; KJ881370.1 *Penicillium brevicompactum*; KJ881371.1 *Penicillium chrysogenum*; JF910284.1 *Penicillium minioluteum*; NR_138358.1 *Penicillium murcianum*. Values in the branch nodes correspond to bootstrap.

tissue (100 mg) was frozen in liquid nitrogen and grounded in 1.2 ml of 3% sulfosalicylic acid. Then, the homogenate was centrifuged at 16,000 g at room temperature for 20 min. An aliquot of the supernatant (1 ml) was mixed with 2 ml ninhydrin reagent [2.5% ninhydrin in glacial acetic acid—distilled water—85% orthophosphoric acid (6:3:1)]. The reaction mixtures were kept in a water bath at 90 °C for 1 h to develop the color. Samples were put on ice, and 2 ml toluene was added to separate the chromophore. The toluene phase was separated and the absorbance was read at 525 nm (Multiskan SkyHigh Microplate Spectrophotometer, Thermo Fisher Scientific). The proline content was calculated using a standard proline curve.

Additionally, to assess whether the fungal endophytes protect plants from water stress, lipid peroxidation, photochemical efficiency, and synthesis of proline were evaluated in shoot tissue of strawberry plants exposed to the condition of simulated drought after 60 days. Lipid peroxidation leads to an increase in the levels of malondialdehyde (MDA) and is considered a useful index for oxidative cell damage (Møller et al., 2007). Shoot tissue (100 mg) was homogenized and the MDA content was evaluated following the procedure previously reported by Barrera et al. (2020).

Antioxidant enzymatic assays

Antioxidative enzyme activity was determined in shoot tissue. Briefly, 500 mg of leaves (fresh weight) was ground in liquid nitrogen and the powder was transferred to tubes containing pre-chilled 50 mmol L^{-1} phosphate buffer (pH 7.8, containing 1% PVP) and centrifuged at 4°C, 10,000 r min^{-1} for 20 min. Then, 5 ml of the supernatant was transferred to new tubes for superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) activity assays according to Erdogan et al. (2016). Determinations were performed in biological triplicates.

Statistical analysis

For the determination of MDA, Fv/Fm, proline content, chlorophylls, carotenoid, biomass, fruit weight, the skin color of the fruit, and diameter, two-way ANOVA was used with the factors water condition (W) and presence of endophytic fungi (E). Normality and homoscedasticity of the data were confirmed before the ANOVA test. Tukey's HSD multiple comparisons analysis was performed to evaluate significant differences between treatments. All analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, United States).

Results

Root colonization, biomass production, and survival of strawberry plants

Under drought stress, microscopic visualization revealed that Antarctic fungal endophytes successfully colonized the root of strawberry plants at 60 days post inoculation (**Supplementary Figure 1**). Spores and hyphae were able to grow inter- and intra-cellularly in both, well-watered and drought-stressed strawberry roots.

Biomass production and survival of plants with and without fungal endophytes were evaluated under drought stress. The survival plant rate was calculated by counting the number of plants that survived divided by the number of plants originally established for each treatment and then multiplied by 100 to express the as the percentage of survival. As was expected, the water-deficient treatment strongly reduced the shoot biomass production in plants without endophytes (W−E−) compared to well-watered plants (W+E−) (See **Supplementary Figure 2**). When the inoculated plants were exposed to a deficient irrigation regime (W−E+), they did not show significant differences compared to those well-watered without inoculation (W+E−), and interestingly, inoculated plants showed a robust increase in shoot biomass under well-watered condition (W+E+) compared to the non-inoculated (W+E−) (**Figure 2**). The inoculated strawberry plants (W+E+) displayed a higher shoot biomass production, increasing by about 38% compared to those well-irrigated plants without endophytes (W+E−). The biomass production was strongly reduced when non-inoculated plants were exposed to a deficient irrigation treatment (W−E−), but the biomass production increased close to 51% in the inoculated plants under water deficiency (W−E+) (See **Table 1**). Additionally, plant survival also increased under drought conditions mainly due to the presence of the fungal endophytes (**Table 1**).

Yield and fruit color of strawberry plants

Concerning fruit color and number, the effect of fungal endophytes in well-watered plants vs. plants under drought stress showed a clear addition-yield response (**Table 2**). The incidence of endophytes addition (E+) in the production showed a positive impact; even when the fruit number was higher in well-watered plants without endophytes (W+E−), the weight parameter between well-watered plants with or without endophytes (W+E− and W+E+) and plants exposed to deficient irrigation with endophytes (W−E+) did not show significant differences between them. Fruit production in uninoculated and drought-exposed plants (W−E−) was significantly decreased compared to the other three conditions. With respect to the quality parameters evaluated, interesting results on the diameter of fruit were evidenced in deficient irrigated plants (W−), where, the addition of endophytes increases the diameter values significantly, reinforcing the best performance obtained in plants when watering conditions are restricted. With respect to the color quality evaluated, redness (a*) and yellowness (b*) exhibited similar values, however a high variability on standard deviation was recorded in plants deficient irrigated without endophytes (W−E−). In the case of the lightness (L*), fruit from inoculated and well-watered plants showed a significant increase in this parameter evaluated compared to the other conditions.

Physiological and biochemical determinations in inoculated strawberry plants under drought stress

To determine whether or not the plants are subjected to stress due to the water deprivation treatment, we carried out three different tests. First, to determine the integrity of the plants, the ecophysiological parameter of the photosynthetic capacity was carried out (**Figure 3A**). Fv/Fm, is a parameter of the potential quantum efficiency of PSII, related to plant photosynthetic performance; when photosynthetic apparatus of plants is under environmental stresses, this value will be found below to 0.80. The result showed that water deprivation treatment negatively affected the photochemical efficiency of strawberry plants without endophytes (W−E−). On the contrary, inoculated plants exposed to drought (W−E+) did not show significant differences to those plants with optimal irrigation regimes (W+E− and W+E +) after 60 days of treatment.

With regard to the plant pigments, total chlorophyll content increased in inoculated plants under water deficit (W−E+) compared to uninoculated plants under water deficit (W−E−). No significant differences were observed between inoculated well-watered (W+E−), uninoculated well-watered (W+E−), and uninoculated plants exposed to drought (W−E−) (**Table 3**). The same behavior was observed in chlorophyll *b* content, indicating that water availability did not affect significantly the total chlorophyll and chlorophyll *b* content. In contrast, when chlorophyll *a* was evaluated, the content was significantly increased in plants with endophytes and exposed to deficient irrigation (W−E+) compared to those without endophytes under drought stress (W−E−) and also to well-watered and uninoculated plants (W+E−). Finally, carotenoid content was evaluated, and the results obtained did not show significant differences between water treatments and plant inoculation (**Table 3**).

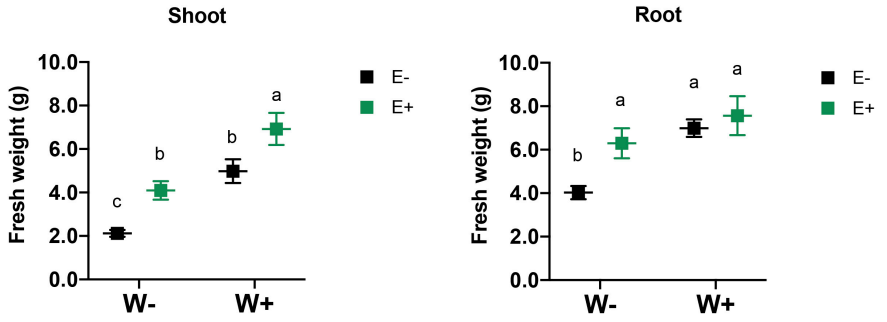

FIGURE 2
Effect of drought stress and fungal inoculation on shoot and root biomass production of inoculated and non-inoculated strawberry plants Different letters indicate significant differences ($P < 0.05$; two-way ANOVA). Bars represent means ± SE from three independent experiments.

TABLE 1 Shoot and root fresh biomass of strawberry plants.

Parameter	W+E−	W+E+	W−E−	W−E+
Shoot fresh biomass (g)	4.98 ± 0.72 [b]	6.92 ± 0.72 [a]	2.12 ± 0.49 [c]	4.09 ± 0.55 [b]
Root fresh biomass (g)	6.99 ± 0.67 [a]	7.55 ± 0.82 [a]	4.03 ± 0.96 [b]	6.29 ± 1.19 [a]
Survival (%)	67.6	71.4	42.9	85.7

Biomass and survival of strawberry plants were evaluated after 60 days of treatment. Plants were well-watered and deficient irrigated (W+ and W−, respectively) and with or without the presence of fungal endophytes (E+ and E−, respectively) isolated from Antarctic plants. Different letters indicate significant differences ($p < 0.05$).

As an additional biochemical approach to evaluate the effects of water deprivation stress and Antarctic fungal endophytes, the free proline content was determined as a response molecule indicative of stress level (Liang et al., 2013). For this reason, we evaluated the proline content in different conditions with and without endophytic fungi (**Figure 3B**). The result of the proline level assessment showed a significant increase in plants with endophytes under drought stress (W−E+) compared with plants without endophytic fungi after 60 days of hydric deficit treatment (W−E−) (**Figure 3B**). In well-watered plants with endophytes (W+E+) or without endophytes (W+E−), the proline accumulation did not show significant differences compared to plants exposed to deficiently irrigated and without endophytes (W−E−).

The oxidative degradation of the lipid was also monitored to evaluate the protective role of fungal endophytes in plants exposed to drought. We evaluated the cell membrane lipid peroxidation (**Figure 3C**). The result showed that the presence of endophytes improved the tolerance to water deficit. Under water deficit, plants with fungal endophytes (W−E+) display a significant decrease in the MDA content compared to the plants without endophytes (W−E−). The MDA accumulation in plants under water deficit show significant differences compared to plants with normal irrigation when fungal endophytes are present, and when the endophytes are absent observed even lower decreased values were observed (**Figure 3C**).

Antioxidant enzymatic activity of plants

With respect to these effects on the antioxidant enzymatic activity triggered by drought, SOD, CAT, APX, and POD activity were evaluated in leaves from inoculated and uninoculated strawberry plants (**Figure 4**). The enzymatic activity was lower in well-watered plants without endophyte (W+E−) compared to other evaluated treatments (W+E+, W−E+ and W−E+). In the case of inoculated plants, SOD increased its activity in well-watered (W+E+) and also in plants exposed to drought (W−E+) compared to uninoculated plants (W−E−). Meanwhile for CAT and APX, the enzymatic activity decreased in inoculated plants exposed to drought (W−E+), but the activity increased in well-watered inoculated plants (W+E+) compared to the uninoculated (W+E−). The activity of POD showed a strong decrease in inoculated plants under drought stress (W−E+) and a significant decrease in well-watered plant uninoculated (W+E−) plants but a slight increase was detected when compared with inoculated well-watered plants (W+E+) (**Figure 4**).

Discussion

In this work, we tested the effects of the functional symbiosis between Antarctic fungal endophytes with strawberry plants, which is well documented stating that deficient irrigation stress shows a detrimental performance in its physiological parameters and triggers a reduction in yield (Mozafari et al., 2019; Cai et al., 2020). We noted that symbiosis is effective in the roots of well-watered and also drought stressed strawberry plants for at least 60 days (**Supplementary Figure 1**). The observations indicate that Antarctic fungal endophytes had the ability to colonize the well-watered and also drought-stressed

TABLE 2 Productive and quality parameters of strawberry plants.

	Fruit number	Weight (g)	CIELAB			Diameter (mm)	Color
			L	a	b		
W+E−	40	5.27 ± 1.30[a]	26.45 ± 3.70[b]	32.70 ± 4.42[a]	16.00 ± 5.24[a]	13.90 ± 2.38[a]	
W+E+	30	5.54 ± 1.95[a]	29.23 ± 1.35[a]	31.85 ± 4.73[a]	15.72 ± 3.42[a]	13.31 ± 3.26[a]	
W−E+	23	4.52 ± 1.96[a]	26.40 ± 2.28[b]	32.97 ± 4.89[a]	18.62 ± 5.10[a]	11.43 ± 1.34[b]	
W−E−	7	3.44 ± 0.11[b]	27.98 ± 13.02[b]	33.12 ± 14.28[a]	18.07 ± 12.01[a]	8.67 ± 0.47[c]	

Fruit number, weight, skin fruit color, and diameter of strawberry plants were evaluated after 60 days of treatment. Plants were well-watered and deficient irrigated (W+ and W−, respectively) and with or without the presence of fungal endophytes (E+ and E−, respectively) isolated from Antarctic plants. Different letters indicate significant differences ($p < 0.05$).

strawberry root cells. In productive and quality parameters, this effective symbiosis was reflected with an increase in yield and maintenance in the fruit size when comparing inoculated plants to uninoculated plants grown under drought stress conditions (**Table 2**). The color was only affected in fruits obtained from inoculated and well-watered plants, in which a moderate but statistically significant increase in the lightness was observed compared to the other treatments. These results could be explained by the role of Antarctic fungal endophytes to enhance nutrient uptake, including nitrogen and phosphorus (Newsham, 2011; Acuña-Rodríguez et al., 2020). The symbiotic interactions increase the availability of essential nutrients for the synthesis of amino acids and proteins in the host plants (Harman et al., 2021). The protective role of Antarctic fungal endophytes has been largely documented in native host plants (Ramos et al., 2018; Barrera et al., 2020; Hereme et al., 2020; Morales-Quintana et al., 2021), as well as in commercial crop species such as lettuce, peppers, and tomato (Molina-Montenegro et al., 2016; Acuña-Rodríguez et al., 2019). Considering the results presented here, in addition to those previously reported, the symbiotic and positive effects on different plant host species could suggest that the two *Penicillium* strains isolated from Antarctic plants are promiscuous mutualists in their plant host selection.

The observed results suggest a positive role of this effective root inoculation on ecophysiological, biochemical, and yield performance on strawberry plants exposed to deficient irrigation regimes and in some parameters even higher than under well watered conditions. Endophyte-inoculated plants showed a higher photochemical efficiency (Fv/Fm), even when the plants were exposed to water restriction. This could be improving the efficiency of excitation energy capture by chloroplasts and the energy cycling among photosynthetic apparatus in strawberry plants under drought stress, similar to that observed in *Ceratonia siliqua* L (Boutasknit et al., 2021). The lowest value of Fv/Fm observed in plants under water stress and non-inoculated (W−E−) could be associated with an increase in energy dissipation in the PSII antennae previously reported by Demming-Adams et al. (1993) as a common response of photosynthetic apparatus to environmental stress. Additionally, drought stress leads to a decrease in chlorophyll content reducing the photosynthetic efficiency of plants (Astolfi et al., 2012). Based on this, the estimation of chlorophyll content is an effective proxy to assess the plant performance under stress. The results showed that the inoculation with Antarctic fungal endophytes improved the chlorophyll concentration of strawberry plants under drought stress (**Table 3**) in accordance to previous reports on the inoculation with fungal endophytes that improved plant chlorophyll concentration under drought stress (Ghorbani et al., 2018). The positive impact of inoculation in yield and photosynthetic pigments could be related with a higher osmotic pressure associated to proline accumulation, and this role has been related previously with an increase in the absorption of water and nutrients, both processes related with photosynthesis and yield in plants (Mafakheri et al., 2010). For other side, the similar values observed in carotenoids in all treatment could be attributed to the protective role and protect chlorophyll from photo-oxidation (Young, 1991).

Free proline content, on the other side, is an amino acid that normally accumulates in plants under drought and salt stress to improve their tolerance (Kishor et al., 2005). Several studies associate the plant response to drought to its role as an osmolyte, and others also indicate that the proline acts as a free-radical scavenger, membrane stabilizer, and regulating the redox potential of cells (Verbruggen and Hermans, 2008; Hayat et al., 2012). The free proline accumulation in plant cells is a phenomenon that is well known to be triggered under water deficit (Hare et al., 1998). Our results show an increase in free proline content in endophyte-inoculated plants under water stress (W−E+) when compared with the other treatment evaluated to exhibit the protective activity of endophyte when restrictive water conditions are applied; however, no significant difference between all treatment was observed; similar result was reported by Sun et al., 2015 when strawberry plants were exposed to mild drought stress and compared to control plant without water restriction. Similar results were observed by Kavroulakis et al. (2018) when tomato plants were subjected to water restriction and evaluated the effect of *Fusarium solani* FsK, another root endophyte. In addition, lipid peroxidation is considered as a hallmark of cell damage, and here it was estimated by evaluating the concentration of MDA using the

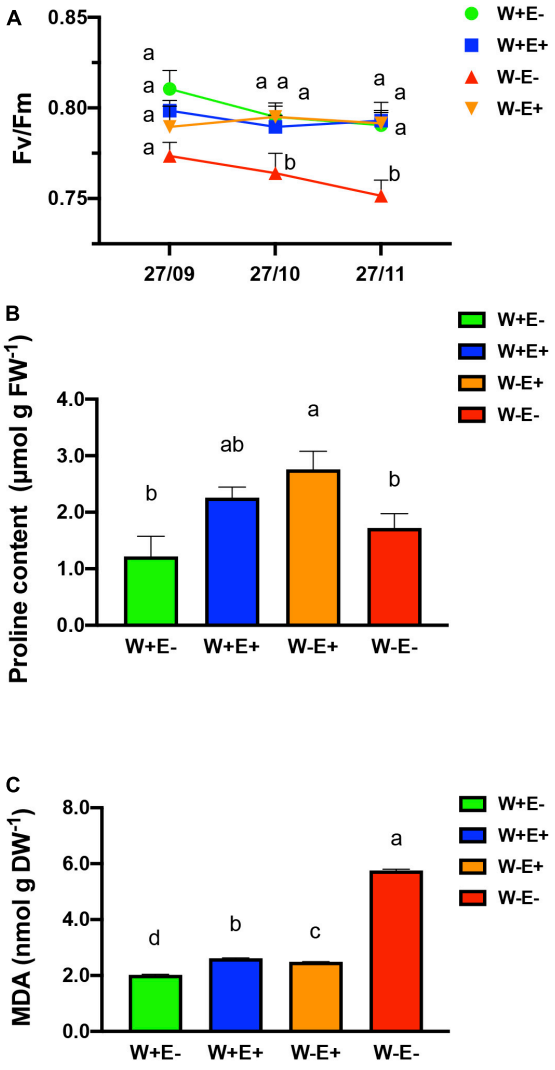

FIGURE 3
Physiological and biochemical effects over plant mediated by endophytic fungi. **(A)** Photosynthetic capacity in strawberry mediated by the presence of endophytic fungi; **(B)** proline concentration in plants, as a molecular marker of the osmoprotective response; **(C)** lipid peroxidation analysis. W−E−: without irrigation and without endophyte fungi; W−E+: without irrigation and with endophyte fungi; W+E−: with irrigation (well-watered) and without endophyte fungi; W+E+: with irrigation (well-watered) and with endophyte fungi. Different letters indicate significant differences ($P < 0.05$; two-way ANOVA). Bars represent means ± SE from three independent experiments.

TABLE 3 Chlorophylls and carotenoid content of strawberry plants.

