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Rhizobacteria inoculation benefits nutrient availability for phytostabilization in copper contaminated soil: Drivers from bacterial community structures in rhizosphere

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) and rhizobia are potentially advantageous in improving plant growth in heavy metal contaminated soils. However, only limited information is available in literature on the manner through which the co-inoculation of PGPR and rhizobia can potentially supply nutrients to benefit plant growth in heavy metal contaminated soil. Accordingly, this study investigated the effects of *Paenibacillus mucilaginosus* (PGPR) and *Sinorhizobium meliloti* (rhizobia) co-inoculation on soil nutrients, enzyme activities, and microbial biomass in copper (Cu) contaminated soil planted with alfalfa (*Medicago sativa*). Moreover, we assessed soil bacterial community structure using high-throughput Illumina sequencing of 16S rRNA genes. Results showed that PGPR and/or rhizobia inoculation improved alfalfa growth. In particular, we found that this co-inoculation approach decreased Cu accumulation (48.6%) in shoots compared to the control (uninoculated). Both partial least squares path modeling (PLS-PM) and the relative importance of regressors in the linear models identified that enzyme activities, microbial biomass, and microbial community structure in Cu contaminated soil were major controlling variables of soil nutrient availability. The co-inoculation treatment significantly increased soil carbon (C) and nitrogen (N) concentrations by increasing urease (55.6%), saccharase (29.5%), and β-glucosidase (31.4%) activities compared to the control. Furthermore, the rhizosphere microbial community structure in the co-inoculation treatment was mainly regulated by soil N concentrations (i.e., both total N and available N) while altering alpha diversity (α-diversity). The relative abundances of *Firmicutes* (including biomarkers of the *Bacillus* genus) and *Acidobacteria* were enriched in the co-inoculated treatment, which can potentially improve soil nutrient availability and subsequently benefit plant growth. These findings indicated that the co-inoculation of PGPR and rhizobia plays an important role in promoting plant growth in Cu contaminated soil. This is because this approach can increase soil nutrient availability by enhancing soil enzyme activities and regulating rhizosphere microbial community structure.

1. Introduction

Phytoremediation is emerging as a low-cost and eco-friendly strategy to clean up metal contamination in soil, also contributing to the health of agricultural soil (Coninx et al., 2017; Waigi et al., 2017). Phytostabilization is one of the many strategies that constitute phytoremediation, which uses specific plant species to stabilize heavy metal pollutants in order to reduce their bioavailability in soil (Burges et al., 2016; Hrynkiewicz et al., 2018). Although phytoremediation is a promising and effective technology compared to physicochemical techniques, it has many limitations, such as being time-consuming, restricted to limited plant biomass, confined by slow growth rates, and

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providing poor overall metal extraction (Chen et al., 2014; Hussain et al., 2018; Ju et al., 2019; Waigi et al., 2017). In recent years, however, numerous studies have found that plant selection in conjunction with associative, suitable microbes (i.e., metal-tolerant and plant growth promoters) is a viable and promising technology that offers several benefits (Hussain et al., 2018; Kamran et al., 2015). Microbe-assisted phytoremediation not only provides plants with natural metal-solubilizing chelators (Dimkpa et al., 2008), but it also promotes plant growth through various mechanisms, including the bacterial production of phytohormones (Hidri et al., 2016) and an overall increase in soil nutrient availability (Ma et al., 2016).

For phytoremediation to be successful, it must effectuate vigorous plant growth and net environmental benefits following remediation; however, it is of utmost importance to maintain soil health and fertility in contaminated soil. Soil enzymes, microbial biomass, and microorganisms are primary mediators in soil biological processes, serving as sensitive indicators of heavy metal stress (Duan et al., 2019; Fang et al., 2019; Yang et al., 2013). Such microbes and their associative processes play an important role in organic matter degradation, mineralization, and nutrient recycling (Ge et al., 2018; Gryta et al., 2019; Wang et al., 2019). Furthermore, soil enzyme activities respond rapidly to metal stress, and their capacity to inhibit such stress includes many factors, including the specific metal species, the enzyme classes, and the soil and plant types involved (Duan et al., 2019; Lyubun et al., 2013). Catalase can break down hydrogen peroxide (H_2O_2) and prevent it from poisoning organisms. Additionally, enzyme activities involved in soil organic carbon (C) (i.e., saccharase and β-glucosidase), nitrogen (N) (urease), and phosphorous (P) (acid phosphatase) cycles are considered useful indices, representative of the potential for soil enzyme synthesis by microbial communities (Cui et al., 2019; Kaurin et al., 2018; Yang et al., 2016).

Moreover, microbial biomass is an important source of soil biological fertility that is involved in biogeochemical nutrient cycling. Microbial biomass, being a good indicator of soil quality, also plays an important role in plant-pathogen suppression, residue decomposition, and pollutant degradation (Kaschuk et al., 2010). Soil microorganisms immobilize C and N by forming new biomass using the energy obtained from the oxidation of C sources, which allows microbial biomass to stock and cycle nutrients (Niemeyer et al., 2012). The rhizosphere, which is involved in soil energy transfer, nutrient cycling, and the resistance and detoxification of metal elements, is more essential to plant growth than bulk soil (Ambrosini et al., 2012; Guo et al., 2019). Accordingly, the diversity and stability of rhizosphere microbial communities significantly influence plant and soil quality and ecosystem sustainability. This demonstrates the importance of investigating the structural and functional properties of rhizosphere microbial communities and the ways in which various factors influence bacterial diversity and microbial activity (Trivedi et al., 2012).