Parameter (µg/mL)	W+E−	W+E+	W−E−	W−E+
Total chlorophyll	10.84 ± 0.75^{ab}	12.47 ± 3.15^{ab}	9.69 ± 0.96^{b}	14.56 ± 2.61^{a}
Chlorophyll *a*	7.69 ± 0.54^{b}	8.96 ± 2.31^{ab}	6.90 ± 0.67^{b}	10.49 ± 1.78^{a}
Chlorophyll *b*	3.15 ± 0.20^{ab}	3.52 ± 0.84^{ab}	2.79 ± 0.28^{b}	4.07 ± 0.83^{a}
Carotenoids	2.74 ± 0.23^{a}	2.94 ± 0.77^{a}	2.57 ± 0.48^{a}	3.25 ± 0.55^{a}

Chlorophylls and carotenoid content of strawberry plants were evaluated after 60 days of treatment. Plants were well-watered and deficient irrigated (W+ and W−, respectively) and with or without the presence of fungal endophytes (E+ and E−, respectively) isolated from Antarctic plants. Different letters indicate significant differences ($p < 0.05$).

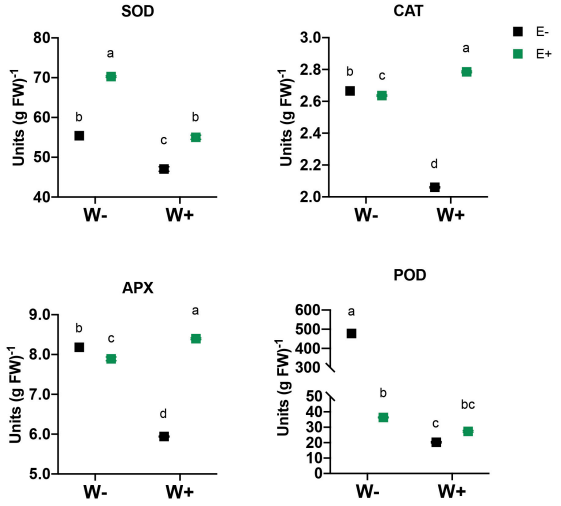

FIGURE 4
Effect of fungal Antarctic endophytes and water deficit treatments on the activities of the antioxidant enzymes (CAT, POD, SOD, and APX) in the leaves of strawberry plants. Different letters indicate significant differences ($P < 0.05$; two-way ANOVA). Bars represent means ± SE from three independent experiments.

assay of thiobarbituric acid reactive substances (TBARS) (Egert and Tevini, 2002). Respect to MDA content, a reliable marker for determining the degree of injury to a stressed plant (Morales and Munné-Bosch, 2019), a lower level of oxidative stress (MDA) in inoculated compared to uninoculated plants was observed. This result is an indicative parameter of the protective role exerted by the fungal endophytes on plants under drought stress (**Figure 3**). The results are in accordance with previous observations in the Antarctic plant *C. quitensis* under a water stress scenario that is likely to be provoked by the global climate change (Hereme et al., 2020; Morales-Quintana et al., 2021).

Reactive oxygen species are oxidizing species, where the hydroxyl radicals (OH) and singlet oxygen (O_2) are the most important molecules. These species are very powerful oxidants that can react with all the components of living cells, producing severe damage to organic macromolecules (lipids, proteins, and nucleic acids) under oxidative stress situations (Sies, 2014). The effect in plant has been related to photosynthesis and photoinhibition, as well as to the environmental responses of photosynthesis (Del Rio, 2015). As is well documented, drought provokes the leakage of electrons during photosynthesis and

respiration that generates cellular oxidative stress enhancing the production of reactive oxygen species (ROS) (Sánchez-Rodríguez et al., 2012). To deal with the cytotoxic effects of ROS generated, plants have developed several antioxidant systems, including SOD, CAT, POD, and APX (Rahnama and Ebrahimzadeh, 2005; Simova-Stoilova et al., 2008; Erdogan et al., 2016). The inoculation with fungal endophytes affected plant physiology and biochemistry (**Figure 3**), and these positive effects were evident in the growth performance of the inoculated plants (**Table 1**). Regarding the antioxidative activity of enzymes, the results are in agreement with previous reports, in which the activities of SOD, CAT, APX, and POD increased under water-deficit stress in the plants (Wu et al., 2006; Erdogan et al., 2016), and this behavior has been related with the scavenging of ROS (Singh et al., 2020). SOD is the first enzyme involved in defense against ROS, and their function is to turn superoxide radical (O_2^-) into O_2 and H_2O_2 (Alscher et al., 2002). These free radicals can react spontaneously with organic molecules provoking membrane lipid peroxidation, protein oxidation, enzyme inhibition, and DNA and RNA damage (Vinocur and Altman, 2005).

Here, we also observed that strawberry plants inoculated with Antarctic fungal endophytes showed high SOD activity, which contributed to enhance plant protection against drought stress. In specific, only SOD enzyme increases its activity in inoculated plants under water deficit and also in plant well-watered (**Figure 4**). Similar results for SOD were reported in drought stress assays in strawberry plants, where SOD was the most effective enzymatic response (Sekmen Cetinel et al., 2021). In the case of CAT, APX, and POD, inoculated plants under drought stress showed a decrease in activity, suggesting that involved molecules could be less affected compared with uninoculated-stressed plants or maybe could be related such that the fungal inoculation not only stimulates their enzymatic defense system but also stimulates a minor production of the stress metabolites.

Conclusion

Here, we observed that it is possible to improve the performance of strawberry plants under water-stress conditions through inoculation of roots with Antarctic fungal endophytes. As was observed previously in Antarctic native plants and now in inoculated strawberry plants, the results here presented strongly suggest that the observed advantage triggered by fungal symbiosis can be transferable to commercial crops to face future conditions, which are a product of the imminent global change.

Data availability statement

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

Author contributions

PR designed the experiments. MM performed the experiments. PR and LM-Q analyzed the data. PR and MM prepared the plant material. PR, LM-Q, CR, and SP wrote the article. LM-Q, RS-M, AC-A, CR, SP, and PR reviewed 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.2022.939955/full#supplementary-material

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Climate-smart agricultural practices influence the fungal communities and soil properties under major agri-food systems

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Fungal communities in agricultural soils are assumed to be affected by climate, weather, and anthropogenic activities, and magnitude of their effect depends on the agricultural activities. Therefore, a study was conducted to investigate the impact of the portfolio of management practices on fungal communities and soil physical–chemical properties. The study comprised different climate-smart agriculture (CSA)-based management scenarios (Sc) established on the principles of conservation agriculture (CA), namely, ScI is conventional tillage-based rice–wheat rotation, ScII is partial CA-based rice–wheat–mungbean, ScIII is partial CSA-based rice–wheat–mungbean, ScIV is partial CSA-based maize–wheat–mungbean, and ScV and ScVI are CSA-based scenarios and similar to ScIII and ScIV, respectively, except for fertigation method. All the scenarios were flood irrigated except the ScV and ScVI where water and nitrogen were given through subsurface drip irrigation. Soils of these scenarios were collected from 0 to 15 cm depth and analyzed by Illumina paired-end sequencing of Internal Transcribed Spacer regions (ITS1 and ITS2) for the study of fungal community composition. Analysis of 5 million processed sequences showed a higher Shannon diversity index of 1.47 times and a Simpson index of 1.12 times in maize-based CSA scenarios (ScIV and ScVI) compared with rice-based CSA scenarios (ScIII and ScV). Seven phyla were present in all the scenarios, where Ascomycota was the most abundant phyla and it was followed by Basidiomycota and Zygomycota. Ascomycota was found more abundant in rice-based CSA scenarios as compared to maize-based CSA scenarios. Soil organic carbon and nitrogen were found to be 1.62 and 1.25 times higher in CSA scenarios compared with other scenarios. Bulk density was found highest in farmers' practice (Sc1); however, mean weight diameter and water-stable aggregates were found lowest in ScI. Soil physical, chemical, and biological properties were found better under CSA-based practices, which also increased the wheat grain yield by 12.5% and system yield by 18.8%. These results indicate that bundling/layering of smart agricultural practices over farmers' practices has tremendous effects on soil properties, and hence play an important role in sustaining soil quality/health.

KEYWORDS

agriculture management, tillage, fungal community, diversity indices, climate smart agricultural practices, soil organic carbon

Introduction

Rice–wheat (RW) system is the dominant cropping system of the Indo-Gangetic Plains of South Asia, spreading over a 13.5 M ha area (Gupta and Seth, 2007). In this system, farmers follow conventional agricultural practices, such as repeated tillage, open field residue burning, indiscreet use of fertilizers, and irrigation waters, which lead to soil health deterioration, groundwater depletion, and environmental pollution (Jat et al., 2021a). In the 21st century, climate change with extreme weather events is likely to be the most serious issue being faced by mankind as per the IPCC predictions (IPCC, 2022). To adapt to extreme events and to manage natural resources, climate-smart agriculture (CSA)-based management practices on the principles of conservation agriculture (CA) (zero tillage, residue retention, and crop rotation) and mitigation co-benefits may prove an excellent option for conventional agriculture to maintain the sustainability of soil and cropping system. In CSA practices, the conventional management practices switch over to resource-conserving management practices by layering with zero/no-tillage, residue retention/incorporation, crop diversification, precise irrigation water, and nutrient management practices to sustain soil and farm productivity and environmental quality (Choudhary et al., 2020). In CSA, residues are managed through retention or incorporation, which are burnt by the farmers under conventional management practices. Crop residues provide an ambient environment for soil fungi, but their density and diversity depend upon the type of residue served. Different types of crop rotations provide different types of root exudates, which influence microbial community compositions (Huang et al., 2014; Jiang et al., 2016). Crop rotation and tillage practices disturb soils and influence the distribution of microbes in the soil layer (Orrù et al., 2021). Fungi play a great role in agricultural practices in nutrient cycling, transformation, and availability.

Soil is the base of all civilizations and mankind, therefore, its functions and structure, directly and indirectly, influence food security. Soil functions are governed by climatic, edaphic, and anthropogenic activities. Soil properties and composition of the soil microbiota are directly interrelated with each other (Wakelin et al., 2008; Bender et al., 2016). Soil microbial communities are essential to biogeochemical cycles, and especially fungi play an important role in the biodegradation of organic matter (Rineau et al., 2012; Qiu et al., 2018). They dominate the microbial biomass in soil habitats (Joergensen and Wichern, 2008) and contribute to nutrient cycling (Stromberger, 2005). Soils have dynamic environments and the microorganisms that live in these soil habitats respond to the changing soil conditions. It has been found that more genera and species of fungi exist in the soil than in any other environment (Nagmani et al., 2006). Fungi are the primary decomposers in soils and secrete various enzymes, such as cellulases, laccases, and xylanases that break down lignocelluloses into simple sugars (Maza et al., 2014; Choudhary et al., 2016). A comprehensive understanding of the fungal community in agricultural soils provides the path to study their roles in the soil ecosystem. The relationship between the biodiversity of soil fungi and ecosystem function is an important issue, particularly in the concern of global climate change and human alteration of ecosystem processes. Fungi play an important role in soil formation, structure, and fertility by contributing to the nutrient cycle and maintenance of the ecosystem (Stromberger, 2005; Hoorman, 2011). Agricultural practices that impact soil conditions can significantly alter soil microbial community composition (Choudhary et al., 2018a,b). The differences in microbial community composition are mainly been attributed to differences in the soil properties (physical and chemical), which are linked to agricultural management practices (IPCC, 2022). Some studies reported the effects of different tillage practices on soil chemical and physical properties and microbial activities, and they reported better soil properties under zero tillage (ZT) and residue retention practices (Choudhary et al., 2018c,d). Agricultural management practices have an impact on different parameters of soil. Under CA practices, better soil physical properties such as lower bulk density (Li et al., 2019), enhanced saturated hydraulic conductivity (Patra et al., 2019), higher infiltration rate (Jat et al., 2018), and increased water-stable aggregates (Bhattacharyya et al., 2012; Jat et al., 2019a) were reported than conventional management practices in cereal-based systems. Increased nutrient availability (Zahid et al., 2020), soil organic carbon (Bera et al., 2017), and nitrogen (Bhattacharyya et al., 2018) were favored under no-tillage/ZT and residue management-based practices. In the western Indo-Gangetic Plains, higher soil biological properties and soil health were reported with CA-based management practices (Choudhary et al., 2018c,d; Sharma et al., 2021).

In any agriculture system, different soil processes depend on microbial community compositions. The diverse nature of fungal communities makes them an important factor for sustainable agriculture in the changing scenarios of climate change (Yadav et al., 2020). The impact of different agricultural management regimes on fungal community composition gained rising interest, although up to date, only a few studies were dedicated to determining the effects of tillage, fertilization, and crop rotation on microbial diversity in cereal systems (Sharma-Poudyal et al., 2017; Choudhary et al., 2018b; Piazza et al., 2019). To date, some studies have applied next-generation sequencing techniques in South Asia to investigate the influence of different tillage regimes and types of crop residue management on soil fungal communities (Choudhary et al., 2018a,b). Our study focused on the changes in the fungal communities under the layering of different management practices, such as soil tillage (conventional vs. zero tillage), residue management (without residue vs. residue retention), crop rotation (rice–wheat vs. maize–wheat), and irrigation (border vs. subsurface drip). The ultimate objective was to identify the impact of

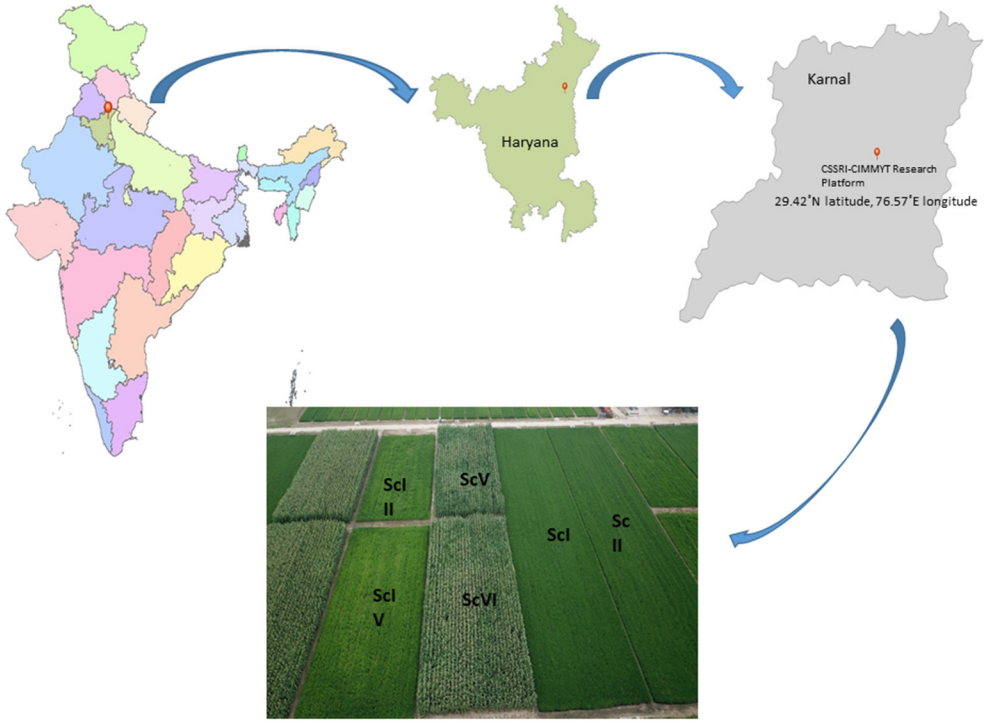

FIGURE 1
Different cereal-based management scenarios at CSSRI-CIMMYT research platform, Karnal, Harayana, India.

layering of management practices on the composition of soil fungal communities.

Materials and methods

Study location, experimental design, and management

A 9-year (2009–2018) long-term production scale fixed plot experiment was conducted at the Indian Council of Agricultural Research, Central Soil Salinity Research Institute (29.42°N latitude, 76.57°E longitude, and at an elevation of 243 m.a.s.l.), Karnal, India. The soil of the experimental field is silty loam in texture and falls under the *Typic Natrustalf* category (Soil Survey Division Staff, 1993). The experiment included six cereal-based scenarios varied with residue, tillage, crop, irrigation, and nutrient supply (Figure 1). The treatments are described as scenarios because they are varied in multiple indicators, namely, tillage, crop establishment, residue management, irrigation management, system intensification, etc. Initially, the experiment started with four scenarios, and later on, in 2016, two more scenarios (ScV and ScVI) were added (Choudhary et al., 2020; Jat et al., 2021b) with minor changes in ScIII and ScIV, and only irrigation management was precise (subsurface drip irrigation was used instead of border irrigation). The details of all the scenarios along with their management practices are presented in Table 1. Scenario III and ScIV were based on the principles of CA practices, so they were described as partial CSA. However, in ScV and ScVI, in addition to ScIII and ScIV, irrigation water and N were precisely managed using subsurface drip irrigation (SDI) and called full CSA. Scenarios were structured in a randomized complete block design and replicated three times. In ScI (farmers' practice or business as usual), both rice and wheat were established with conventional practice. Rice was planted by manual transplanting with 25–30 days old seedlings in puddled fields and wheat was planted by manual broadcasting in tilled soil under ScI. In ScII, manual transplanting for rice in a random geometry (20 × 15 cm) was done. In other scenarios (zero tillage (ZT) conditions), all the crops (rice, wheat, and mungbean) were planted with a row spacing of 22.5 cm using Happy Seeder with an inclined plate seed metering mechanism. However, maize was seeded by Happy Seeder at a row spacing of 67.5 cm. In the farmers' practice (ScI), all the crop residues of rice and wheat were removed from the ground level. However, in other scenarios, it was managed as per the details presented in Table 1. The total amount of crop residue in different scenarios ranged from 100.5 to 119.25 Mg ha^{-1} in 9 years of study (Table 1). Maize-based scenario received the highest (~119 Mg ha^{-1}) amount of crop

TABLE 1 Description of different cereal-based management scenarios.

Scenarios	Crop rotations	Tillage	Crop establishment method	Residue management options	Water management options	Residue load
ScI	Rice–Wheat–Fallow	CT–CT	Rice: Transplanting Wheat: Broadcast	All residue removed	Border irrigation	–
ScII	Rice–Wheat–Mungbean	CT–ZT–ZT	Rice: Transplanting Wheat: Drill seeding Mungbean: Drill/relay	Full (100%) rice and anchored wheat residue retained on soil surface; full mungbean residue incorporated	Border irrigation	104.67[b]
ScIII	Rice–Wheat–Mungbean	ZT–ZT–ZT	Rice: Drill seeding Wheat: Drill seeding Mungbean: Drill/relay	Full (100%) rice and mungbean; anchored wheat residue retained on soil surface	Border irrigation	100.5[c]
ScIV	Maize–wheat–Mungbean	ZT–ZT–ZT	Maize: Drill seeding Wheat: Drill seeding Mungbean: Drill/relay	Maize (65%) and full mungbean; anchored wheat residue retained on soil surface	Border irrigation	119.25[a]
ScV	Rice–Wheat–Mungbean	ZT–ZT–ZT	Same as in scenario 3	Same as in scenario 3	Subsurface drip irrigation	100.95[c]
ScVI	Maize–wheat–Mungbean	ZT–ZT–ZT	Same as in scenario 4	Same as in scenario 4	Subsurface drip irrigation	119.11[a]

[a,b,c]Means followed by similar lowercase letters within a column are not significantly different at 0.05 level of probability using Tukey's HSD test.

residue, while the rice was based on the range of 100–105 Mg ha^{-1}. The NPK dose (nitrogen, phosphorus, and potash) was given as per the recommendation of CCS Haryana Agricultural University for all the crops. In the subsurface drip-irrigated scenario (ScV and ScVI), 80% of the total N as urea (minus N added through DAP and NPK complex) was applied through irrigation (fertigation). In rice, maize, and wheat, irrigation was applied based on soil moisture potential (SMP) using a tensiometer.

Soil sampling

The soil samples were collected from all six scenarios (three from each scenario) at 0–15 cm soil depth after harvesting of wheat crop in May 2018. The soil surface was cleaned by removing crop residues and samples were taken randomly aseptically using an auger. Collected soil samples (18 samples) were sieved with 2 mm mesh to eliminate large soil aggregates and plant roots. Samples were divided into three parts, one part was immediately transferred to the laboratory, stored at −20°C until DNA was extracted, and the second part is stored in the refrigerator at 4°C for further analysis of biological properties. The third part was air-dried, ground, and stored in glass containers for chemical and physical analysis.

DNA extraction, amplification, and sequencing

From the soil samples, DNA was extracted by MO BIO's PowerSoil® DNA Isolation Kit as per the instructions of the manufacturer. The quantity and quality of DNA were measured by Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis, respectively. ITS1 and ITS2 sequencing libraries were constructed by a two-step PCR-based workflow. For round one PCR, using a template of 10–100 ng metagenomic DNA, ITS1 and ITS2 regions were amplified using region-specific proprietary primers developed at Genotypic Technology Pvt. Ltd., Bangalore, India. The protocol also includes overhang adapter sequences that were appended to the primer pair sequences for compatibility with Illumina index and sequencing adapters. PCR was carried out for 26 cycles (hot-start 95°C/3 min, denaturation 95°C/30 s, annealing 55°C/30 s, extension 68°C/1 min, elongation 68°C/5 min, and infinite hold at 4°C) using 0.5 μM primers. The resultant amplicons were analyzed on 1.5% agarose gel whereby the desired amplicons were observed to be of sizes ∼350–450 bp for ITS1 and ∼450–500 bp for ITS2. For the second round of PCR, 1 μl of 1:2 diluted round one PCR amplicons were taken and amplified for 10 cycles to add Illumina sequencing barcoded adaptors (Nextera XT v2 Index Kit, Illumina, USA). Second round PCR amplicons (sequencing libraries) were analyzed on 1.5% agarose gel, then purified using Ampure XP magnetic beads (Beckman Coulter, USA), and concentrations were measured using Qubit

dsDNA HS assay (Thermo Fisher Scientific, USA). The ITS1 and ITS2 amplicons were generated separately by individual PCRs and libraries were constructed off these amplicons individually. The sample libraries were normalized based on the qubit concentrations, then multiplexed on the MiSeq flowcell, and sequenced using a 300PE read length chemistry.

The forward and reverse primers (White et al., 1990; Fujita et al., 2001) were as follows:

ITS1_Forward TCCGTAGGTGAACCTGCGG.
ITS1_Reverse GCTGCGTTCTTCATCGATGC.
ITS2_Forward GCATCGATGAAGAACGCAGC.
ITS2_Reverse TCCTCCGCTTATTGATATGC.

We have adapted our primer design based on fungal rDNA sequencing literature targeting the conserved regions of 5.8S rDNA and 28S rDNA. ITS1 primers were used to amplify the intervening 5.8S rDNA and the adjacent ITS1 region. ITS2 primers were used to amplify a longer region across the 5.8S rDNA through the intervening ITS2 region and the large 28S subunit.

Analysis of soil properties

The organic carbon (OC) content of the soils was determined using the wet oxidation method (Walkley and Black, 1934). The available nitrogen (N) in soil was determined by the alkaline permanganate method of Subbiah and Asija (1956), available phosphorus (P) by the ascorbic acid reductant method of Olsen et al. (1954), and available potassium (K) by flame photometer using neutral 1N ammonium acetate extractant as described by Jackson (1973). Mean weight diameter (MWD) and water-stable aggregates (WSA) were determined following the method of Jat et al. (2019b). Soil bulk density (BD) was measured by core sampler method (Blake and Hartge, 1986).

Crop yield

At maturity, crops were harvested manually for grain and straw yields according to residue management protocols. Grain yield was expressed as Mg ha^{-1} at 14, 12, and 14% grain moisture content for rice, wheat, and maize, respectively. Grain yields of maize, rice, wheat, and mungbean were converted to the rice equivalent yield (REY) by using Equation 1.

$$\text{Rice equivalent yield } \left(\text{Mg ha}^{-1}\right) = \left[\text{maize/wheat/mungbean yield } \left(\text{Mg ha}^{-1}\right) \times \text{MSP of respective crop } \left(\text{INR Mg}^{-1}\right)\right] / \left[\text{MSP of rice } \left(\text{INR Mg}^{-1}\right)\right] \quad (1)$$

where, MSP, Minimum support price of Govt. of India; INR, Indian Rupee.

Bioinformatics and statistical analysis

The Illumina paired-end reads were de-multiplexed using Bc12fastq software v2.20 and FastQ files were generated based on the unique dual barcode sequences. The sequencing quality was assessed using FastQC v0.11.8 software (Andrews, 2010). Raw reads with primer sequence were processed using the Cutadapt tool (Martin, 2011). The adapter sequences were trimmed and bases above Q30 were considered, and low-quality bases were filtered off during read preprocessing and used for downstream analysis. The high-quality R1 and R2 reads were filtered and stitched using Fastq-join (Aronesty, 2013). ITS1 and ITS2 sequences were merged and considered for further analysis using the QIIME pipeline (Caporaso et al., 2010). The query sequences were clustered using the UCLUST (Edgar, 2010) method against the reference UNITE (Abarenkov et al., 2010) database (ver7) with 97% similarity. The reads, which did not hit the reference database, were then clustered using the *de novo* method where reads are clustered against one another without any external reference sequence collection. Taxonomy was assigned through the BLAST method (Altschul et al., 1990), with an *e*-value of 0.001. The BIOM file generated was taken ahead for further advanced analysis and visualization. The rarefied biom at a depth of 177,000 for merged ITS sequences/samples was used for the calculation of alpha diversity indices using various metrics. Sequences from all six agricultural management scenarios have been submitted to NCBI with the Bio project: PRJNA563827.

Biplot and principal component analysis (PCA) were done with JMP 14.1 software. The results were submitted to PCA to determine the common relationships between fungi classes, soil physical properties, and SOC, N, P, and K contents of the soil. The crop yield data were analyzed by the analysis of variance (ANOVA) technique for a completely randomized block design using SAS 9.1 software (SAS Institute, Cary, NC). Differences among treatment means were compared using Tukey's HSD test at the 5% probability level.

Results

Fungal diversity influenced by management scenarios

From the 18 soil samples, three of each from six scenarios, a total of 12,468,774 paired-end reads were obtained after processing of sequences, and a total of 5,033,723 sequences were obtained. Fungal diversity was calculated by different indices, such as Shannon's diversity index, Simpson's diversity index, and Chao1. Diversity indices were found to be significantly affected by agricultural management systems, as Shannon's diversity index, Simpson's diversity index, Chao1, and observed

TABLE 2 Diversity indices of fungi in different scenarios of crop management systems.

Scenarios*	Shannon	Simpson	Chao1	Observed species/OTU
ScI	$5.71^{B**} \pm 0.195$	$0.93^{A} \pm 0.010$	$2{,}891^{AB} \pm 96$	$2{,}529^{AB} \pm 87$
ScII	$6.80^{A} \pm 0.551$	$0.95^{A} \pm 0.016$	$3{,}223^{A} \pm 256$	$2{,}874^{A} \pm 229$
ScIII	$4.38^{C} \pm 0.150$	$0.85^{B} \pm 0.013$	$2{,}289^{C} \pm 66$	$1{,}929^{C} \pm 46$
ScIV	$6.60^{A} \pm 0.164$	$0.96^{A} \pm 0.004$	$2{,}416^{BC} \pm 132$	$2{,}087^{BC} \pm 128$
ScV	$4.46^{C} \pm 0.051$	$0.85^{B} \pm 0.034$	$2{,}472^{BC} \pm 126$	$2{,}150^{BC} \pm 121$
ScVI	$6.42^{A} \pm 0.163$	$0.94^{A} \pm 0.028$	$2{,}077^{C} \pm 220$	$1{,}829^{C} \pm 222$

A* Refer Table 1 for scenarios description.
B** Means followed by similar uppercase letters within a column are not significantly different at 0.05 level of probability using Tukey's HSD test.

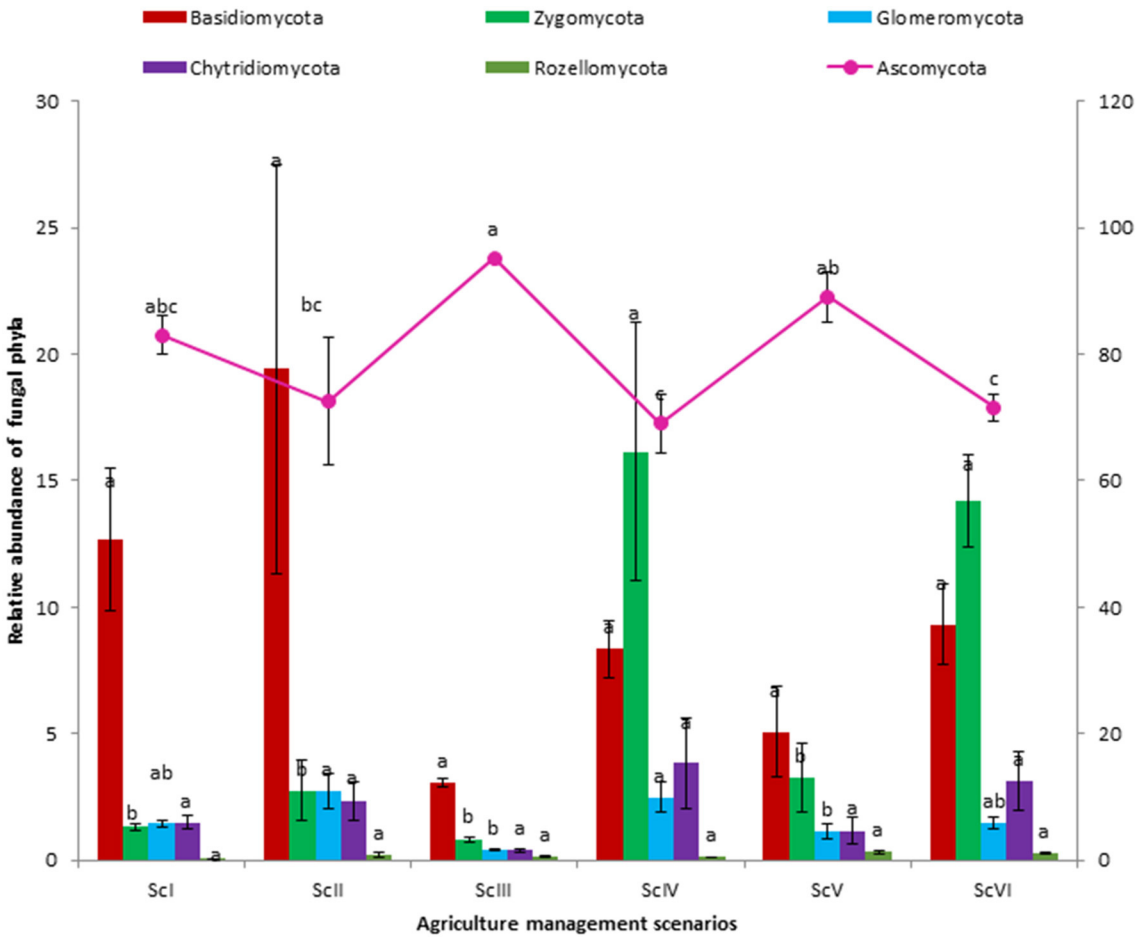

FIGURE 2
Abundance of phyla in different crop management-based scenarios. a,b,c Means followed by similar lowercase letters are not significantly different at 0.05 level of probability using Tukey's HSD test.

species/OTU were varied in scenarios (Table 2). Shannon diversity index was found to be 1.47 times higher in maize-based scenarios (ScIV and ScVI) as compared to rice-based CSA scenarios (ScIII and ScV). A similar pattern was observed for the Simpson index, as it was 1.12 times higher in maize-based scenarios compared with rice-based CSA scenarios. Shannon's diversity index (6.80 ± 0.551), Simpson's diversity index (0.95 ± 0.016), Chao1 (3,223 ± 256), and observed species/OTU (2,874 ± 229) were found highest in the partial CSA scenario (ScII). Our results showed ahigher Shannon diversity index (1.55

times), Simpson index (1.18 times), Chao1 (1.41 times), and observed species (1.49) in ScII than in ScIII.

A total of seven fungal phyla were present in all six scenarios. Ascomycota is the dominating phyla followed by Basidiomycota and Zygomycota (Figure 2). A higher abundance of Ascomycota was observed in rice-based CSA scenarios (ScIII and ScV) as compared to maize-based scenarios (ScIV and ScVI). In rice-based scenarios (ScI, ScII, ScIII, and ScV), Ascomycota was followed by Basidiomycota but in maize-based scenarios (ScIV and ScVI), it was followed by Zygomycota. Higher Ascomycota (1.31 times) was found in ScIII than ScII, whereas, Glomeromycota was found 7 times higher in ScII than ScIII and 1.66 times higher in ScIV than ScVI. Chytridiomycota was found non-significantly higher in maize-based scenarios as compared to rice-based scenarios. In all six scenarios, a total of 48 classes were observed. Sordariomycetes, an unidentified class of Ascomycota, and Dothideomycetes are among the dominating classes (Table 3). The highest abundance was observed for Sordariomycetes (25.45–43.47%), except for ScV in which an unidentified class of Ascomycota (39.30%) was found in the highest abundance. The abundance of Sordariomycetes was found at par among all the scenarios. The relative abundance of the unidentified class of Ascomycota was found to be 7.8 times higher in CSA-based rice systems compared with maize systems. Although the difference in class Agaricomycetes was non-significant among scenarios, it was two times higher in maize-based scenarios (7.72%) as compared to rice-based scenarios (3.83%). The abundance of Mortierellomycotina_cls_Incertae_sedis class of Zygomycota was recorded 8.4 times higher in maize-based scenarios than in rice-based scenarios. Ascomycota unidentified class was respectively higher in ScIII (2.72 times) than that in ScII and 1.42 times higher in ScV than ScIII. Pezizomycetes class was 6.84 times, Glomeromycetes was 6.97 times, Saccharomycetes was 3.75 times, and Blastocladiomycetes was 8.5 times higher in ScII than that in ScIII. At the level of order, Hypocreales was the dominating order followed by Pleosporales, Pezizales, and Sordariales (Supplementary Table 1).

Effect of agriculture management scenarios on soil chemical and physical properties

Differences among the scenarios were recorded for soil chemical and physical parameters. Soil organic carbon (SOC) and available nitrogen (N) were 1.43 and 1.34 times higher in CSA-based scenarios (ScII, ScIII, ScIV, ScV, and ScVI) compared with farmers' practice (ScI). Available phosphorus and potassium were found to be 1.65 and 1.60 times higher, respectively, in the soil of CSA-based scenarios than that in the CT scenario/farmers' practice. All four SOC (5.7 $\pm$ 0.01 g kg^{-1}), N (119.01 $\pm$ 0.58 kg ha^{-1}), P (16.1 $\pm$ kg ha^{-1}), and K (137 $\pm$ kg ha^{-1}) were lowest with farmers' practice (ScI). Bulk density was observed highest in ScI (1.58) followed by ScII (1.52) (Table 4). It was lower in rice-based CSA scenarios (1.36) as compared to maize-based scenarios (1.445). The highest mean weight diameter was found in ScIII (2.95 mm) and ScV (2.70 mm) and the lowest was observed in ScI (1.45 mm). Water-stable aggregate was non-significant in full/CSA scenarios but significantly higher in ScII (68.3%) and farmers' practice (62.7%) than that in other scenarios.

Crop and system yield

The total residue load in ScI, ScII, ScIII, ScIV, ScV, and ScVI was 105, 101, 119, 101, and 119 Mg ha^{-1}, respectively. During the year 2018, a higher yield of wheat was recorded with all the CSA-based scenarios (ScIII–ScVI) and ranged from 6.58 to 6.79 Mg ha^{-1}. The lowest yield was recorded with farmers' practice, that is, 5.88 Mg ha^{-1}. A similar trend was also observed for the system yield in all the scenarios (Table 4). The higher system yield was recorded with ScVI (16.85 Mg ha^{-1}) followed by ScII (16.19 Mg ha^{-1}) and ScIV (16.04 Mg ha^{-1}). The lowest yield was found with farmers' practice or ScI (13.33 Mg ha^{-1}).

Correlations between soil properties

The results from the principal component analysis provided evidence that the different scenarios were diverse regarding the fungi classes, soil physical properties, and SOC, N, P, and K contents of the soil (Figure 3). According to the factor loadings, the first PC, which explains 48.8% of the total variance, had higher positive correlations with Agaricomycetes, Saccharomycetes, Blastocladiomycetes, and bulk density (BD), and a negative correlation with SOC, N, WSA, and MWD, while the second PC, which explains 16.76% of the total variance, was strongly correlated with Chytridiomycetes, Mortierellomycotina_cls_Incertae_sedis, and Pezizomycotina_cls_Incertae_sedis. The third PC explains 12.6% of the total variance and is correlated with Dothideomycetes (Supplementary Table 2). The PCA biplot in Figure 3 shows both PC scores of samples and the loadings of variables.

Significant correlations were observed among the fungi classes, soil physical properties, and SOC, N, P, and K contents of soil irrespective of scenarios (Table 5). An unidentified class of fungi was significantly negatively correlated to Pezizomycetes, Chytridiomycetes, and Pezizomycotina_cls_Incertae_sedis. Dothideomycetes was significantly negatively correlated to WSA and MWD. Agaricomycetes was significantly positively correlated to Pezizomycetes, Saccharomycetes, and Blastocladiomycetes, and negatively correlated to the available N content of the soil. Eurotiomycetes was significantly positively correlated to Pezizomycetes, Tremellomycetes, and

TABLE 3 Relative abundance of classes in different crop management scenarios.