Plant-growth-promoting rhizobacteria (PGPR)-assisted phytoremediation is not only beneficial to plant growth but also beneficial to soil quality (Ju et al., 2019; Song et al., 2015). Inoculation of PGPR *Pseudomonas* sp. GHD-4 can reduce lead (Pb) concentrations in soil, improve soil enzyme activities, and enrich bacterial community diversity (Yu et al., 2019). Hidri et al. (2019) found that PGPR *Bacillus subtilis* inoculation increased the activities of soil enzymes such as urease, alkaline phosphatase, β-glucosidase, and dehydrogenase compared to the uninoculated control, and subsequently improved soil quality. Kumar et al. (2016) reported that soil enzymes and microbial diversity were significantly modulated in singular PGPR treatments and in plants with microbial consortia. Marques et al. (2013) also reported that bacterial community diversity decreased with increasing levels of cadmium (Cd) and zinc (Zn); however, greater bacterial diversity was maintained throughout the experimental period for *Helianthus annuus* (common sunflower) inoculated with PGPR strains in the rhizosphere.

Resistant rhizobia used for phytoremediation not only directly acts on metals under certain mechanisms but also possesses plant growth-promoting traits, including N fixation, P solubilization, phytohormone synthesis, and siderophore release (Hao et al., 2014). Biological N fixation within a legume-rhizobia system is regulated by the exchange of organic C and N nutrients between host plants and N-fixing bacteria (Li et al., 2019). PGPR also stimulates symbiotic N fixation by inducing the production of phytohormone synthesis and the solubilization and uptake of soil nutrients (Gomez-Sagasti and Marino, 2015). However, few studies have investigated the effects of PGPR and rhizobia co-inoculation on soil nutrient cycling, enzyme activities, and microbial community structure in metal contaminated soil.

In this study, we hypothesized that PGPR and rhizobia can improve soil nutrient cycling by enhancing soil enzyme activities and regulating the microbial community structure of the rhizosphere, thereby providing a suitable environment for plant growth. We selected the PGPR *Paenibacillus mucilaginosus* (strain ACCC10013) and the copper (Cu) resistant rhizobia *Sinorhizobium meliloti* (strain CCNWSX0020) as experimental objects in which to investigate the interaction between soil nutrient cycling and soil biochemical properties in Cu contaminated soil following the co-inoculation treatment. Results from this study will help to provide an efficient strategy to improve the nutrient availability for the phytoremediation of metal contaminated soil.

2. Materials and methods

2.1. Experimental design

Contaminated soil (obtained from the top 20-cm layer) was collected from farmland surrounding a copper smelter in Huangshi City (30°42′ N, 114°54′ E), Hubei Province, China. Selected physicochemical soil properties are shown in Table S1. The soil is acidic (pH = 5.65), and Cu (685 mg kg^{-1}) is the major contaminant. Total nitrogen (TN), available potassium (AK), and soil organic matter (SOM) concentrations in the soil were 1.37 g kg^{-1}, 69.8 mg kg^{-1}, and 24.4 g kg^{-1}, respectively. The experimental strains used in this study were heavy metal resistant rhizobia (*S. meliloti*, strain CCNWSX0020) and PGPR (*P. mucilaginosus*, strain ACCC10013) (Ju et al., 2019). A strain of *S. meliloti* was isolated from the root nodules of *M. lupulina* plants growing in the tailings of a lead-zinc mine in China. A strain of *P. mucilaginosus* was provided by the Agricultural Culture Collection of China. Through our pre-experimental design stage, we determined that the rhizobia *S. meliloti* and the PGPR *P. mucilaginosus* were able to endure higher Cu concentrations. The rhizobia *S. meliloti* was grown in a tryptone/yeast liquid medium and the PGPR *P. mucilaginosus* was grown in an optimised liquid medium (Ju et al., 2019). Bacterial cells were then harvested by centrifugation (8000 *g* at 4 °C for 10 min), and the pellets were immediately washed three times with a phosphate buffer (pH 7.0). Bacterial suspensions in 0.85% sterilised NaCl were adjusted to an optical density of 0.8 at 600 nm. Alfalfa (*Medicago sativa*) seeds (obtained from Beijing Rytway Ecotechnology Co., Ltd.) were sterilised in a 20% *v*/v sodium hypochlorite (NaClO) solution for 10 min and then washed four separate times with deionised (DI) water. Approximately 20 pre-germinated seedlings were transplanted into each plastic pot (diameter 12 cm and height 17 cm; with 1.30 kg of contaminated soil per pot). Additionally, moisture content was maintained at ~70% of the water-holding capacity by adding DI water when necessary to ensure optimal conditions for alfalfa growth. After the first leaves appeared, bacterial suspensions were sprayed once per week (for a total of three weeks) into plant roots of the inoculated treatments. Specifically, each plastic pot contained approximately 20 seedlings, and each seedling was inoculated with 1 mL of the suspension. Bacterial suspensions were irrigated into seedling roots once per week over a three week period (i.e., a total of three times). Five treatments were used in this study: blank soil (BS, i.e., only contaminated soil), soil + alfalfa (A, i.e., the control), soil + alfalfa + PGPR (AP), soil + alfalfa + rhizobia (AR), and soil + alfalfa + PGPR + rhizobia (APR). Three replicates were produced for each treatment. The same DI water volume was added to