Scenarios*	Sordariomycetes	Ascomycota; unidentified	Dothideomycetes	Agaricomycetes	Eurotiomycetes	Pezizomycetes	Tremellomycetes	Glomeromycetes	Saccharomycetes	Mucoromycotina_cls_Incertae_sedis	Chytridiomycetes	Mortierellomycotina_cls_Incertae_sedis	Chytridiomycota; unidentified	Blastocladiomycetes	Pezizomycotina_cls_Incertae_sedis
ScI	29.55	21.65^{B**}	22.84	10.48	3.17^{B}	4.00^{AB}	1.66	1.46^{AB}	1.39^{A}	0.61^{A}	0.97	0.65^{B}	0.31	0.19^{A}	0.15
ScII	34.54	10.21^{C}	13.47	8.79	6.67^{A}	5.06^{A}	10.12	2.72^{A}	1.05^{AB}	0.16^{B}	1.81	2.53^{B}	0.32	0.17^{AB}	0.4
ScIII	42.65	27.77^{B}	21.43	2.42	1.86^{B}	0.74^{C}	0.47	0.39^{B}	0.28^{C}	0.08^{B}	0.2	0.72^{B}	0.16	0.02^{C}	0.09
ScIV	31.37	3.85^{C}	22.19	7.14	3.71^{B}	5.07^{A}	0.65	2.47^{A}	1.08^{AB}	0.66^{A}	2.27	15.45^{A}	1.51	0.05^{C}	1.3
ScV	25.45	39.30^{A}	19.91	4.23	1.06^{B}	1.94^{BC}	0.55	1.12^{B}	0.37^{C}	0.19^{B}	0.72	3.05^{B}	0.33	0.09^{BC}	0.13
ScVI	43.47	4.72^{C}	14.45	8.03	2.90^{B}	3.95^{AB}	0.78	1.48^{AB}	0.74^{BC}	0.42^{AB}	2.57	13.74^{A}	0.47	0.08^{BC}	0.8
Mean	34.5	17.92	19.05	6.85	3.23	3.46	2.37	1.61	0.82	0.35	1.42	6.02	0.52	0.1	0.48
p-value	0.3821	<0.0001	0.4627	0.0599	0.0189	0.0413	0.3542	0.0267	0.0198	0.0176	0.2938	0.0013	0.1215	0.0242	0.0866
CV(%)	33.47	26.66	36.92	43	48.27	46.45	249.18	45.8	43.28	55.69	94.99	61.98	108.81	56.13	107.04
SE(d)	9.428	3.9	5.742	2.404	1.273	1.312	4.822	0.601	0.29	0.16	1.105	3.048	0.46	0.046	0.416
LSD at 5%	NS	8.6907	NS	NS	2.836	2.9242	NS	1.34	0.6453	0.3567	NS	6.792	NS	0.1014	NS

*Refer Table 1 for scenarios description.

**Means followed by similar uppercase letters within a column are not significantly different at 0.05 level of probability using Tukey's HSD test.

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TABLE 4 Effect of management practices on soil chemical and physical properties and grain yield.

Scenarios*	SOC (g kg^{-1})	N (kg ha^{-1})	P (kg ha^{-1})	K (kg ha^{-1})	BD	WSA (%)	MWD (mm)	Grain yield (Mg ha^{-1})		
								Rice/maize	Wheat	System
ScI	5.7^{D**}	119^{D}	16.1^{C}	137^{C}	1.58^{A}	62.7^{C}	1.45^{D}	6.37^{BC}	5.88^{C}	13.33^{C}
ScII	7.1^{C}	144^{C}	23.1^{B}	226^{A}	1.52^{B}	68.3^{B}	1.81^{C}	6.63^{B}	6.06^{BC}	16.19^{AB}
ScIII	9.0^{A}	171^{A}	24.9^{B}	218^{B}	1.35^{D}	75.7^{A}	2.95^{A}	6.60^{B}	6.58^{AB}	14.86^{BC}
ScIV	8.3^{AB}	156^{B}	29.7^{A}	226^{A}	1.43^{C}	72.5^{A}	2.50^{B}	6.85^{B**}	6.49^{AB}	16.04^{AB}
ScV	8.1^{B}	171^{A}	30.5^{A}	210^{B}	1.37^{D}	76.3^{A}	2.70^{A}	6.86^{B}	6.61^{AB}	15.61^{AB}
ScVI	8.5^{AB}	157^{B}	24.2^{B}	218^{B}	1.46^{C}	73.7^{A}	2.28^{B}	7.67^{B**}	6.79^{A}	16.85^{A}

WSA, water stable aggregates; MWD, mean weight diameter.
*Refer Table 1 for scenarios description.
**Means followed by similar uppercase l.etters within a column are not significantly different at 0.05 level of probability using Tukey's HSD test.

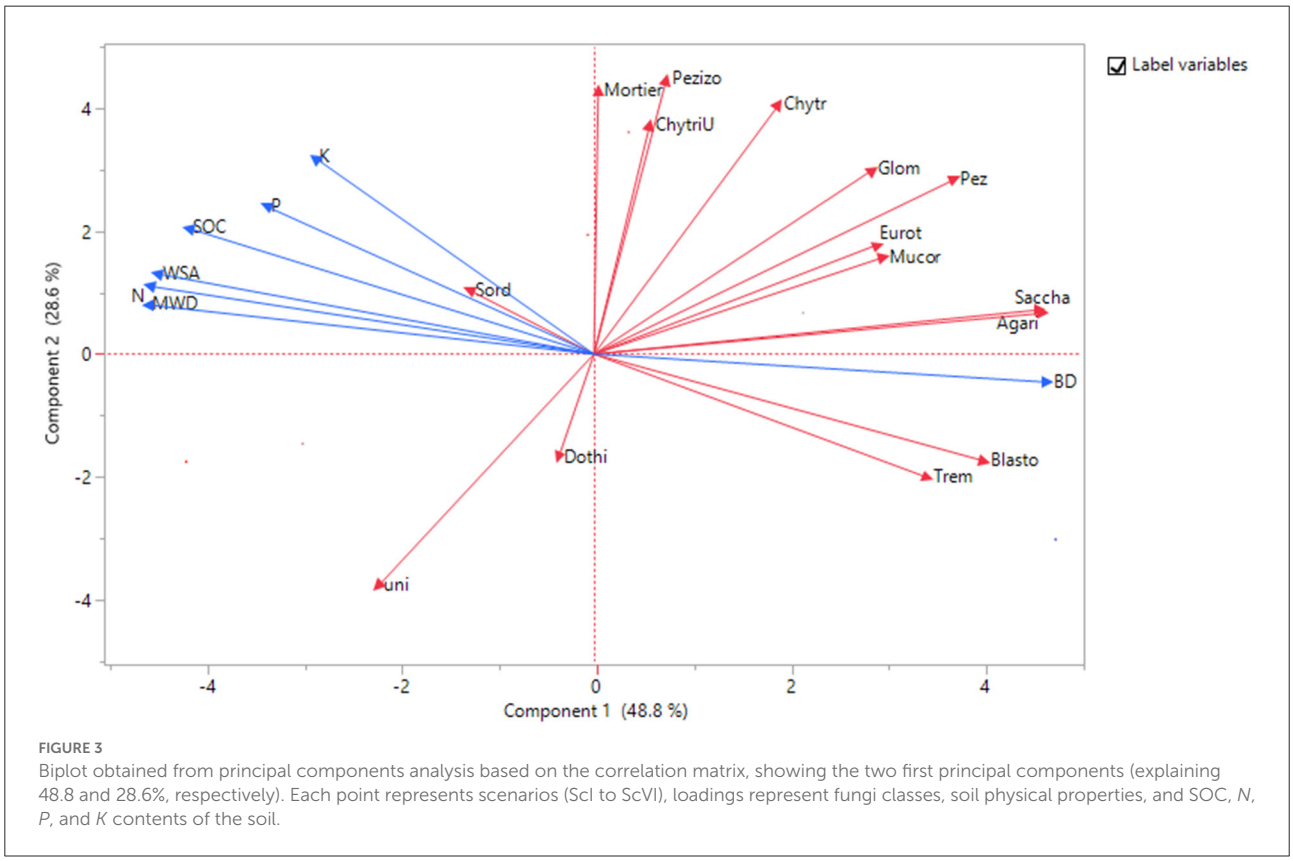

FIGURE 3
Biplot obtained from principal components analysis based on the correlation matrix, showing the two first principal components (explaining 48.8 and 28.6%, respectively). Each point represents scenarios (ScI to ScVI), loadings represent fungi classes, soil physical properties, and SOC, *N*, *P*, and *K* contents of the soil.

Glomeromycetes. Pezizomycetes was significantly positively correlated with Glomeromycetes, Saccharomycetes, and Chytridiomycetes. Glomeromycetes was positively significantly correlated to Chytridiomycetes. Saccharomycetes was positively correlated to Mucoromycotina_cls_Incertae_sedis and Blastocladiomycetes, and negatively correlated to N. Mucoromycotina_cls_Incertae_sedis, which was positively correlated to Chytridiomycota;unidentified. Chytridiomycetes was positively correlated to Mortierellomycotina_cls_Incertae_sedis, Pezizomycotina_cls_Incertae_sedis, and WSA. Mortierellomycotina_cls_Incertae_sedis was significantly positively correlated to Chytridiomycota;unidentified and Pezizomycotina_cls_Incertae_sedis, and WSA. Chytridiomycota;unidentified was significantly positively correlated to Pezizomycotina_cls_Incertae_sedis. Blastocladiomycetes was positively correlated to BD and negatively correlated to the C, N, and P contents of the soil. WSA was significantly positively correlated to MWD and C and K contents. MWD was positively correlated to the C and K contents of the soil. BD was significantly negatively correlated to C, N, P, and K contents of the soil. Soil carbon was positively

correlated to the N, P, and K contents of the soil. The available N was significantly positively correlated with available P and K content of the soil. The available P was significantly positively correlated to the available K content of the soil.

Discussion

Conservation agriculture-based practices showed higher Shannon and Simpson diversity indices in maize-based scenarios as compared to rice-based scenarios due to the inclusion of maize in the crop rotation system. Although sampling of soil was done in all scenarios after harvesting the common crop among them (wheat), the residue and root system of the previous crop can impact soil properties. Fungal diversity is directly or indirectly affected by plant and soil properties (Yang et al., 2017). Maize has a different root system than rice (tap root instead of fibrous root system); the amount and composition of root exudates vary with crops/plants, which can influence the microbial diversity of soil (Sasse et al., 2018). Moreover, the quantity and quality of residue also play a critical role in the microbial properties of soil (Moore et al., 2000). Maize residue provides higher amounts of lignocellulosic material, which can harbor more types of residue-decomposing fungi, hence showing higher fungal diversity than rice-based CA practices. Similar results were reported in our previous study (Choudhary et al., 2018b) based on 5 years of continuous CA-based practices. This combination of residue retention and incorporation in one calendar year may be the reason for the highest diversity indices in this scenario II instead of only crop residue retention in a similar cropping system. Through the tillage practices, crop residues mix well in the soil and alter the microclimate and distribution of nutrients, which resulted in higher fungal diversity under partial CA practice than that in other practices where only crop residues were retained.

Ascomycota, found as a dominating phylum in all management scenarios, is well known as one of the most abundant fungal phyla of soils globally (Choudhary et al., 2018a,b; Egidi et al., 2019; Maguire et al., 2020). Ascomycota is the largest phylum of fungi and is ubiquitous in soil (Money, 2016; Egidi et al., 2019). Most Ascomycota members are saprophytic and the main decomposers of plant residue in the soil, hence they dominate the fungal community composition in soils. In our study, a higher abundance of Ascomycota was observed in rice-based CA scenarios as compared to maize-based scenarios due to the difference between residues. Since soil samples were taken after the harvesting of the wheat crop (common crop in all scenarios) and by that time ample duration (5 months for maize and rice residue) has been received by the residue to decompose. Initially, the residue decomposes fast due to the presence of water-soluble compounds in the residues, which are easily decomposable (Diochon et al., 2016), but in later stages, decomposition of more recalcitrant compounds such as lignin and cellulose takes place. The composition of residue and decomposition, which varies with crop type, are the main factors in harboring different types of microbial communities. This might also lead to the high abundance of Basidiomycota in rice-based scenarios and Zygomycota in maize-based scenarios. The type of crop, size, and type of residue and tillage practices vary in different management systems leading to variation in soil moisture also. Chytridiomycota are reported in aquatic ecosystems as well as in terrestrial ecosystems (Gleason et al., 2004), but in our study, these were found in different management systems and favored in maize-based scenarios. Not only residue type and decomposition duration but also variation in moisture played an important role in deciding the abundance of different groups of fungi (Miura et al., 2015). Sordariomycetes and Eurotiomycetes were among the dominating classes in all the scenarios, which were previously reported in soils of conservation agricultural practices (Wang et al., 2016; Choudhary et al., 2018b). Sordariomycetes is one of the largest classes of Ascomycota, which includes pathogens, endophytes, and saprobes. Members of the Sordariomycetes are ubiquitous and cosmopolitan. Saprobic Sordariomycetes have the potential to produce cellulolytic enzymes, and its member *Chaetomium* is a well-known cellulolytic organism responsible for the destruction of paper and fabrics (Zhang et al., 2006). Members of Sordariomycetes class play an important role in the decomposition and nutrient cycling of plant residues. Dothideomycetes also represents one of the largest and dominant classes of the phylum Ascomycota, which is mostly found as endophytes or saprobes (Kirk et al., 2008). Members of this class can cause disease in almost every crop plant including maize and rice (Goodwin, 2013). Members of this class are also known to degrade cellulose and other complex carbohydrates of dead or partially digested plant organic matter (Hyde et al., 2013). One unidentified class of Ascomycota is also among the most dominating classes found in scenarios. Although its identification is not known to class level, these unidentified and unknown fungal groups may be important to community compositions. Many of these previously unknown groups are later found as major components of communities of interest (Rosling et al., 2011). The abundance of some classes found to be high in maize-based scenarios can be correlated with the difference in crop type, root system, and residue depending on plant species, and biochemical characteristics of plant materials differ considerably (Xu et al., 2006). The effect of tillage practices on the abundance of fungi was observed at both phyla and class levels. Some phyla and classes were found higher in ZT, which indicates that the hyphal network in CT practices got disturbed due to intensive tillage practices, which resulted in limited growth. Whereas, the abundance of some phyla/classes was reported higher in CT than ZT, which might be due to the higher growth rate of broken mycelia of these phyla/classes under CT than that in other management practices. Wang et al. (2017) concluded that tillage influenced the distribution

TABLE 5 Correlation between fungal class abundance, soil chemical and physical properties in different management scenarios.

	Sord	Unid	Dothi	Agari	Eurot	Pez	Trem	Glom	Saccha	Mucor	Chytr	Mortier	ChytriU	Blasto	Pezizo	WSA	MWD	BD	C	N	P	K
Sord	1																					
Unid	−0.40																					
Dothi	−0.39	0.40																				
Agari	−0.19	−0.57	−0.23																			
Eurot	0.06	−0.65	−0.54	0.61																		
Pez	−0.18	**−0.79***	−0.33	**0.84***	**0.77***																	
Trem	−0.03	−0.27	−0.64	0.40	**0.89***	0.49																
Glom	−0.27	−0.68	−0.38	0.64	**0.83***	**0.93****	0.64															
Saccha	−0.29	−0.56	0.04	**0.93****	0.62	**0.84***	0.34	0.68														
Mucor	−0.27	−0.51	0.38	0.64	0.07	0.62	−0.31	0.39	**0.74***													
Chytr	0.18	**−0.89***	−0.52	0.56	0.50	**0.81***	0.20	**0.72***	0.46	0.51												
Mortier	0.20	−0.72	−0.16	0.16	0.06	0.49	−0.28	0.41	0.14	0.56	**0.84***											
ChytriU	−0.20	−0.57	0.26	0.15	0.15	0.55	−0.21	0.55	0.34	**0.67***	0.59	**0.79***										
Blasto	−0.42	−0.05	−0.23	**0.80***	0.54	0.51	0.6	0.43	**0.71***	0.19	0.08	−0.4	−0.30									
Pezizo	0.11	**−0.81***	−0.09	0.26	0.28	0.65	−0.10	0.60	0.33	0.61	**0.84***	**0.94****	**0.90***	−0.29								
WSA	0.4	−0.52	**−0.71***	−0.07	0.18	0.28	0.11	0.33	−0.25	−0.10	**0.72***	**0.72***	0.31	−0.36	0.60							
MWD	0.19	−0.33	**−0.70***	−0.11	0.1	0.21	0.12	0.30	−0.32	−0.18	0.62	0.61	0.22	−0.28	0.47	**0.96****						
BD	−0.18	−0.42	−0.18	0.96**	0.62	0.71	0.48	0.53	0.91*	0.51	0.34	−0.09	−0.05	0.89*	0.02	−0.28	−0.32					
C	0.51	−0.06	−0.11	−0.78	−0.39	−0.46	−0.38	−0.33	−0.79	−0.39	0.04	0.41	0.18	**−0.94****	0.28	**0.61***	**0.55***	**−0.89***				
N	0.25	0.29	−0.07	**−0.89***	−0.49	−0.59	−0.33	−0.39	**−0.91***	−0.56	0.17	0.18	0.03	**−0.84***	0.05	0.49	0.53	**−0.75***	**0.88***			
P	−0.16	0.11	0.01	−0.63	−0.3	−0.18	−0.26	0.04	−0.57	−0.20	0.10	0.44	0.47	**−0.69***	0.39	0.54	0.62	**−0.81***	**0.94****	**0.83***		
K	0.36	−0.26	−0.48	−0.49	0.16	−0.02	0.18	0.21	−0.52	−0.44	0.32	0.43	0.29	−0.61	0.42	**0.79***	**0.75***	**−0.88***	**0.79***	**0.76***	**0.76***	1

Sor, Sordariomycetes, Unid, Ascomycota; unidentified, Dothi, Dothideomycetes, Agari, Agaricomycetes, Eurot , Eurotiomycetes, Pez, Pezizomycetes, Trem, Tremellomycetes, Glom, Glomeromycetes, Saccha, Saccharomycetes, Mucor, Mucoromycotina_cls_Incertae_sedis, Chytr, Chytridiomycetes, Mortier, Mortierellomycotina_cls_Incertae_sedis, ChytriU, Chytridiomycota; unidentified, Blasto, Blastocladiomycetes, Pezizo, Pezizomycotina_cls_Incertae_sedis, WSA, water-stable aggregates, MWD, Mean weight diameter, BD, bulk density, C, organic carbon, P, available phosphorus, N, available nitrogen, K, available potassium.

*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level; ***Correlation is significant at the 0.001 level.

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of fungal communities with more stable communities under ZT conditions. Irrigation management and soil moisture had a great impact on fungal communities in arid and semi-arid areas where water availability is limited (Vargas-Gastélum et al., 2015; Deng et al., 2022). But in our study, irrigation was given to all the scenarios based on the tensiometer to maintain the soil moisture as per crop requirement to minimize the effect of irrigation management on fungal community composition.

Crop residue retention enriches soils with a significant amount of nutrients (Jat et al., 2018, 2019b; Lohan et al., 2018). It is a major reason behind high SOC, N, P, and K contents in residue retention scenarios. Moreover, under zero tillage practices, the decomposition of residue also does not disturb due to the non-disturbance of soil. Residue not only harbors different types of microfauna but also provides nutrients to them, which in turn release different types of enzymes such as phosphatase, glucosidase, dehydrogenase, etc., responsible for the conversion of unavailable to the available forms of nutrients (Maguire et al., 2020). The activity of different enzymes is reported to be more in CA/CSA-based practices and have a higher soil quality index over farmers' practices (Choudhary et al., 2018c,d). CA practices not only improve nutrient availability but also improve overall soil quality (Jat et al., 2021a). It was observed by many researchers that tillage and residue had a positive effect on bulk density (Gathala et al., 2011). In farmers' practice, the residue is either burnt or removed, which resulted in more compactness in soils. Lower BD in the rice-based system as compared to maize-based CA scenarios might be due to higher conversion of crop residue carbon to SOC leading to good soil structure as evidenced through higher WSA and MWD (Mandal et al., 2007).

The results clearly showed the differential benefits of CSA-based management practices in both rice- and maize-based systems. The highest wheat and systems yield (rice equivalent yield basis) were recorded with all CSA-based scenarios. Tillage and cropping systems, crop residue management, soil type, and climate control the magnitude at which SOC affects crop yields (Blanco-Canqui et al., 2013). About 0.1% increase in SOC concentration enhanced wheat yield by 0.04 Mg ha^{-1} at 0 kg N ha^{-1} (Blanco-Canqui et al., 2012). A direct relationship between SOM stocks and crop productivity was observed by Oldfield et al. (2018) through variables for soils and amendments. In a global meta-analysis, zero tillage (ZT) when combined with residue retention and crop rotation can produce equivalent or greater yields than conventional tillage, by minimizing its negative impacts. Higher system productivity in CSA-based scenarios might be due to the integration of mungbean (Gathala et al., 2013), less terminal heat effects on the wheat crop (Gathala et al., 2013; Sharma et al., 2015), higher carbon mineralization (Kirk et al., 2008; Datta et al., 2019) with better nutrient availability (Sasse et al., 2018), and improved soil biological properties (Olsen et al., 1954; Choudhary et al., 2018b,d).

Some classes are negatively correlated while some are positively correlated with each other, and it is due to the ecological relationship between them, which may influence each other's position in the community. The groups, which have similar modes of nutrition, compete for the same biochemical fraction of the crop residues, and this might show a negative correlation. Biochemical qualities of residue are the main drivers in the composition of fungal communities. Specific function during the decomposition of residue linked to specific taxa (Rezgui et al., 2021). In residue decomposition, the end product of one class/group may be acted as food for another group. In such a situation, these may be positively correlated as they do not have competition for food. Due to the preferences of microbes for specific residue compounds, organic matter decomposition and transformation under different agricultural managements influence the abundances of the specific microbial taxa (Zheng et al., 2022). Different type of residue management over the years strongly influences the soil's microbial, chemical, and physical properties. In this 9-year-old experiment, the residue is accumulated over the years in all CSA scenarios, and according to Fontaine et al. (2011), the soil microbes can decompose old recalcitrant soil organic matter by using fresh carbon as a source of energy, which can lead to a different type of distribution of soil fungal taxa in these scenarios. A significant positive correlation between SOC and MWD and WSA was due to good soil structure rendered by higher SOC in CA-based scenarios. Higher MWD and WSA were found under crop residue retention scenarios in cereal systems (Jat et al., 2019a). There is a strong link between microbial communities and SOC (Zheng et al., 2022), which is influenced by the different tillage and crop establishment practices. ZT improved C fractions over CT and these C pools can directly impact the activities of microbes in soils (Rakesh et al., 2021). Soil properties and soil fungal communities also showed different types and magnitudes of relationships because of robust associations between them (Yang et al., 2017). Both the diversity and composition of the fungi community directly correlated with the soil properties and indirectly correlated with management practices (Li and Zhang, 2022).

Conclusion

Agricultural practices in isolation do not prove good for soil and crop sustainability in cereal systems. Crop management practices, such as tillage, residue management, nutrient, water management, and crop diversification have a strong influence on the physical, chemical, and biological properties of soil. Therefore, the combination of different management practices led to differences in fungal community composition and soil chemical properties under climate-smart agriculture (CSA) systems. Maize-based scenarios are more diverse in terms of fungal communities than rice-based CSA scenarios. Rice-based scenarios with CSA practices showed a more abundance of Ascomycota phyla over the maize-based CSA scenarios. Ascomycota is followed by Basidiomycota in rice-based scenarios and Zygomycota in maize-based scenarios.

CSA-based scenarios improved the soil chemical properties (organic carbon, nitrogen, and potassium) across the different cereal-based CSA scenarios compared with other scenarios (CT and partial CSA). Soil physical properties such as bulk density, mean weight diameter, and water-stable aggregate were also improved under CSA scenarios. Improved soil properties under CSA-based practices resulted in improved crop and system yield. Results indicate that the bundling/layering of smart agricultural practices not only influences the soil properties but also played an important role in deciding the microbial community composition.

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/, PRJNA563827.

Ethics statement

The experiment was conducted after taking proper approval from the Institute Research Committee of ICAR-CSSRI, Karnal. Guidelines of the ICAR-CSSRI were followed for taking data on crops/weeds/plants.

Author contributions

HJ, PS, and MJ conceptualized and designed the experiment. MJ and PS did the funding acquisition. HJ and PS did the management of the experiment. HJ and MC conducted the research. MC wrote the manuscript. HJ provided critical comments on the manuscript. All authors 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.2022.986519/full#supplementary-material

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In-depth characterization of phytase-producing plant growth promotion bacteria isolated in alpine grassland of Qinghai-Tibetan Plateau

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The use of plant growth promoting bacteria (PGPB) express phytase (*myo*-inositol hexakisphosphate phosphohydrolase) capable of hydrolyzing inositol phosphate in soil was a sustainable approach to supply available phosphorus (P) to plants. A total of 73 bacterial isolates with extracellular phytase activity were selected from seven dominant grass species rhizosphere in alpine grassland of Qinghai-Tibetan Plateau. Then, the plant growth promoting (PGP) traits of candidate bacteria were screened by qualitative and quantitative methods, including organic/inorganic Phosphorus solubilization (P. solubilization), plant hormones (PHs) production, nitrogen fixation, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and antimicrobial activity. Further experiment were conducted to test their growth promoting effect on Lolium perenne L. under P-limitation. Our results indicated that these bacteria as members of phyla Proteobacteria (90.41%) and Actinobacteria (9.59%) were related to 16 different genera. The isolates of *Pseudomonas* species showed the highest isolates number (36) and average values of phytase activity (0.267 ± 0.012 U mL^{-1}), and showed a multiple of PGP traits, which was a great candidate for PGPBs. In addition, six strains were positive in phytase gene (β-propeller phytase, *bpp*) amplification, which significantly increased the shoot length, shoot/root fresh weight, root average diameter and root system phytase activity of *Lolium perenne* L. under P-limitation, and the expression of phytase gene (*bppP*) in root system were verified by qPCR. Finally, the PHY101 gene encoding phytase from *Pseudomonas mandelii* GS10-1 was cloned, sequenced, and recombinantly expressed in *Escherichia coli*. Biochemical characterization demonstrated that the recombinant phytase PHY101 revealed the highest activity at pH 6 and 40°C temperature. In

particular, more than 60% of activity was retained at a low temperature of 15°C. This study demonstrates the opportunity for commercialization of the phytase-producing PGPB to developing localized microbial inoculants and engineering rhizobacteria for sustainable use in alpine grasslands.

KEYWORDS

phytase, plant growth-promoting bacteria, plant growth promotion, β-propeller phytase, *Pseudomonas mandelii* GS10-1, heterologous expression

Introduction

Over the past few decades, to prevent food shortage worldwide, meat and milk consumption have increased markedly. In particular, the grasslands are important contributors to global food security by supplying proteins and energy to ruminants (Sattari et al., 2016; Stromberg and Staver, 2022). However, grassland degradation has accelerated given the accelerated growth of animal husbandry, soil nutrient loss from overgrazing, weed encroachment, and desertification (Granada et al., 2018). Sustainably meeting forage grass demand is one of the grand challenges of agricultural production, and phosphorus (P) is increasingly found to be a new global sustainability issue given the increasing depletion of extractable P rocks (Dhillon et al., 2017; Granada et al., 2018). Similar to nitrogen, P is an essential nutrient in agriculture and is removed from grassland soils by grass withdrawal and erosion (Sattari et al., 2016). The P removed during the harvest of grass needs to be replaced by inputs through organic and mineral fertilizers to sustain grass production.

Phytate (*myo*-inositol hexakisdihydrogen phosphate, IHP) is a class of inositol phosphate found widely in the natural environment (Turner et al., 2002). They are synthesized in terrestrial ecosystems by plants and strongly complexed in soils, where they accumulate to form the dominant component of organic phosphorus (P_o) (Menezes-Blackburn et al., 2013). The survey of global P storage suggested that the potential of the use of monoester P (mostly inositol phosphates) (George et al., 2018) is greater for grasslands than for arable soils (Menezes-Blackburn et al., 2018). However, due to the lack of active substances that hydrolyze phytate, it is not easy for plants to obtain P from them (Gerke, 2015). In contrast, microorganisms have been shown to efficiently degrade it (Li et al., 2018; Ding et al., 2021; Li J. T. et al., 2021). The P deficiency can significantly enhance the ability of microbial communities to extract P from soil (Jarosch et al., 2019) and increase the abundance of phosphohydrolase-related genes, such as phytase (Yao et al., 2018; Park et al., 2022). Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) produces P by hydrolyzing phytate (Lei et al., 2013). Many bacteria have been reported to secrete phytases, such as *Pseudomonas*, *Bacillus*, *Achromobacter*, *Acinetobacter*, *Klebsiella*, *Micrococcus*, *Burkholderia*, and *Serratia* (Jorquera et al., 2018; Neal et al., 2018; Wan et al., 2020; Rix et al., 2021), which are isolated in different habitats, and support its great potential as a suitable phytase producer with desired properties. Three groups of phytase encoding β-propeller phytases (BPPhy), cysteine phytases (CPhy), and histidine acid phytases (HAPhy) were found in bacteria (Singh et al., 2020), among which BPPhy was the most widely distributed and was considered a good biomarker for evaluating soil organic P transformation (Kumar et al., 2017; Lin et al., 2021). However, despite their importance in soil P mineralization, the study of phytase addressing the P cycle in grasslands soil remains poorly understood. Its diversity, bacterial communities involved in its secretion, and conditions that may influence its production and reactivity need to be elucidated (Devi et al., 2022).

Plant growth-promoting bacteria (PGPB) is a group of well-known microorganisms that can promote plant growth, which can enhance nitrogen fixation, synthesize growth regulators or plant hormones, dissolve organic/inorganic phosphates, protect against harmful effects of soil-borne plant pathogens, etc. (Tapia-García et al., 2020; Hu et al., 2021; Li M. Y. et al., 2021). However, most global studies mainly focus on arable land and ignore the huge biotechnological potential of PGPBs in grasslands (Haskett et al., 2021; Pankievicz et al., 2021). Additionally, little attention has been given to the screening for PGPBs by identifying microbial-containing phosphatase encoding genes (Wan et al., 2020). The plants in combination with PGPBs possessing the ability to produce phytase and promote plant growth is an efficient and environmentally sustainable strategy used in the restoration of degraded grasslands (Granada et al., 2018) since it presents the potential to reduce the amount of the most important synthetic inputs applied on grasses, which is the paramount importance of fertilizers obtained from limited sources (Alori et al., 2017). The availability and stability of PGPBs are affected by various factors, such as soil physicochemical properties, climatic conditions, and the composition of microbial communities. Therefore, the understanding of native bacterial populations, finding region-specific microbial, and collecting and establishing a database of

isolates are necessary to advance the wider commercial use of PGPBs (Goh et al., 2019; Vasseur-Coronado et al., 2021).

The Qinghai-Tibet Plateau is the largest and highest plateau on earth and also the main distribution area of alpine grasslands in China that has unique climatic characteristics, such as low temperature, hypoxia, and nutritional deficiencies, and breeds abundant microbial resources (Ma et al., 2020). Therefore, PGPBs living there potentially represent new species and develop unique physiological adaptation mechanisms, ultimately producing bioactive compounds. The aims of the present study are as follows: (1) using the culture-dependent approach to investigate the cultivable phytase-producing bacterial diversity in the rhizosphere; (2) assessing the PGP traits of isolates quantitatively under suitable conditions; (3) comparing the effect of inoculation of strains on plant growth under P-limitation; and (4) cloning, sequencing, and expressing the phytase genes from strains, and evaluating its biochemical properties. We anticipate that several bacteria with phytase activity and PGP traits will be obtained in this study, and the new phytase with activities will be revealed clearly.

Materials and methods

Sample collection

The samples were collected from seven dominant types of grass, including *Astragalus chilienshanensis*, *Avena sativa*, *Bupleurum chinense*, *Kobresia kansuensis*, *Oxytropis ochrocephala*, *Poa pratensis*, and *Stipa capillata* from the Menyuan County, Qinghai Province, China (37°39′51″N, 101°10′44″W) in August 2019, which is located in the northeast side of the Qinghai-Tibet Plateau. The healthy individual plants were collected by using sterile tools, and the whole roots were carefully dug out and shaken to remove the bulk soil while retaining the tightly bound soil. Samples were kept in sterile plastic bags, transported to the laboratory on ice, and used immediately for the isolation of strains of bacterial cultures.

Isolation and screening of phytase-producing bacteria

The isolates were screened from samples using the dilution-plate method in modified medium M1 containing sucrose 10 g, $(NH_4)_2SO_4$ 0.5 g, NaCl 0.3 g, KCl 0.3 g, $MnSO_4{\cdot}4H_2O$ 0.03 g, $FeSO_4{\cdot}7H_2O$ 0.03 g, yeast extract 0.4 g, calcium phytate 5 g, and agar 18 g and medium M2 containing glucose 10 g, $(NH_4)_2SO_4$ 0.1 g, $MgCl_2{\cdot}6H_2O$ 5 g, $MgSO_4{\cdot}7H_2O$ 0.25 g, KCl 0.2 g, calcium phytate 5 g, and agar 18 g (Nautiyal, 1999). Approximately 1 g of fresh root samples was added to 9 ml of 0.9% normal saline and serially diluted to 10^{-3}, 10^{-4}, and 10^{-5} dilutions. Then, 100 μl of diluted samples were spread-plated on the medium and incubated at 28°C for 4–6 days. The strains were selected by their color morphology and surface smoothness, purified two to three times to obtain pure cultures, and cryopreserved in 20% glycerol at −80°C.

The isolates were inoculated in Luria-Bertani (LB) liquid medium for 24 h. The cultures were centrifuged and suspended in sterile water until the optical density (OD) reached 1.0 at 600 nm. The cell suspension was inoculated in phytase-screening medium containing glucose 20 g, NH_4NO_3 2 g, KCl 0.5 g, K_2HPO_4 0.04 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $MnSO_4{\cdot}4H_2O$ 0.01 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, and $CaCl_2{\cdot}2H_2O$ 0.04 g at pH 7.0 and incubated at 15°C, 200 rpm for 5 days. Then, 10 ml of each culture was centrifuged at 12,000 × *g* for 15 min and the supernatant was used to assay extracellular phytase activity.

Phytase activity was measured by the ferrous sulfate-molybdenum blue colorimetry method (Bae et al., 1999). Under standard conditions, the reaction mixture contained 0.1 ml of culture supernatant, 2 mM $CaCl_2$, 1.5 mM sodium phytate (Sigma-Aldrich P3168, DE, USA), and 0.1 M Tris–HCL buffer (pH 7.0) in order to bring the final volume to 1 ml. The reaction mixture was incubated for 30 min at 37°C and the reaction was terminated by the addition of 1 ml 10% (w/v) trichloroacetic acid (TCA). Then, 2 ml of color reagent containing 1% (w/v) ammonium molybdate, 3.2% (v/v) sulfuric acid, and 7.32% (w/v) ferrous sulfate was added. The released inorganic phosphate was analyzed by standing at room temperature for 10 min followed by centrifugation (13,000 × *g*, 4°C) for 10 min. One unit of phytase activity was defined as the amount of enzyme catalyzing the release of 1 μmol of P per min under the above-specified conditions. Each experiment was replicated three times.

16S rRNA genes amplification and phylogenetic analysis

The genomic DNA of isolates was extracted using Bacteria Genomic DNA Extraction Kit (Takara No. 9763, Beijing, China). The 16S rRNA gene of strains was amplified using the universal primer 27F-1492R following the PCR conditions described by Frank et al. (2008). Both genomic DNA and PCR product quality and quantity were determined in NanoPro (2010) (DHS Life Science and Technology, Beijing, China). Samples were sent to Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China) for sequencing using the Sanger method. The sequences of strains of the 16S rRNA gene were identified, and the most similar strains were downloaded by Ezbiocloud[1] (Yoon et al., 2017). Then, the phylogenetic tree of the 16S rRNA gene was aligned by ClustalX and constructed by the Neighbor-joining method (Saitou and Nei, 1987) with bootstrap based on 1,000

1 https://www.ezbiocloud.net

replications in MEGA 7.0, and the nucleotide sequences of the strains 16S rRNA gene fragments were deposited into GenBank under the accession numbers OP412497-OP412569.

Determination of *in vitro* PGP traits

The isolates belonging to *Pseudomonas* species (n = 36) were selected to determine *in vitro* plant growth-promoting (PGP) traits.

Phosphorus solubilization

The isolates were grown in Pikovskaya's and Mongina's liquid medium (Li M. Y. et al., 2021) at 15°C, 180 rpm for 10 days, and centrifuged at 10,000 × g for 20 min to obtain the supernatant. The inorganic (tricalcium phosphate) and organic (egg-yolk lecithin) P solubilization activities were quantified using phospho-molybdate blue color method (Nautiyal, 1999) at 700 nm. Each experiment was replicated three times.

Plant hormones production

The isolates were grown in LB liquid medium at 15°C, 180 rpm for 5 days in dark conditions. The production of plant hormones (PHs) was quantified by high-performance liquid chromatography (HPLC). The supernatant was extracted using ethyl acetate and analyzed on an HPLC system (Agilent Technologies 1260 Infinity, CA, USA) using Perrig et al. (2007) methods. The chromatographic column was Agilent ZORBAX Eclipse Plus C18 Column (250 × 4.6 mm, 5 μm) and the mobile phase was 45:55:1 (v:v:v), which included methanol, ultrapure water, and acetic acid in 254 nm. The standard PHs for testing, namely, trans-Zeatin (t-Z), gibberellin acid (GA_3), and indoleacetic-3-acid (IAA), were purchased from Merck (Darmstadt, Germany), and the chromatographic peak times were 3.788 min, 5.382 min, and 10.076 min, respectively. Each experiment was replicated three times.

ACC deaminase activity

The isolates were suspended in 7.5 ml DF salts minimal medium, and 45 μl sterile 0.5 M 1-aminocyclopropane-1-carboxylic acid (ACC) solution was added to the culture at 15°C, 200 rpm for 24 h to induce ACC deaminase production, and the activity of isolates was determined using 2, the 4-dinitrophenylhydrazine colorimetric method by Penrose and Glick (2003) described, and the protein concentration was determined by the method of Bradford (Spector, 1978). Each experiment was replicated three times.

Nitrogen fixation

The isolates were inoculated into malate Ashby's nitrogen-free medium semi-solid medium vials at 15°C for 48 h. Nitrogen fixation activity was calculated based on the results of the acetylene reduction assay (ARA) (Hardy et al., 1968). Acetylene (5% v/v) was injected into the vials and incubated at 15°C for 24 h, 50 μl of gas samples from the vials were analyzed on a gas chromatography (GC) system (Aglient Technologies 7890B, CA, USA), and the protein concentration determination method is the same as mentioned previously. Each experiment was replicated three times.