the uninoculated treatment (A). Plants were harvested after 60 days, and rhizosphere soil was collected for further analysis.

2.2. Soil and plant characteristics

We determined soil pH, TN, available nitrogen (AN, i.e., ammonium N and nitrate N), AK, and SOM concentrations following the method by Lu (2000). Soil pH was determined using a pH meter (Model 225, Denver Instrument, NY, USA) with a soil-water suspension of 1:2.5 (*w/v*). Total nitrogen concentration was determined using the Kjeldahl method, and ammonium N (NH_4^+-N) and nitrate N (NO_3^--N) concentrations were determined using an AutoAnalyzer (SEAL, AutoAnalyzer 3, Germany). Additionally, AK was determined using inductively coupled plasma atomic emission spectrometry (ICP-AES) (Optima 3300 DV, PerkinElmer, Inc., MA, USA). The external heating method for potassium dichromate was used to determine SOM concentration. Soil samples were digested using a modified version of "Method 3051A" from the United States Environmental Protection Agency (USEPA) to measure total Cu concentration (Duan et al., 2019; Ju et al., 2019). Plant dry biomass was measured after oven-drying at 70 °C for 3 d. Plant samples were digested in a 10-mL acid mixture (HNO_3:$HClO_4$, 4:1) to quantify total Cu concentrations using atomic absorption spectrophotometry (Hitachi, FAAS Z-2000, Japan). The transportability of Cu from plant roots to shoots was ascertained using the translocation factor (TF), "$TF = Cu_{shoot} / Cu_{root}$", where Cu_{shoot} and Cu_{root} are the Cu concentrations in shoots and roots, respectively.

2.3. Soil enzyme activities and microbial biomass

Soil enzyme activities (urease, saccharase, catalase, acid phosphatase, and β-glucosidase) were determined as previously described (Eivazi and Tabatabai, 1988; Guan et al., 1986). Urease (EC 3.5.1.5) and saccharase (EC 3.2.1.26) activities were determined using urea and sucrose as substrates and quantified by a spectrophotometer (UV-2450, Shimadzu Corporation, Japan) at 587 and 508 nm, respectively. Catalase (EC 1.11.1.6) activity was determined using potassium permanganate titration and expressed in milliliters of 0.02 mol L^{-1} $KMnO_4$ per gram of soil per 20 min. Acid phosphatase (EC 3.1.3.2) activity was determined using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate, quantified at 400 nm based on *p*-nitrophenol (PNP) release, and expressed as PNP μg g^{-1} h^{-1}. We also quantified β-glucosidase (EC 3.2.1.21) activity at 400 nm based on PNP release and expressed it as PNP μg g^{-1} h^{-1} (Eivazi and Tabatabai, 1988). Soil microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN) were determined using the chloroform fumigation extraction (CFE) method (Brookes et al., 1985; Vance et al., 1987).

2.4. DNA extraction and Illumina MiSeq high-throughput sequencing

Bacterial DNA was extracted from 0.5 g of the soil samples using the FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA, USA). The 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) universal primer set was used to amplify the V4–V5 regions of the 16S rRNA gene, with a 12-bp barcode on the reverse primer. Polymerase chain reaction (PCR) amplification was used according to a method previously described (Fang et al., 2019; Wang et al., 2019). PCR reactions were performed in a volume of 30 μL, containing 2 μL of sterile ultrapure water, 15 μL of Phusion Master Mix with a GC Buffer (New England Biolabs, USA), 3 μL of primer, and 2 μM and 10 μL of template DNA (10 ng μL^{-1}). The initial denaturation step for the PCR procedure was 98 °C for 1 min, followed by 30 cycles at 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s, and a final elongation was conducted at 72 °C for 5 min, which was sustained at 4 °C. PCR amplification was verified by 2% agarose gel electrophoresis. Composite DNA was gel purified using the GeneJET Gel Extraction Kit (Thermo Scientific) and sequenced using an Illumina MiSeq platform at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China).