Antimicrobial activity

The antimicrobial activity of isolates against two plant pathogenic fungi (*Rhizoctonia solani* and *Fusarium oxysporum*) was estimated by the plate confrontation method (Li et al., 2020) in potato dextrose agar (PDA) medium. The growth inhibition ratio (%) was calculated by I = (D-d)/D × 100%, where "D" and "d" shows the colony diameters of the pathogen in the control and the treatment groups, respectively. Each experiment was replicated three times.

Detection of phytase gene using PCR amplifications

The degenerate PCR methods used to detect the presence of the phytase gene included β-propeller phytase (*bpp*) (Huang et al., 2009), histidine acid phytase (*hap*) (Huang et al., 2011), and cysteine phytase (*cp*) (Huang et al., 2006). The primers' information is shown in **Supplementary Table 2**. The touchdown PCR conditions are as follows: 95°C for 4 min, then 10 (*bpp*)/8 (*hap*, *cp*) cycles of 94°C for 30 s, 50°C (*bpp*)/58°C (*hap*, *cp*) for 30 s (decreasing 0.5°C of *bpp* and 1°C of *hap* and *cp* for each cycle), and 72°C for 30 s, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 10 min, 4°C hold. The 50-μl PCR reaction systems contained 25 μl Premix (1.25 U of DNA polymerase, 0.4 mM each of the dNTPs, 4 mM Mg^{2+}), 0.05 mM of each primer, and 50–100 ng of bacterial genomic DNA, and verified by agarose gel electrophoresis (1.5%) using gel imager (Bio-Rad GelDoc XR, Hercules, CA, USA). The amplified fragments with appropriate size (160–200 bp) were cut by rubber recycling and ligated into the pTOPO-TA vector (Aidlab CV14, Beijing, China). The positive clones were sent to Sangon Bio for sequencing, and the results were searched using BLSATn in NCBI.[2]

PGP potential of strains under P-limitation

Six strains containing the phytase gene were selected to test their PGP potential under P limitation. *Lolium perenne* L. "227" (Grass Germplasm Innovation Laboratory, Gansu Agricultural

2 https://www.ncbi.nlm.nih.gov/

University, China) seeds were washed, surface-sterilized for 15 min in 2% (v/v) sodium hypochlorite, washed four times with sterile water, and the floating seeds were discarded (Ramakrishna et al., 1991). The sterilized seeds were sown in the germination box and germinated in the dark at 15°C for 4 days. Then, the seedlings were transplanted into the hydroponics unit and incubated in a biotron (Eshengtaihe Ctrl Tech, Beijing, China) under controlled conditions (15°C day/night, 18 h light/6 h dark, light intensity 1,250 μmol m^{-2} s^{-1}) at Pratacultural College, Gansu Agricultural University, in each device in five replicates. Half-strength P-free Hoagland sterile nutrient solution and 100 mg L^{-1} sodium phytate were added to each device. The bacterial suspension with an OD (600 nm) value of 1.0 was added to the device after the seedlings were grown for 2 days. The Hoagland's was changed every 7 days. The plants were harvested on day 28, and the following indicators were measured: shoot length, root length, fresh weight shoot, fresh weight root, dry weight shoot, dry weight root, and root/shoot ratio. The root morphology was obtained by using Expression 12000XL (Epson, Suwa, Japan). Then, different treatments of roots with plants were collected to determine the phytase activity and used for quantitative PCR (qPCR) detection.

The RNA of the root sample was extracted using RNAprep Pure Bacteria Kit (TIANGEN DP430 Beijing, China), and the cDNA was synthesized using a reverse transcription kit (PrimeScript RT reagent Kit with gDNA Eraser, TaKaRa No. RR047A, Beijing, China). The TB Green Premix Ex Taq^{TM} II (TaKaRa No. RR820Q, Beijing, China) on the LightCycler 96 PCR System (Roche, Basel, Switzerland) was used to perform the qPCR procedure. The primer *bpp*P (**Supplementary Table 2**) was employed to evaluate the expression of strains *bpp* gene in the root system. The abundance of the 16S rRNA gene was used as inner control to calculate the relative expression. The PCR reaction conditions were referenced by Dong et al. (2020) method.

Cloning of phytase gene

The full-length sequences of phytase gene was cloned from strain *Pseudomonas mandelii* GS10-1. The 5′ and 3′ flanking regions of the phytase gene were obtained using the Genome Walking Kit (Takara No. 6108, Beijing, China) by the manufacturer's instructions, and the nested gene-specific primers are shown in **Supplementary Table 2**. The amplified PCR products were ligated into the pTOPO-TA vector for sequencing. Both upstream and downstream corrected sequences and known sequences were spliced using the Seqman program to assemble into a complete sequence, and the complete open reading frame (ORF) was predicted for the sequence using an ORF finder in the NCBI program. The similarity of the sequence encoding amino acid to other homologous sequences was compared using a Blast tool in the UniProt protein database,[3] and the similar sequences to multiple alignments were downloaded using a ClustalX program. The SWISS-MODEL[4] was used to perform protein structure homology modeling. The obtained phytase gene was named PHY101 (GenBank accession number: OM935859).

Production and purification of recombinant protein

The recombinant PHY101 protein was expressed using an *Escherichia coli* expression system. The mature protein-coding gene sequence (remove signal peptide) of PHY101 and PCR *Pfu* DNA polymerase (Vazyme Biotech P525, Nanjing, China) were used for amplification. The PCR product was purified by gel extraction and sub-cloned between the *Bam*HI and *Hind*III sites of the pET-28a(+) vector. The 6 × His-tag at the N-terminal end of the target gene, and the vector named as pET-PHY101. The vector pET-PHY101 was transformed into *E. coli* BL21 competent cells by heat shock method. The recombinants were selected on LB agar medium containing kanamycin (Kana, 50 μg mL^{-1}). The white colonies that appeared on plates were extracted using Plasmid Mini Kit (Omega Bio-Tek D6943, CT, USA), and PCR methods were performed for commercial sequencing identification.

The *E. coli* strain (BL21/pET-PHY101) was grown in LB liquid medium containing 50 mg L^{-1} Kana at 37°C, 220 rpm. Recombinant protein expression was induced using 1 mM IPTG until the cultures' OD (600 nm) reached 0.8 (log phase), induction culture at 20°C for 6 h. Cells were harvested by centrifugation at 4°C and pellets were suspended in Tris–HCl (pH 7.0). The his-tagged recombinant protein PHY101 was purified using Ni-NTA Fast Start Kit (QIAGEN No. 30600, Hilden, Germany). The fraction containing the purified protein was collected for 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The phytase activity and protein concentration were assayed as previously described. All activity assays were performed in the presence of 5 mM Ca^{2+} unless otherwise stated.

Enzymatic properties of recombinant phytase analysis

Effects of pH and temperature

The optimum pH of PHY101 was determined by measuring the relative activity of the enzyme under different pH conditions (3.0–10.0), The buffers used were 0.1 M citric acid-sodium

3 https://www.uniprot.org/

4 https://swissmodel.expasy.org/

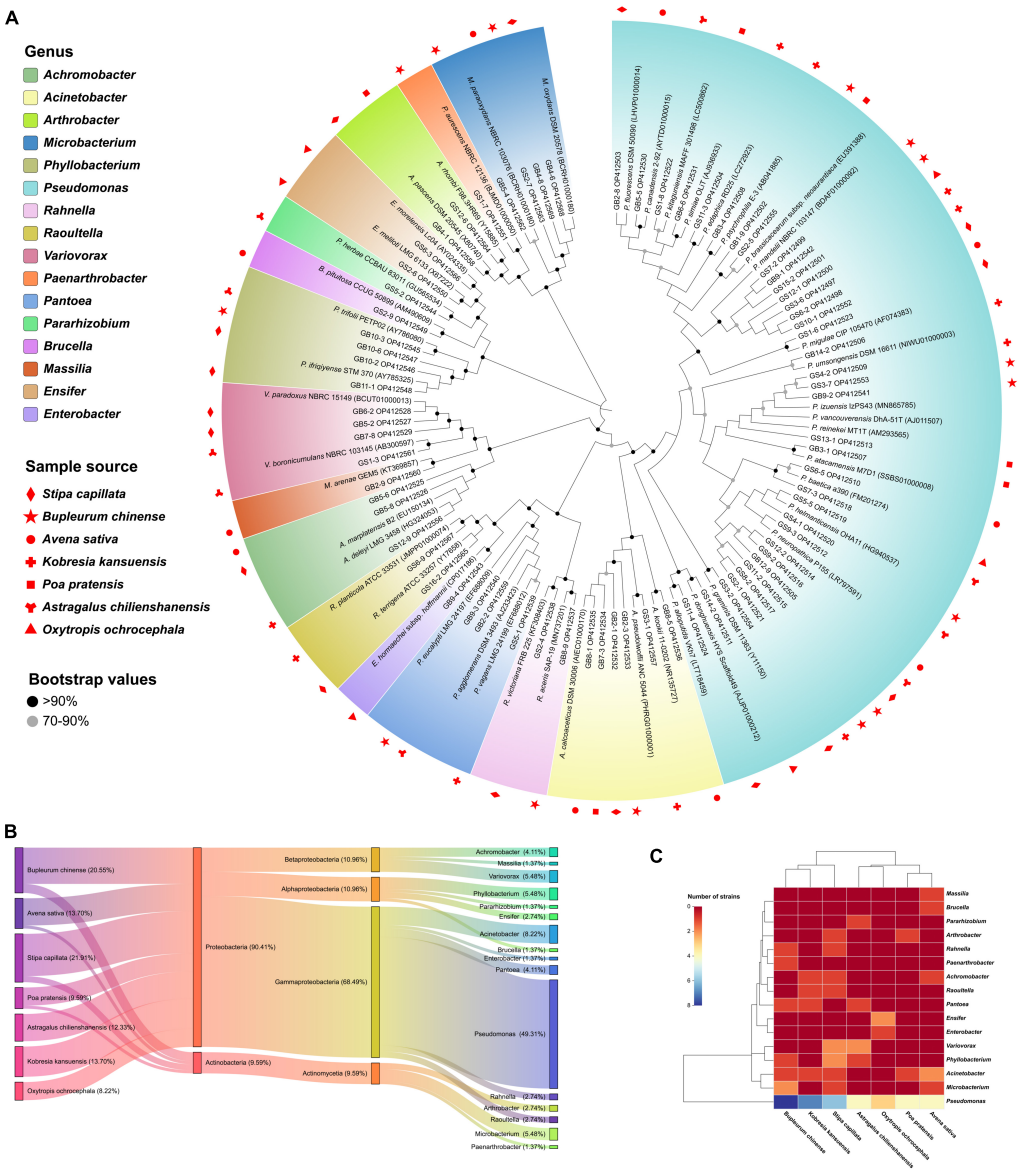

FIGURE 1
Diversity and taxonomic analysis of phytase-producing bacteria. **(A)** Neighbor-joining tree based on 16S rRNA gene sequences showing relationships between the 73 isolates and their closest relatives, the effective sequence length was 1,200 bp and the bootstrap values were 1,000 replications. The serial number in parentheses denotes the GenBank accession number of strains. **(B)** Sankey diagram of the community composition of bacteria isolates. From left to right: Host plant, phylum level, class level, and genus level. **(C)** Abundance and hierarchical clustering analysis of bacteria isolates under different plants treatment at genus level.

citrate buffer at pH 3.0–5.0; 0.1 M acetic acid-sodium acetate buffer at pH 5.0–7.0; 0.1 M Tris–HCl buffer at pH 7.0–8.0; and 0.1 M glycine-sodium hydroxide buffer at pH 9.0–10.0. The stability of pH was determined by optimum pH at 37°C after the enzyme solution was treated in different pH buffers (2.0–10.0) at 37°C for 1 h.

The optimum temperature was determined by different temperature conditions (10–60°C) under optimum pH. The stability of temperature was determined by different temperatures from 10 to 60°C for 20 min, and the thermal stability was determined by optimum pH after 10–60 min for 40, 50, and 60°C.

Effect of metal ions and chemical reagents

The different concentrations (1 and 5 mM) of metal ions (Mg^{2+}, Na^{+}, K^{+}, Mn^{2+}, Cr^{3+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+}) and chemical reagents (EDTA and SDS) were added to the enzymatic reaction system to study their effects on the enzymatic activity at optimum pH and temperature, and treatment without any reagent was used as the control.

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) by SPSS 25.0 software. The mean comparison was carried out by using Duncan's multiple range tests. Statistically significant data was determined at $p \leq 0.05$. Data are presented as means ± SD.

Results

Phytase-producing bacteria from plant samples

A total of 114 bacteria isolates were selected and purified from seven plants using enriched media, and 73 isolates (64.04%) of them have extracellular phytase activity. The 16S rRNA gene (~1,200 bp) sequences BLAST alignment validated the identity with 99.41–100% similarity (**Figure 1A**). The sample details as shown in **Supplementary Table 1**. The cultivable phytase-producing bacteria belong to two phyla, four classes, and sixteen genera. Proteobacteria (90.41%) was the dominant phyla and the other was Actinobacteria (9.59%) (**Figure 1B**). *Pseudomonas* was the most abundant genus for 36 isolates (49.31%) followed by *Acinetobacter* for six isolates (8.22%). In addition, *Stipa capillata* rhizosphere yielded the highest number of isolates (16) including *Pseudomonas*, *Phyllobacterium*, *Variovorax*, *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Rahnella*, *Raoultella*, and *Microbacterium*, followed by *Bupleurum chinense* (15), and the lowest number of them was *Oxytropis ochrocephala* (6). Only *Pseudomonas* species isolates were identified in all plants tested (**Figure 1C**).

The phytase activity assay results of isolates are shown in **Supplementary Table 1**. The mean value of phytase activity of *Pseudomonas* species isolates showed a higher average of 0.267 ± 0.012 U mL^{-1}, among *Pseudomonas baetica* GS6-5 from *Avena sativa* showed the highest activity (0.391 U mL^{-1}), and the mean value of others genus was 0.168 ± 0.13 U mL^{-1}, among *Acinetobacter calcoaceticus* GB2-3 from *Bupleurum chinense* showed the highest activity (0.350 U mL^{-1}) (**Figure 2**).

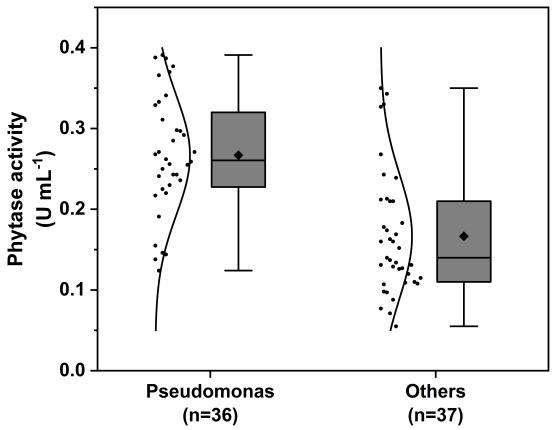

FIGURE 2
Comparison analysis of phytase activity between genera of *Pseudomonas* and others.

PGP traits of bacterial isolates

All the isolates from *Pseudomonas* (36) species were selected to evaluate different PGP traits, the antagonism of two pathogenic fungi, and the presence of three encoding phytase genes (*bpp*, *hap*, *cp*). The results showed that they had multiple PGP traits as shown in **Table 1**. Among these, all the isolates showed an excellent P-solubilization in the range 49.08–410.73 μg mL^{-1} ability when tricalcium phosphate was the P source, and *Pseudomonas mandelii* GS10-1 showed the highest solubilization ability, followed by *Pseudomonas mandelii* GS3-6. A total of 27 isolates (75.00%) showed P-solubilization ability in the range 0.97–27.76 μg mL^{-1}when egg-yolk lecithin was the P source, *Pseudomonas baetica* GS7-3 showed the highest solubilization ability, followed by *Pseudomonas neuropathica* GS4-1.

The ability of PHs production (t-Z, GA_3, IAA) was detected by HPLC. All the isolates can produce at least one or more PHs. Among them, a total of 15 isolates (41.67%) produce three, 17 isolates (47.22%) produce two, and 4 isolates (11.11%) produce one PH (**Figure 3**). A total of 26 isolates (72.22%) showed t-Z-producing ability in the range 3.98–17.45 μg mL^{-1}, 32 isolates (88.89%) showed GA_3-producing ability in the range 23.16–173.22 μg mL^{-1}, and 25 isolates (69.44%) showed IAA-producing ability in the range 0.09–2.27 μg mL^{-1} (**Table 1**).

Only five isolates (13.89%) showed ACC deaminase activity in the range 0.32–3.14 μmol mg^{-1} protein h^{-1}. The maximum activity of ACC deaminase by bacteria strains was observed in *Pseudomonas helmanticensis* GS8-2.

Nitrogen fixation of isolates was determined by indirect measurement of the amount of acetylene reduced to form ethylene. A total of 25 isolates (69.44%) were capable of growing on NFM medium and showed nitrogen fixation ability in the range of 51.53–94.01 nmol (C_2H_4) mg^{-1} protein h^{-1}. *Pseudomonas helmanticensis* GS5-5 showed the highest nitrogenase activity, followed by *Pseudomonas graminis* GS3-2 (**Table 1**).

Antimicrobial activity of isolates of *R. solani* and *F. oxysporum* showed low inhibitory, as shown in **Table 2**. Eight isolates (22.22%) exhibited antimicrobial activity against *R. solani* in the range 6.89–25.94% and seven isolates (19.44%) against *F. oxysporum* in the range 8.60–32.90% (**Table 1**).

The presence of functional genes related to phytase (*bpp*, *hap*, *cp*) was used to evaluate the enzyme class of isolates, the *bpp* gene was detected in six isolates by PCR amplification and

TABLE 1 Screening the *in vitro* plant growth-promoting (PGP) traits in genus of *Pseudomonas*.

Strain no.	P-solubilization[a] (μ g mL^{-1})		Plant hormones production[b] (μ g mL^{-1})			ACCD activity[c] (μ mol mg^{-1} protein h^{-1})	Nitrogen fixation (nmol C_2H_4 mg^{-1} protein h^{-1})	Antimicrobial activity[d]		PCR[e]		
	I-P	O-P	t-Z	GA_3	IAA			*RS*	*FO*	*bpp*	*hap*	*cp*
GB1-9	49.08	0.97	–	62.22	0.11	–	51.53	–	–	–	–	–
GB2-8	247.98	–	–	53.43	0.33	–	69.93	15.77%	32.90%	–	–	–
GB3-1	85.32	8.88	8.71	29.37	0.09	–	–	–	–	–	–	–
GB3-4	179.64	10.23	4.44	41.97	1.02	–	–	–	–	–	–	–
GB5-5	99.32	1.94	6.47	56.25	–	–	85.20	–	–	+	–	–
GB6-6	234.85	9.08	–	95.67	0.14	–	66.92	12.47%	22.54%	–	–	–
GB9-1	111.41	3.15	12.36	–	–	–	80.72	–	–	–	–	–
GB9-2	271.43	2.65	–	74.98	–	–	77.66	–	–	–	–	–
GB12-9	255.54	6.13	12.01	63.39	0.20	–	90.33	24.80%	18.55%	–	–	–
GB14-2	305.55	–	–	38.55	–	0.85	69.94	–	–	–	–	–
GS1-6	309.87	6.98	11.17	–	0.17	–	88.41	6.89%	20.46%	–	–	–
GS1-8	212.63	17.45	7.50	99.40	–	–	77.07	–	–	–	–	–
GS2-1	83.95	–	9.08	–	0.32	–	–	–	–	–	–	–
GS2-5	80.90	–	–	39.62	–	–	–	–	–	–	–	–
GS3-2	278.05	4.80	16.67	67.44	1.39	–	93.20	–	–	–	–	–
GS3-6	380.01	12.37	15.91	109.51	0.87	–	–	10.37%	–	+	–	–
GS3-7	189.62	–	–	48.60	0.30	–	–	–	–	–	–	–
GS4-1	247.99	25.44	–	28.65	1.05	–	88.82	–	–	–	–	–
GS4-2	174.35	19.08	9.19	48.60	0.23	1.09	74.50	21.86%	8.60%	–	–	–
GS5-5	77.90	9.80	14.50	–	0.62	–	94.01	–	–	–	–	–
GS6-2	185.44	–	12.50	80.94	–	–	–	–	–	–	–	–
GS6-5	342.33	–	11.61	130.09	0.66	–	–	–	–	+	–	–
GS7-2	197.40	11.93	6.93	98.51	0.98	–	74.93	–	–	–	–	–
GS7-3	278.05	26.76	–	66.50	2.27	–	84.47	25.94%	20.80%	–	–	–
GS8-2	366.39	11.51	8.63	23.16	0.37	3.14	75.25	–	–	–	–	–
GS9-2	211.50	7.83	15.90	90.51	–	–	88.05	–	–	–	–	–
GS9-3	300.98	2.94	3.98	110.50	0.85	–	90.58	–	–	–	–	–
GS10-1	410.73	14.85	10.64	173.22	0.69	–	–	–	–	+	–	–
GS11-2	186.05	7.80	17.45	44.23	0.73	2.29	92.33	–	–	–	–	–
GS11-3	224.44	8.65	12.84	144.22	0.23	–	68.92	–	–	–	–	–
GS11-4	310.25	10.86	13.07	35.36	–	–	–	–	–	–	–	–
GS12-1	291.71	–	8.60	80.44	–	0.32	74.48	–	–	–	–	–
GS12-2	234.56	6.22	7.77	56.73	0.49	–	84.20	19.22%	15.50%	–	–	–
GS14-1	114.52	1.88	–	118.65	0.19	–	–	–	–	+	–	–
GS14-2	201.90	–	7.54	50.45	0.38	–	75.65	–	–	–	–	–
GS15-2	212.30	6.76	10.15	33.97	–	–	71.97	–	–	+	–	–

Each values is a mean of three independent trials.

[a]"I-P" means tricalcium phosphate, "O-P" means egg yolk lecithin.

[b]"t-Z" means trans-Zeatin, "GA_3" means gibberellins, "IAA" means indoleacetic-3-acid, "–" means showed no detected on this hormones.

[c]1-aminocyclopropane-1-carboxylate deaminase activity.

[d]"*RS*" means *Rhizoctonia solani*, "*FO*" means *Fusarium oxysporum*.

[e]"–" means indicates the absence of PCR products and "+" means indicates the presence of PCR products for the phytase functional genes: *bpp*, β-propeller phytase; *hap*, histidine acid phytase; *cp*, cysteine phytase.

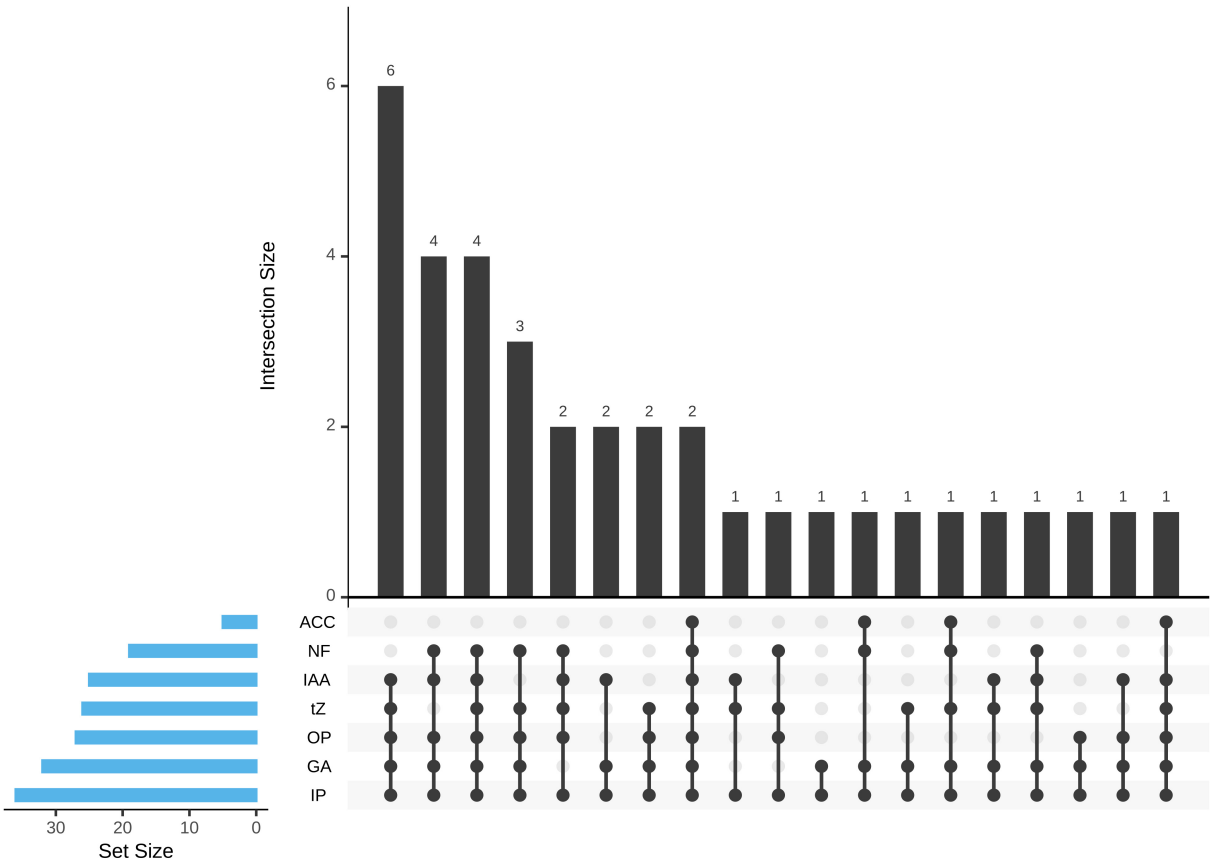

FIGURE 3
The upset plot representation of the 36 *Pseudomonas* sp. strains was allocated to seven different plant growth-promoting (PGP) traits. ACC, ACC deaminase activity; NF, nitrogen fixation; IAA, IAA production; tZ, t-Z production; OP, organic phosphate solubilization; GA, GA_3 production; IP, inorganic phosphate solubilization.

confirmed by sequencing sequence alignment. The *hap* and *cp* gene PCR products showed non-specific amplification in this study (Table 1).

Plant growth promotion potential under P-limitation

The isolates (GB5-5, GS3-6, GS6-5, GS10-1, GS14-1, and GS15-2) were tested for their potential of growth-promoting for *Lolium perenne* L. when sodium phytate was the P source, and a non-inoculated control experiment was set. The strains had variable effects on the shoot and root in *Lolium perenne* L. All strains inoculation significantly enhanced the length and fresh weight of the shoot and reduced the root length to the control ($P < 0.05$). Among them, GS10-1 and GS3-6 increased by 82.74 and 30.95% the root fresh weight to the control, and also root/shoot ratio was the highest except for the control ($P < 0.05$). *Lolium perenne* L. promotes P uptake by regulating root morphology and configuration under P-limitation. In this study, all strains inoculation significantly ($P < 0.05$) reduced the total root length to the control (Figure 4A). Except for GS10-1, which was increased by 7.08%, the others significantly ($P < 0.05$) reduced the root surface area to the control. GS10-1 and GS14-1 were increased by 33.07 and 12.60% with the root volume. In addition, all treatments increased the root average diameter to the control. The highest growth promotion was GS10-1 compared to the others tested, which had significantly increased ($P < 0.05$) aboveground and underground biomass of *Lolium perenne* L. (Table 2).

We investigated the effect of strains inoculation on phytase activity in the *Lolium perenne* L. root system; the inoculation treatment significantly ($P < 0.05$) increased the root system activity to the control, among which GS10-1 showed the highest activity with 0.344 $\mu mol\ g^{-1}$ plant min^{-1} (Figure 4B). In addition, we designed primers *bpp*P based on the conserved sequence of the phytase gene. The qPCR results showed that the phytase gene can be effectively expressed in the *Lolium perenne* L. root system (Figure 4C), and related to the results of root

TABLE 2 Effects of strains inoculation on the growth of *Lolium perenne* L. seedlings.

Treatments	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g plant^{-1})	Root fresh weight (g plant^{-1})	Root/Shoot ratio	Total root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Root average diameter (mm)
Control	14.01 ± 1.52d	21.52 ± 2.44a	0.071 ± 0.007e	0.168 ± 0.021c	2.37 ± 0.13a	265.15 ± 8.31a	20.61 ± 1.85b	0.127 ± 0.007c	0.247 ± 0.016e
GB5-5	17.69 ± 1.41c	8.80 ± 1.85e	0.079 ± 0.011d	0.136 ± 0.019e	1.99 ± 0.21d	53.28 ± 6.97g	6.22 ± 1.69f	0.058 ± 0.011f	0.371 ± 0.024ab
GS3-6	20.40 ± 1.01a	13.92 ± 1.51c	0.102 ± 0.009b	0.220 ± 0.028b	2.17 ± 0.15c	189.41 ± 8.48c	17.96 ± 2.22c	0.135 ± 0.039bc	0.302 ± 0.030c
GS6-5	18.83 ± 2.32b	13.42 ± 1.89c	0.091 ± 0.010c	0.137 ± 0.019e	1.70 ± 0.09e	162.87 ± 6.66d	14.63 ± 1.86d	0.105 ± 0.008d	0.286 ± 0.017d
GS10-1	20.65 ± 1.85a	17.61 ± 2.12b	0.137 ± 0.015a	0.307 ± 0.024a	2.24 ± 0.24bc	229.89 ± 14.98b	22.07 ± 2.09a	0.169 ± 0.027a	0.306 ± 0.049c
GS14-1	17.75 ± 0.69c	10.99 ± 1.31d	0.091 ± 0.013c	0.154 ± 0.026d	1.51 ± 0.10f	118.41 ± 5.54e	14.56 ± 2.28d	0.143 ± 0.018b	0.392 ± 0.023a
GS15-2	19.28 ± 1.75b	9.06 ± 0.78e	0.085 ± 0.008cd	0.157 ± 0.014d	1.84 ± 0.14d	87.17 ± 4.65f	9.19 ± 1.58e	0.077 ± 0.010e	0.336 ± 0.034b
LSD ($P \leq 0.05$)	2.00	2.28	0.01	0.03	0.65	10.77	2.51	0.03	0.04

Each values is a mean ± SD of three independent trials.

system phytase activity ($R^2 = 0.748$; $P < 0.05$), GS10-1 showed highest relative expression of the phytase gene.

Cloning and sequence analysis of phytase

The phytase PHY101 was amplified from the genome of *Pseudomonas mandelii* GS10-1 using gene-specific degenerate primers by PCR method. The ORF length of PHY101 was 1,941 bp and it encoded a polypeptide of 647 amino acids. The secreted proteins were predicted using TMHMM 2.0.[5] The residue protein was a theoretical molecular mass of 70.1 kDa and a theoretical pI of 4.86. A UniProt BLAST search was performed using the deduced amino acid sequence and the identified gene/protein was a member of the phytase superfamily. The analysis of homologous amino acids revealed that PHY101 shared 79.4% similarity with 3-phytase (Q4KAB7) from *Pseudomonas fluorescens* and 75.9% similarity with 3-phytase (C3JXS5) from *Pseudomonas fluorescens* (**Figure 5**). The PHY101 was determined consisting of three parts based on the prediction of signal peptide and multiple alignments: a signal peptide of 35 amino acids, an inactive site N-terminal domain of 162 amino acids (66–228), and a BPPhy C-terminal domain of 325 amino acids. The alignment results of PHY101 with the five highest known homologous of closest sequences in UniProt are shown in **Figure 6**, in which the primary structure showed significant identities with *Pseudomonas* phytases and predicted it to be a typical BPPhy with six-bladed propeller shape by the 3D modeling results (**Supplementary Figure 1**).

Production and purification of recombinant PHY101

His-tagged versions of recombinant protein were successfully expressed and purified from the supernatant of cell lysate after induction, and SDS-PAGE showed that the molecular mass of PHY101 was around 67 kDa (without signal peptide) (**Figure 7**). The purified protein of PHY101 displayed a specific activity of 208 U mg^{-1} on sodium phytate.

Biochemical profiles of recombinant PHY101

Phytase activity was assayed at different pH and temperature conditions. The enzyme had more than 70% activity at pH 5–8 and was highest at pH 6 (**Figure 8A**). The stability of the enzyme at pH 4–9 was more than 60% after 1 h at 37°C (**Figure 8A**).

5 https://services.healthtech.dtu.dk/

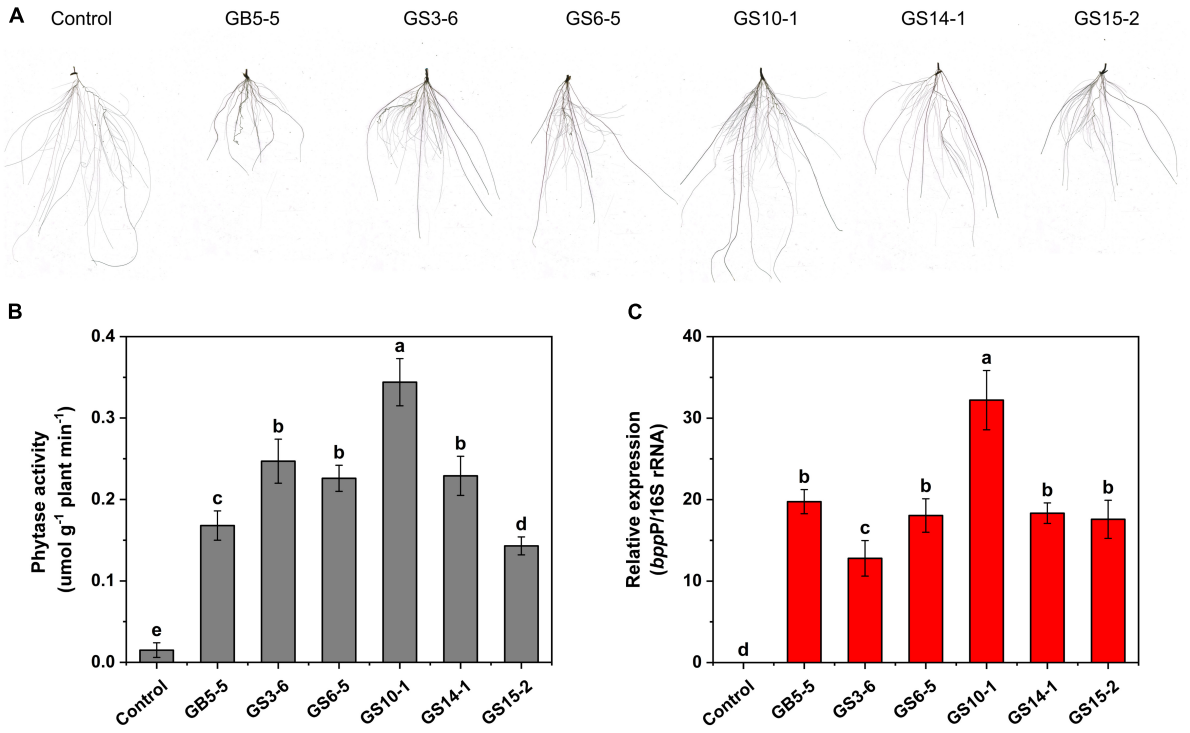

FIGURE 4
Effect of strains inoculation on *Lolium perenne* L. root system after 28 days for **(A)** root morphology, **(B)** root system phytase activity, and **(C)** phytase gene (*bpp*P) relative abundance. Control: root system of uninoculated strains. Each value is a mean ± SD of three independent trials.

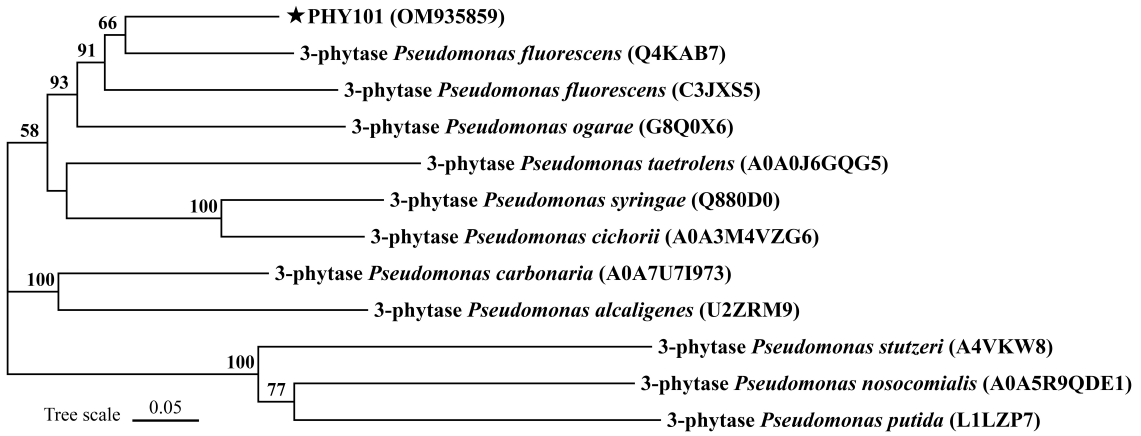

FIGURE 5
Neighbor-joining phylogenetic tree of PHY101 based on sequence similarity. The numbers at the nodes indicate the levels of bootstrap support based on data for 1,000 replicates. The scale bar represents 5% sequence divergence.

The optimal temperature of the enzyme was 40°C, whereas a rapid decline with the temperature increases (Figure 8B). Notably, the enzyme still had 34.41% activity of its maximal activity at 10°C and 63.31% at 15°C. The activity of the enzyme was stably stable above 90% between 10 and 50°C at optimal pH (Figure 8B). The thermal stability results showed that the remaining activity of the enzyme was 69.09% at 40°C for 60 min, and 18.44% at 50°C for 60 min, and it lost all activity at 60°C for 40 min (Figure 8C). At optimal temperature and pH, Na^+ and K^+ ions have a stimulating effect on the PHY101 activity, 1 mM Mg^{2+} stimulated and 5 mM Mg^{2+} inhibited the activity. Respectively, Mn^{2+}, EDTA slightly and Cr^{3+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, SDS strongly inhibited of enzyme activity (Figure 8D).