2.5. Statistical analysis

One-way analysis of variance (ANOVA) was used for all statistical calculations to assess the effects and interactions of the five treatments (SPSS 20.0, SPSS Inc., Chicago, IL, USA). Least Significant Difference (LSD) multiple comparison was used to assess significant differences ($P < 0.05$) in the treatments. Values are means ± standard errors (SE) ($n = 3$). A correlation heat map was generated to measure the pairwise relationship between different variables based on Pearson correlation coefficient analysis using the "ggcorrplot" package in R software. The relative abundance of bacterial taxonomy was further analysed using Statistical Analysis of Taxonomic and Functional Profiles (STAMP) software v.2.1.3 (Parks et al., 2014), where Welch's *t*-test was used to compare differences in the relative abundance of the bacterial genus level between treatments. Linear discriminant analysis (LDA) effect size (LEfSe) was used to detect potential biomarkers (across five taxonomic levels, i.e., from phylum to genus for bacterial communities) within soil microbiomes, based on a calculated probability of significance at a 0.05 probability level and an LDA threshold score of 4.0 using the "MASS" package. The "Vegan" package was used for canonical correspondence analysis (CCA) to determine the most important factors that affect bacterial community structure. Moreover, the "Vegan" package was also used for principal coordinates analysis (PCoA) to determine the beta diversity (β-diversity) of bacterial communities based on Bray–Curtis dissimilarity. We determined the relative importance of predictor variables that explained variation in the soil nutrient cycle using the "relaimpo" package in R software. Partial least squares path modeling (PLS-PM) was used to identify the major pathways of the influential predictor variables on the soil nutrient cycle using the "innerplot" function of the "plspm" package in R software. Variation partitioning analysis (VPA) was used to determine the contribution of the predictor variables to the soil nutrient cycle using the "Vegan" package. All statistical analyses were performed using R software v.3.5.2.

3. Results

3.1. Soil physicochemical properties and plant biomass

The singular rhizobia inoculation treatment (i.e., the AR treatment)

Table 1
Soil physicochemical properties.

Treatments	pH	TN (g kg^{-1})	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	AK (mg kg^{-1})	SOM (g kg^{-1})	Total Cu (mg kg^{-1})
A	5.83 ± 0.08 a	1.25 ± 0.01 c	1.24 ± 0.03 c	0.02 ± 0.01 c	50.6 ± 2.51 a	27.5 ± 0.34 c	676 ± 3.04 a
AP	5.77 ± 0.16 ab	1.30 ± 0.01 b	1.20 ± 0.05 c	0.07 ± 0.02 c	43.1 ± 4.91 a	27.9 ± 0.21 c	674 ± 2.51 a
AR	5.72 ± 0.09 ab	1.35 ± 0.01 a	1.93 ± 0.04 b	0.81 ± 0.16 b	40.6 ± 3.19 a	29.8 ± 0.20 a	675 ± 3.03 a
APR	5.59 ± 0.19 b	1.35 ± 0.01 a	2.80 ± 0.07 a	1.20 ± 0.07 a	45.7 ± 3.18 a	28.7 ± 0.12 b	674 ± 4.03 a

Note: TN: total nitrogen; AK, available potassium; SOM: soil organic matter. Treatments: A (soil + alfalfa), AP (soil + alfalfa + PGPR), AR (soil + alfalfa + rhizobia), and APR (soil + alfalfa + PGPR and rhizobia). Values are means ± SE ($n = 3$). Different letters indicate significance at a 0.05 probability level ($P < 0.05$) using the LSD test.

Table 2
Dry biomass, copper concentrations, and copper uptake in plant tissues.

Treatments		Dry biomass (g pot^{-1})		Concentration (mg kg^{-1})		Total uptake (μg pot^{-1})		Translocation factor
		Shoot	Root	Shoot	Root	Shoot	Root	
Cu	A	3.04 ± 0.12 d	2.22 ± 0.07 c	53.7 ± 3.33 a	58.6 ± 8.80 a	163 ± 6.25 a	129 ± 15.2 a	0.94 ± 0.10 a
	AP	3.69 ± 0.06 c	2.60 ± 0.09 b	36.3 ± 1.55 b	52.3 ± 10.2 a	134 ± 7.68 b	137 ± 30.9 a	0.74 ± 0.11 ab
	AR	4.20 ± 0.09 b	2.70 ± 0.10 b	31.2 ± 1.13 bc	56.4 ± 4.88 a	131 ± 5.43 b	151 ± 8.23 a	0.56 ± 0.03 bc
	APR	4.84 ± 0.12 a	3.02 ± 0.12 a	27.6 ± 1.06 c	61.9 ± 8.73 a	133 ± 3.33 b	188 ± 32.6 a	0.46 ± 0.08 c

Note: Treatments: A (soil + alfalfa), AP (soil + alfalfa + PGPR), AR (soil + alfalfa + rhizobia), and APR (soil + alfalfa + PGPR and rhizobia). The transport capacity of metals from plant roots to shoots is derived from the translocation factor, namely, "translocation factor = shoot metals/root metals", where shoot metals and root metals are the metal concentrations in shoots and roots, respectively. Values are means ± SE (n = 3). Different letters indicate significance at a 0.05 probability level ($P < 0.05$) using the LSD test.

significantly increased soil TN, AN, and SOM concentrations, while the singular PGPR inoculation treatment (i.e., the AP treatment) significantly increased soil TN concentrations (Table 1, $P < 0.05$). Soil TN concentrations were greater by a factor of 1.04 in the AP treatment compared to the control (A), while TN, NH_4^+-N, NO_3^--N, and SOM concentrations were greater by a factor of 1.08, 1.56, 40.5, and 1.08 in the AR treatment compared to the A treatment, respectively. The highest TN, NH_4^+-N, and NO_3^--N concentrations were observed in the APR treatment, which was greater by a factor of 1.08, 2.26, and 60.0 compared to the A treatment, respectively. Interestingly, the APR treatment had the lowest pH level (5.59). Shoot and root biomass were significantly higher in the AP, AR, and APR treatments compared to the A treatment (Table 2). Biomass was 21.4% and 38.2% greater in shoots and 17.1% and 21.6% greater in roots in the AP and AR treatments compared to the A treatment, respectively. The highest biomass was observed in the APR treatment, namely, 4.84 g pot^{-1} and 3.02 g pot^{-1} in shoots and roots, respectively.

3.2. Copper concentrations and uptake in plant tissue

Although the PGPR and rhizobia co-inoculation treatment (APR) did not significantly affect Cu root concentrations, this treatment did significantly decrease Cu root concentrations in shoots (Table 2, $P < 0.05$). Moreover, Cu concentrations were 32.4%, 41.9%, and 48.6% lower in shoots in the AP, AR, and APR treatments relative to the A treatment, respectively. Similar to Cu shoot concentrations, total Cu uptake in shoots was significantly lower in the singular PGPR (AP) and rhizobia (AP) treatments and the co-inoculate (APR) treatment compared to the control (A). Total Cu uptake was 17.8%, 19.6%, and 18.4% lower in shoots in the AP, AR, and APR treatments compared to the A treatment, respectively. However, the co-inoculation (APR) treatment did not significantly increase total Cu uptake in roots. Total Cu uptake in roots was greater by a factor of 1.46, 1.37, and 1.25 in the APR treatment compared to the A, AP, and AR treatments, respectively. Additionally, the Cu translocation factor (TF) was lower by a factor of 1.27 and 1.68 in the AP and AR treatments compared to the A treatment, respectively. The lowest TF was observed in the APR treatment (0.46).

3.3. Soil enzyme activities

The singular PGPR (AP) and rhizobia (AR) inoculation treatments did not significantly affect urease, saccharase, catalase, acid phosphatase, and β-glucosidase activities (Table 3, $P < 0.05$). However, the co-inoculation treatment (APR) did significantly increase urease, saccharase, and β-glucosidase activities. Urease, saccharase, and β-glucosidase activities were greater by a factor of 1.56, 1.30, and 1.31 in the APR treatment compared to the A treatment, respectively. Catalase activity was significantly positively correlated to SOM, soil TN, and AN (NH_4^+-N and NO_3^--N) concentrations (Fig. 1; $P < 0.05$). Urease activity was also significantly positively correlated to AN ($P < 0.01$) concentrations. Moreover, saccharase and β-glucosidase activities were significantly positively correlated to TN ($P < 0.01$) and Cu ($P < 0.05$) concentrations, respectively.

3.4. Soil microbial biomass

All three inoculation treatments (excluding the BS and A treatments) increased soil MBN concentrations while significantly decreasing MBC concentrations (Table 3, $P < 0.05$). MBN concentrations were greater by a factor of 1.79, 3.29, and 2.71 in the AP, AR, and APR treatments compared to the A treatment, respectively. MBC concentrations were significantly lower by a factor of 1.37, 2.59, and 1.47 in the AP, AR, and APR treatments compared to the A treatment, respectively. MBN concentrations were significantly positively correlated to TN ($P < 0.01$), NO_3^--N, and SOM ($P < 0.05$) concentrations (Fig. 1). In contrast to MBN concentrations, MBC concentrations were significantly negatively correlated to NO_3^--N ($P < 0.05$), TN, and SOM ($P < 0.01$) concentrations.

3.5. Rhizosphere microbial communities

Rhizosphere communities corresponding to blank soil (BS), control (A), PGPR inoculated (AP), rhizobia inoculated (AR), and co-inoculated (APR) treatments were primarily composed of *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Acidobacteria* at a phylum level and *Bacillus* and *Sphingomonas* at a genus level (Fig. 2). Compared to the blank soil (BS) treatment, the relative abundance of *Proteobacteria* and *Actinobacteria* significantly increased in the treatment planted with alfalfa alone (A, the control), whereas the relative abundance of *Acidobacteria* significantly decreased (Table S2, $P < 0.05$). The singular PGPR and rhizobia inoculation (i.e., AP and AR) treatments significantly increased the relative abundance of *Acidobacteria* while decreasing the relative abundance of *Proteobacteria*. The relative abundances of *Firmicutes* and *Acidobacteria* were significantly higher in the APR treatment compared to the A treatment (i.e., by 187% and 39.8%, respectively), while the relative abundances of *Proteobacteria* and *Actinobacteria* were significantly lower (i.e., by 25.3% and 23.9%, respectively). The relative abundances of the genera *Bacillus* and *Gemmatimonas* significantly increased in the PGPR inoculation treatment relative to the uninoculated treatment (Table S2). Moreover, the highest *Bacillus* abundance was observed in the co-inoculation (APR) treatment. STAMP analysis revealed that the inoculation of PGPR mainly enhanced the abundances of *Bacillus* and *Gemmatimonas* compared to the control (Fig. 3). Rhizobia inoculation (i.e., the AR treatment) mainly enhanced the abundances of *Gemmatimonas*, *Ralstonia*, *Roseiflexus*, *Streptomyces*, and *Massilia*. *Bacillus* showed a high frequency of abundance in the AP treatment, while *Ralstonia* showed a high frequency of abundance in the AR treatment. With respect to N-fixing bacteria, *Bradyrhizobium* and *Mesorhizobium* were significantly negatively correlated to TN concentrations (Table S3). *Ensifer* was significantly positively correlated to TN, NO_3^--N, and SOM concentrations. LEfSe identified eight