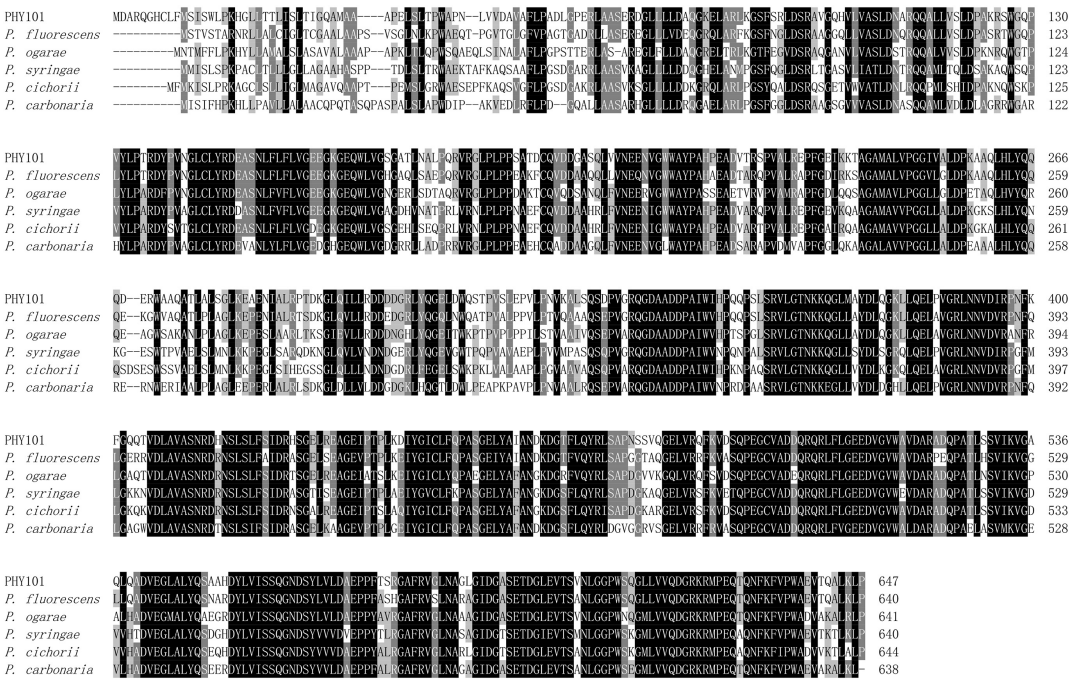

FIGURE 6
Multiple protein sequence alignment of phytase between PHY101 (OM935859) from *Pseudomonas mandelii* GS10-1 and other similarly species. The DNAMAN was utilized to perform the alignment for *Pseudomonas fluorescens* (Q4KAB7), *Pseudomonas fluorescens* (C3JXS5), *Pseudomonas ogarae* (G8Q0X6), *Pseudomonas syringae* (Q880D0), *Pseudomonas cichorii* (A0A3M4VZG6), and *Pseudomonas carbonaria* (A0A7U7I973). The consensus residues are identical in all sequences and the majority of sequences are shaded separately in a black and gray background.

Discussion

In the next few decades, the required quantum of global grass production and P uptake will increase substantially (Sattari et al., 2016). Assuming no area expansion of grassland, the raising increase in grass yields can only be possible if the P status of the soil under grasslands is improved. On the other hand, although plants can only uptake inorganic orthophosphate anions, a considerable portion of P of grassland soil is present in the organic phosphorus (P_o) form (Gerke, 2015; Menezes-Blackburn et al., 2018). Mineralization of the P_o forms requires these into plant available P through the action of phosphatase enzymes (Menezes-Blackburn et al., 2013). The PGPBs express extracellular phytases that hydrolyze recalcitrant phytate, which are excellent biotechnological substances and can be used as potential substitutes for P-fertilization (Zhuang et al., 2021). Therefore, understanding the PGPB isolates associated with the soil P cycle and conferring sustainability in agriculture to delineate their function in grasslands has become important.

All plant species may establish a relationship with some strains, and the selection of bacterial isolates able to mineralize phytate *in vitro* is an important and necessary step. In the present study, we used a combination of molecular biological and extracellular phytase activities for the selection of environmentally adaptable PGPBs from the rhizosphere soil of seven dominant types of grasses in the Qinghai-Tibet Plateau. The 16S rDNA sequencing analysis suggested 73 phytase-producing bacteria isolates grouped into two phyla which consisted of 16 different genera. Proteobacteria (90.41%) was the most dominant, including *Pseudomonas*, *Acinetobacter*, *Variovorax*, *Achromobacter*, *Pantoea*, *Arthrobacter*, etc., and more than 49% (36) of isolates belongs to *Pseudomonas*, which also confirmed that the γ-Proteobacteria was the main bacterial group producing phytase, as reported by Park et al. (2022). In addition, we compared the extracellular phytase activity between genera of *Pseudomonas* and others to confirm the dominant genus, and the results indicated that isolates of *Pseudomonas* in the present work had a higher average activity, which could be representative within strains colonized in grass rhizosphere in the soils studied. *Pseudomonas* was one of the common ecological groups in the rhizosphere of plants with various PGP traits such as dissolved soil insoluble P, fixed nitrogen, and secreted plant growth hormone (Zhuang et al., 2021). Previous literature has reported that some phosphorus solubilizing bacteria (PSB) belong to these genera (Bargaz et al., 2021; Li J. T. et al., 2021; Bi et al., 2022; Li et al., 2022), but most studies have not reported phytase production abilities. Our work suggests that screening by using calcium phytate as the P source

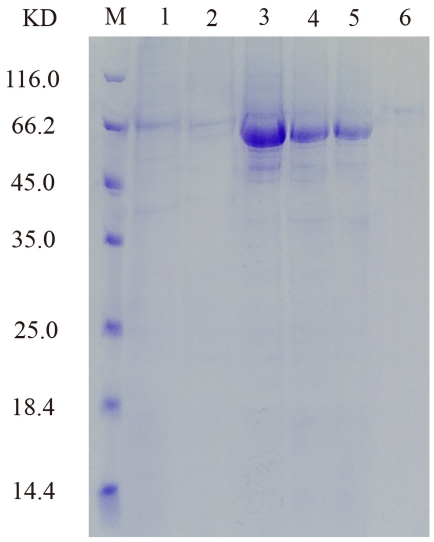

FIGURE 7
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the recombinant PHY101. M: Protein molecular weight marker; Lane 1: 96 h of culture to secrete supernatant; Lane 2: Effluent solution through column; Lane 3–6: Elution solution through column.

provides an efficient approach to obtaining phytase-producing bacteria. Interestingly, no *Bacillus* species were identified in this work. *Bacillus* is the main group of soil microorganisms that secrete phytase, especially β-propeller phytases (BPPhy), and has been obtained and reported in different species (Jorquera et al., 2011; Zhao et al., 2021). Speculated reasons were due to the nutrients of the medium, the geographical environment differences, and the composition of plant species.

This study focuses on phytase, but it is evident that select PGPBs should be considered PGP traits that can be used in bio-fertilizers (Tchakounte et al., 2018), such as P-solubilization, plant hormones production, ACC deaminase activity, nitrogen fixation, and antimicrobial activity. In the present study, the genus of *Pseudomonas* isolates (36) was performed to text *in vitro* PGP characteristics, and we found that some isolates were multifunctional and showed no less than two or more traits, which were similar to the results of previous reports on PGPB research (Bargaz et al., 2021; Hazarika et al., 2021). Tricalcium phosphate and lecithin were commonly used as a medium for P sources to detect the ability of PGPB to dissolve organic/inorganic P (Wan et al., 2020). The amount of P solubilization by strains in this work was comparable to other PGPBs reported in the literature (Yahya et al., 2021). Moreover, all the strains showed an excellent solubilization ability on tricalcium phosphate, and a majority showed a digesting effect on lecithin, which was similar to that reported by Wan et al. (2020), the PSB harboring abilities in utilizing multiple P sources. Plant hormones (PHs) are a kind of trace organic compounds that play important roles in regulating plant growth, metabolism, and more. PHs produced by microorganisms mainly include indoleacetic-3-acid (IAA), gibberellic acid (GA_3), and cytokinins (CTKs) (Streletskii et al., 2016). However, the previous method of PHs was mainly aimed at measuring IAA production, which has certain deficiencies in the detection accuracy and detection type. In the present study, we used HPLC to detect the contents of IAA, GA_3, and t-Z in the strains' fermentation products. The results showed that all strains to be tested can produce one or more PHs, but many strains produced far lower concentrations of IAA than the same species of other reports (Hazarika et al., 2021; Li M. Y. et al., 2021; Alotaibi et al., 2022), speculated that the reason is related to the different detection methods. HPLC is a world-recognized detection method for the detection of PHs and has the advantages of rapidity, sensitivity, high detection accuracy, and simultaneous determination of multiple components. However, studies have rarely reported on the characteristics of secreting different PHs using the HPLC method in PGPBs to our knowledge. Our method improves detection accuracy and expands the scope of detection range with PHs. ACC deaminase activity bacteria effectively protect plants against growth inhibition and make plants more resistant to pathogens, it is one of the indicators to determine the ability of PGP. We observed that few strains obtained in the present study contain ACC deaminase activity, and speculate that they are potentially related to less biotic or abiotic stresses in the environment of strains. For example, Li M. Y. et al. (2021) screened PGPB from the rhizosphere of four grass species in alpine grasslands of the Qilian Mountains, and their PGP properties results showed that 5 (7.46%) of the 67 isolates contain ACC deaminase. PGPB potentially converts free nitrogen in the air into compound nitrogen (ammonia/nitrate nitrogen) by nitrogen fixation which serves as a source of stimulating plant growth (Li et al., 2020; Alotaibi et al., 2022). They are divided into three kinds for abiogenous azotobacter, symbiotic nitrogen-fixing bacteria, and associative nitrogen-fixing bacteria, and *Pseudomonas* is a species of associative nitrogen-fixing bacteria (Mahmud et al., 2020). In the present study, most of the strains have the ability to fix nitrogen, with a little difference in nitrogenase activity, which is similar with the Hazarika et al. (2021), who reported that PGPB can escalate plant biomass by the accumulation of nitrogen. Soil microorganisms contained a few bacteria with antimicrobial activity. Eight antagonistic bacteria (22.22%) against *R. solani* and *F. oxysporum* were screened from all strains, which suggests that the *Pseudomonas* species have poor antibacterial activity in this study. These potentially related to the strains species, as reported by Penha et al. (2020) and Salazar et al. (2022) that *Bacillus* was the dominant antagonistic bacteria, and showed higher antibacterial activity than other species.

To enhance the understanding of phytase-encoding genes of PGPBs, we used degenerate PCR to amplify the phytase gene (*bpp*, *hap*, *cp*). Six strains (16.7%) were found positive for

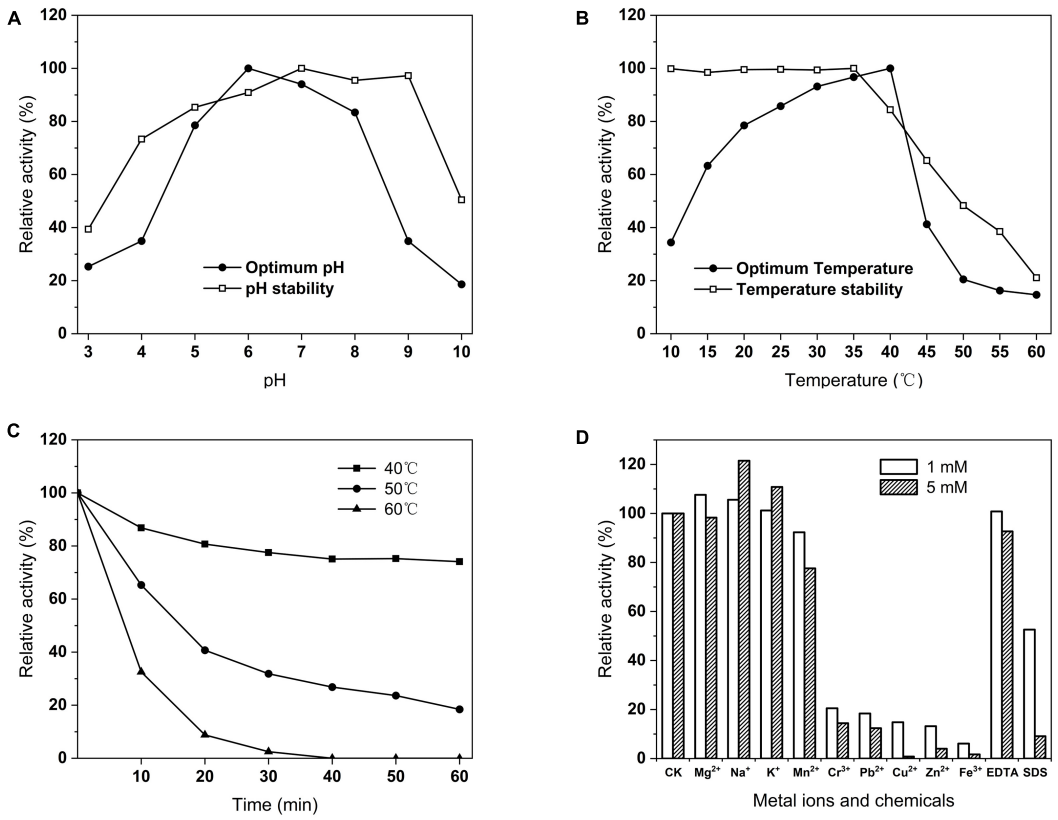

FIGURE 8
(A) Optimum and stability of pH on PHY101 activity from 3.0 to 10.0. **(B)** Optimum and stability of temperature on PHY101 activity from 10 to 60°C. **(C)** Thermal stability on PHY101 activity by different temperatures from 40 to 60°C for 10–60 min. **(D)** Effect of metal ions and chemical reagents on PHY101 activity. Each value is a mean of three independent trials.

the *bpp* gene in PCR products. Previous literature showed that BPPs were the most widely distributed phytase in nature, and also the only phytase observed under neutral and alkaline pH conditions, in which optimal reaction conditions are similar to the rhizosphere environment (Jorquera et al., 2013; Cotta et al., 2016; Stout et al., 2016). Therefore, it is considered to be the most important phytase in the rhizosphere (Kumar et al., 2017). Our results also proved the above-reported conclusion. However, no *hap* and *cp* gene was amplified, the reason presumably is related to other pathways for the degradation of phytate or the existence of new undiscovered phytase encoding genes, and needs to be further studied.

To evaluate the growth-promoting performance of phytase-producing PGPB, and explain the process of P-metabolism of strains, we select *Lolium perenne* L. to measure the effect of strains inoculation on the growth of plants under P-limitation when sodium phytate was the P source, which is suitable for planting in cold regions (Ren et al., 2016). Strains inoculation produced beneficial functions to promote *Lolium perenne* L. growth, which significantly increased the aboveground biomass accumulation and the root system phytase activity. Similar to that reported by Barra et al. (2019), due to the Hoagland's does not provide any plant available P in this work, the growth promoting of the plants may derived from the release of P by strains through mineralized phytates. Plant roots have response mechanisms such as root configuration changes (root hair length increase, density increase, root/shoot ratio increase, etc.) to improve the uptake efficiency of phosphate when they are under a P-deficient environment (Kavanova et al., 2006). In the present study, plants responded differently to strain inoculation, which is presumably related to the plant characteristics and the colonization ability of strains. Among them, *Pseudomonas mandelii* GS10-1 performed best and significantly increased the fresh weight, root surface area, root volume, and root average diameter of *Lolium perenne* L. In addition, qPCR proved that the *bpp* gene was effectively expressed in plant rhizosphere, and positively correlated with the root system phytase activity (R^2 = 0.748). The primers designed by this study can be used for qPCR research of BPPs in *Pseudomonas* species. The qPCR verification of BPPs has been reported in several bacterial strains, such as Shen et al. (2016), who reported a BPP gene (phyPf) cloned from *Pseudomonas fluorescens* JZ-DZ1, where the results of qPCR proved that the secretion of phytase was regulated by phyPf. In particular, some reports indicate that

the activity of microbial phosphatases was determined by the interaction between functional gene abundance, soil available P content, and substrate availability (Suleman et al., 2018; Rasul et al., 2021; Yahya et al., 2022). Therefore, screening of PGPBs with phytase genes can help identify the new microbial taxa capable of mineralizing organic P.

In order to reveal the basic characteristics of enzymes and discover novel phytases with desirable properties, the enzyme must be purified and identified. In recent years, a considerable number of phytases have been isolated and cloned in microorganisms, which have achieved heterologous expression, and their enzymatic properties are tested (Nam et al., 2014; Zhang et al., 2015; Rix et al., 2021). However, the phytases that possess high activity at low temperatures are still uncommon (Jang et al., 2018), which play a role in P cycling and potentially be suitable for use in cold areas. In the present study, we emphatically studied *Pseudomonas mandelii* GS10-1 and cloned a novel BPPhy (PHY101) with a high activity of phytase. The molecular weight of the recombinant protein was around 67 kDa, which is larger than most reported of BPPs. The optimal pH of PHY101 was 6.0, stable at pH 5.0–9.0, and the optimum temperature was 40°C, where the temperature is lower than most reported strains of *Pseudomonas* sp. (50°C) (Nam et al., 2014) and *Bacillus* sp. (55–60°C) (Borgi et al., 2014). In particular, the high activity of PHY101 at low temperatures (more than 60% of maximum enzyme activity at 15°C) is potentially suitable for being applied to agricultural production in the region since the temperature of the soil in alpine grasslands in below 20°C all year round (Kun, 2021). Strains growing in alpine habitat is a source of low temperature-tolerant enzymes. In addition, Jang et al. (2018) first reported a BPP with an N-terminal domain from *Pseudomonas* sp. FB15, and verified that the presence of the N-terminal domain improved the catalytic efficiency and low-temperature tolerance of BPPs (Jang et al., 2019). Kumar et al. (2017) reviewed the current developments in genes sources, diversity, biochemical properties, and structural elucidation of BPPhy, and reported and found that the C-terminal of BPPs is relatively conserved while the N-terminal motifs are more diverse, which is consistent with the results of our study findings. The effect of metal ions and chemical reagents on enzyme activity showed that Cr^{3+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{3+} are potent inhibitors of PHY101 activity. According to Maenz and Classen (1998) reports, this inhibitory effect may be related to the formation of insoluble mineral-phytate complexes by phytate at neutral pH.

Conclusion

This is the first report about combined phytase activity and plant growth-promoting traits for the screening of efficient PGPB candidates associated with alpine grasslands on the Qinghai-Tibetan Plateau. The phytase-producing PGPBs inoculated exhibited plant-growth promoting effect under P-limitation. Our results reveal the potential of selected indigenous bacteria to serve as biological inoculants for the restoration of degraded grasslands and P-deficient soils to enhance grass growth and productivity. Additionally, this study cloning and the function of a novel low-temperature resistance phytase form *Pseudomonas mandelii* GS10-1, which should be defined as a functional gene that participated in promoting plant growth. We make the case established that the alpine grasslands in the Qinghai-Tibetan Plateau are one of the effective sources of low temperature-tolerant active enzymes. Furthermore, future studies will focus on the use of whole genome sequencing and multi-omics technologies to study the composition, characteristics, and catalytic mechanisms of the bioactive compounds produced by those PGPBs, and the development of engineered rhizobacteria for practical applications.

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

QL and JL designed the experiments and contributed to the writing and revision of the manuscript. QL and XY conducted all the experiments. QL, XY, and CL analyzed the data and wrote the manuscript. ML 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.2022.1019383/full#supplementary-material

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