Table 3
Soil enzyme activity and microbial biomass.

Treatments	Urease (mg NH_3 -N g^{-1} 24 h^{-1})	Saccharase (mg glucose g^{-1} 24 h^{-1})	Catalase (ml $KMnO_4$ g^{-1} (20 min) $^{-1}$)	Acid phosphatase (μg PNP g^{-1} h^{-1})	β-glucosidase (μg PNP g^{-1} h^{-1})	Microbial biomass (mg kg^{-1})	
						C	N
A	0.09 ± 0.01 b	6.61 ± 0.51 b	4.26 ± 0.55 ab	114 ± 3.4 a	60.9 ± 1.6 bc	157 ± 16 a	1.89 ± 0.68 b
AP	0.08 ± 0.01 b	7.99 ± 0.37 ab	3.87 ± 0.37 b	138 ± 14 a	77.0 ± 11 ab	115 ± 7.7 b	3.38 ± 0.62 ab
AR	0.09 ± 0.01 b	7.73 ± 0.31 ab	5.00 ± 0.12 ab	116 ± 13 a	55.6 ± 2.9 c	60.7 ± 10 c	6.21 ± 0.85 a
APR	0.14 ± 0.02 a	8.56 ± 0.61 a	5.31 ± 0.24 a	126 ± 17 a	80.0 ± 1.9 a	107 ± 2.6 b	5.13 ± 1.68 ab

Note: Treatments: A (soil + alfalfa), AP (soil + alfalfa + PGPR), AR (soil + alfalfa + rhizobia), and APR (soil + alfalfa + PGPR and rhizobia). Values are means ± SE (n = 3). Different letters indicate significance at a 0.05 probability level ($P < 0.05$) using the LSD test.

biomarkers in the AR treatment and four in the APR treatment (Fig. 4). The most differentially abundant bacteria in the AR and APR treatments belonged to the genera *Ensifer* and *Bacillus*, respectively. Moreover, CCA showed that variations in the composition of bacterial communities were well accounted for (42.8%) by soil physicochemical properties (Fig. 5). It showed that soil TN and AN content were the major contributing factors for bacterial community variation based on ANOVA ($P < 0.05$).

The Shannon and phylogenetic diversity (PD) indices were significantly lower in the treatment planted with alfalfa alone relative to the blank soil treatment (Table S4). Compared to the control, the singular PGPR and rhizobia inoculation treatments (AP and AR, respectively) significantly increased the Shannon and PD indices, which was also supported by results from rarefaction curve analysis (Fig. S1). However, the co-inoculation treatment did not significantly affect the Shannon, Simpson, Chao1, abundance-based coverage estimator (ACE), and PD indices. Additionally, soil physicochemical properties did not significantly affect rhizosphere bacterial alpha diversity (α-diversity) (Table S5). PCoA was used to identify the rhizosphere bacterial community structure in all samples (Fig. 6A), which revealed significant differences in rhizosphere bacterial communities among the blank soil (BS), control (A), and inoculated treatments (AP, AR, and APR). For the inoculation treatments, the bacterial community structure did not show any clear difference between the AP, AR, and APR treatments, which was confirmed by the Venn diagram (Fig. 6B).

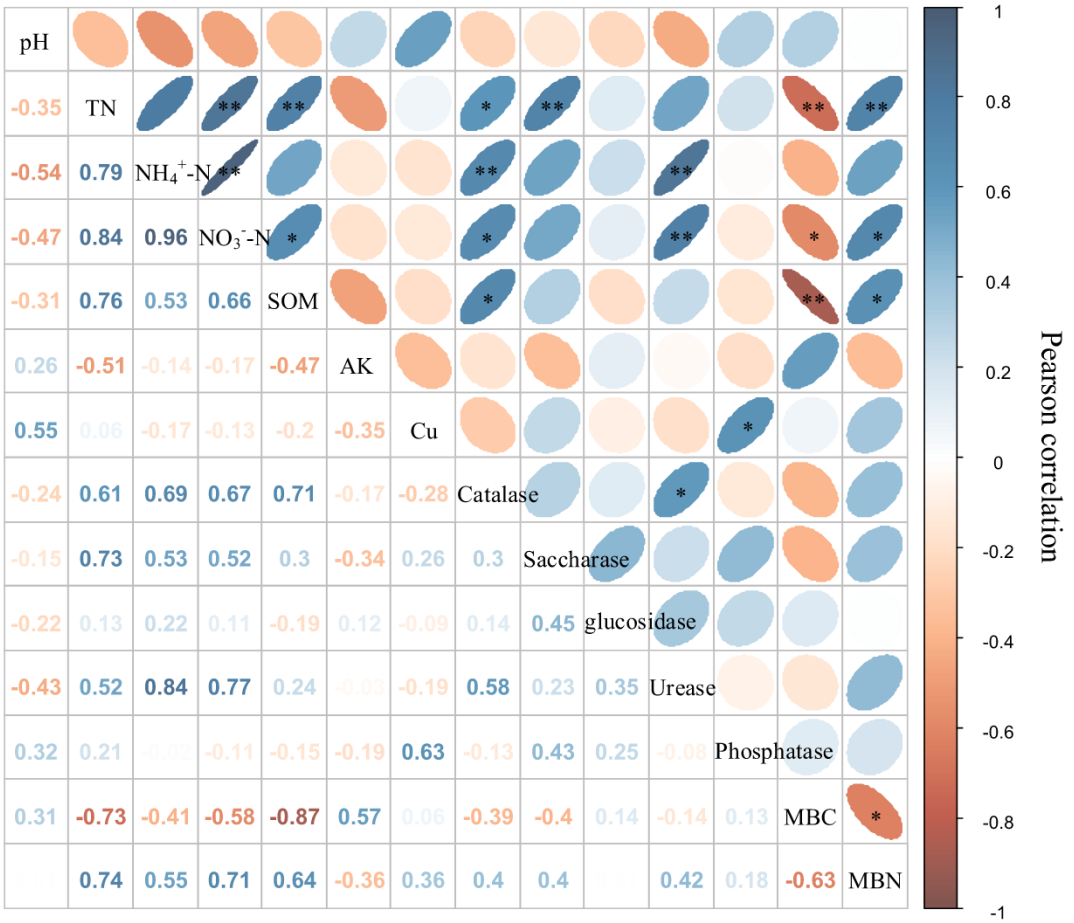

Fig. 1. A correlation heat map illustrating pairwise relationships between soil physicochemical properties and enzyme activities based on Pearson correlation coefficient analysis. TN: total nitrogen; AK: available potassium; SOM: soil organic matter; MBC: microbial biomass carbon; MBN: microbial biomass nitrogen. * denotes significance at a $P < 0.05$ probability level; ** denotes significance at a $P < 0.01$ probability level; *** denotes significance at a $P < 0.001$ probability level.

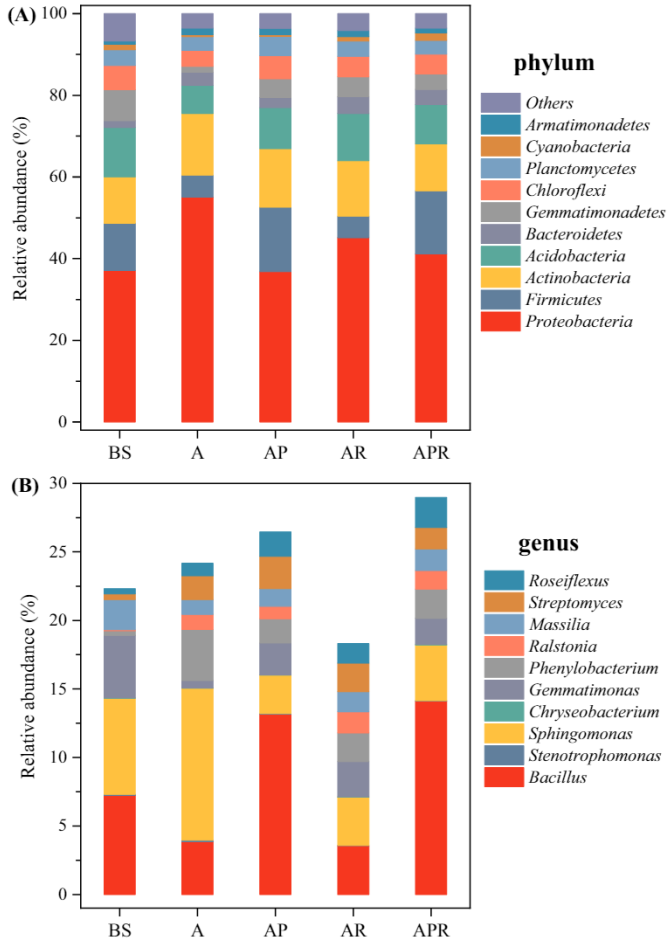

Fig. 2. Relative abundance (%) of the top 10 phylum (A) and genus (B) in the rhizosphere. Treatments: BS (blank soil, i.e., only soil), A (soil + alfalfa, i.e., the control), AP (soil + alfalfa + PGPR), AR (soil + alfalfa + rhizobia), and APR (soil + alfalfa + PGPR and rhizobia).

3.6. Divergent effects of environmental variables on soil nutrient cycling

The Shannon index, MBC, soil enzyme activities, and bacteria associated with soil C cycling used as environmental variables explained 78.9% of SOM (Fig. 7A), and MBC was identified as the most important variable (with a relative influence = 52.7%). The relative abundance of *Acidobacteria* was also found to be a key C cycling soil variable. The Shannon index, MBN, soil enzyme activities, and bacteria associated with soil N cycling used as environmental variables explained 75.4%, 80.7%, and 80.1% of the variation in soil TN, NH_4^+-N, and NO_3^--N, respectively (Fig. 7B-D). MBN was identified as the most important variable for variation in soil TN (with a relative influence = 27.8%) and NO_3^--N (with a relative influence = 26.1%), and urease was identified as the most important variable for soil NH_4^+-N (with a relative influence = 31.2%). MBN, urease, and the relative abundance of *Actinobacteria* were key variables in soil N cycling. Soil enzyme activities, microbial biomass, and bacterial composition collectively explained most variation associated with soil nutrient cycling (Fig. 8). Moreover, PLS-PM showed that microbial biomass had a significant direct effect (-0.63; $P < 0.05$) on soil nutrients (Fig. 8A). Both soil enzyme activities (with a total effect = 0.43) and the relative abundance of bacteria (with a total effect = 0.80) associated with soil C and N cycling had a positive total effect on soil nutrients, whereas microbial biomass (with a total effect = -0.69) resulted in a negative total effect (Fig. 8B). VPA explained 83% of the variation in the selected variables (Fig. 8C). Soil enzyme activities, microbial biomass, and bacterial composition explained 75.3%, 42.2%, and 48.7% of soil nutrient variation. Interactions among these three variables accounted for 5.41% of the variation, which was higher compared to the other two variables, namely, microbial biomass (none) and bacterial composition (none).

4. Discussion

4.1. Regulatory mechanisms of PGPR and rhizobia co-inoculation in improving plant growth

Inoculation with both the PGPR and rhizobia treatments (i.e., the AP and AR treatments) had beneficial effects on plant growth by increasing plant biomass under metal stress (Table 2). This is because PGPR and rhizobia can individually produce higher concentrations of plant growth-promoting active biomolecules (indole acetic acid, siderophore, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase) as well as a rich nutritional source (Chen et al., 2018; Rizvi and Khan, 2018). Additionally, inoculation, particularly with rhizobia, also increased soil nutrient availability (i.e., available N and SOM; Table 1) which in turn improved plant nutrient absorption. Furthermore, several studies have reported that utilizing PGPR and rhizobia for plant inoculation can stimulate plant efficiency in retrieving soil nutrients, while helping plants to cope with abiotic stress, which prevents plant diseases (Pattnaik et al., 2020; Pii et al., 2015; Poole et al., 2018). PGPR and rhizobia co-inoculation was found to be more effective than their singular inoculation in improving plant growth. This phenomenon could be due to several reasons, such as the higher nutrient availability of the co-inoculation treatment, that PGPR has the potential in enhancing nodulation as well as the growth of legume-rhizobia symbiosis (Sanchez et al., 2014), and that co-inoculation provides biologically-fixed N at a low cost (Egamberdieva et al., 2013).

Furthermore, Cu concentrations and its total uptake in shoots were significantly lower in the inoculated treatments relative to the uninoculated treatment under Cu stress (Table 2), suggesting that PGPR and/or rhizobia inoculation reduced phytotoxicity induced by Cu accumulation (Dary et al., 2010; Rizvi and Khan, 2018). Additionally, total Cu uptake was more pronounced in roots than in shoots, and a very low level of Cu was translocated to shoots as confirmed by the TF of Cu. These results provide evidence for the usefulness of PGPR and rhizobia for heavy metal phytostabilization (Chen et al., 2018; Dary et al., 2010; Ju et al., 2019), and subsequently decrease the risk of Cu entering the food chain. In brief, the abovementioned results demonstrated that inoculation, particularly co-inoculation with PGPR and rhizobia, can potentially be used to improve plant growth by reducing phytotoxicity and providing plants with an adequate amount of nutrients.

4.2. The role of soil enzyme and microbial activities in soil nutrient cycling after co-inoculation

Enzyme activities are also associated with microbial metabolism and reflect the interaction between microorganisms and their associative environment (Cui et al., 2019; Wei et al., 2018). In this study, the singular inoculation of PGPR and rhizobia did not significantly affect soil enzyme activities (Table 3). This finding, however, is not in agreement with previous studies that reported that the singular inoculation of either PGPR or rhizobia increased soil enzyme activities (i.e., urease, saccharase, catalase, and phosphatase) (Gong et al., 2012; Hidri et al., 2019; Yu et al., 2019), which was likely the result of differences in bacterial type, inoculation time, and inoculation environment. However, co-inoculation significantly increased soil urease, saccharase, and β-glucosidase activities relative to the uninoculated treatment. A recent study of ours also showed that co-inoculation was more effective than singular inoculation in increasing soil enzyme activities, namely, by 90 days following co-inoculation (Ju et al., 2019). Moreover, PGPR and rhizobia co-inoculation produced synergistic

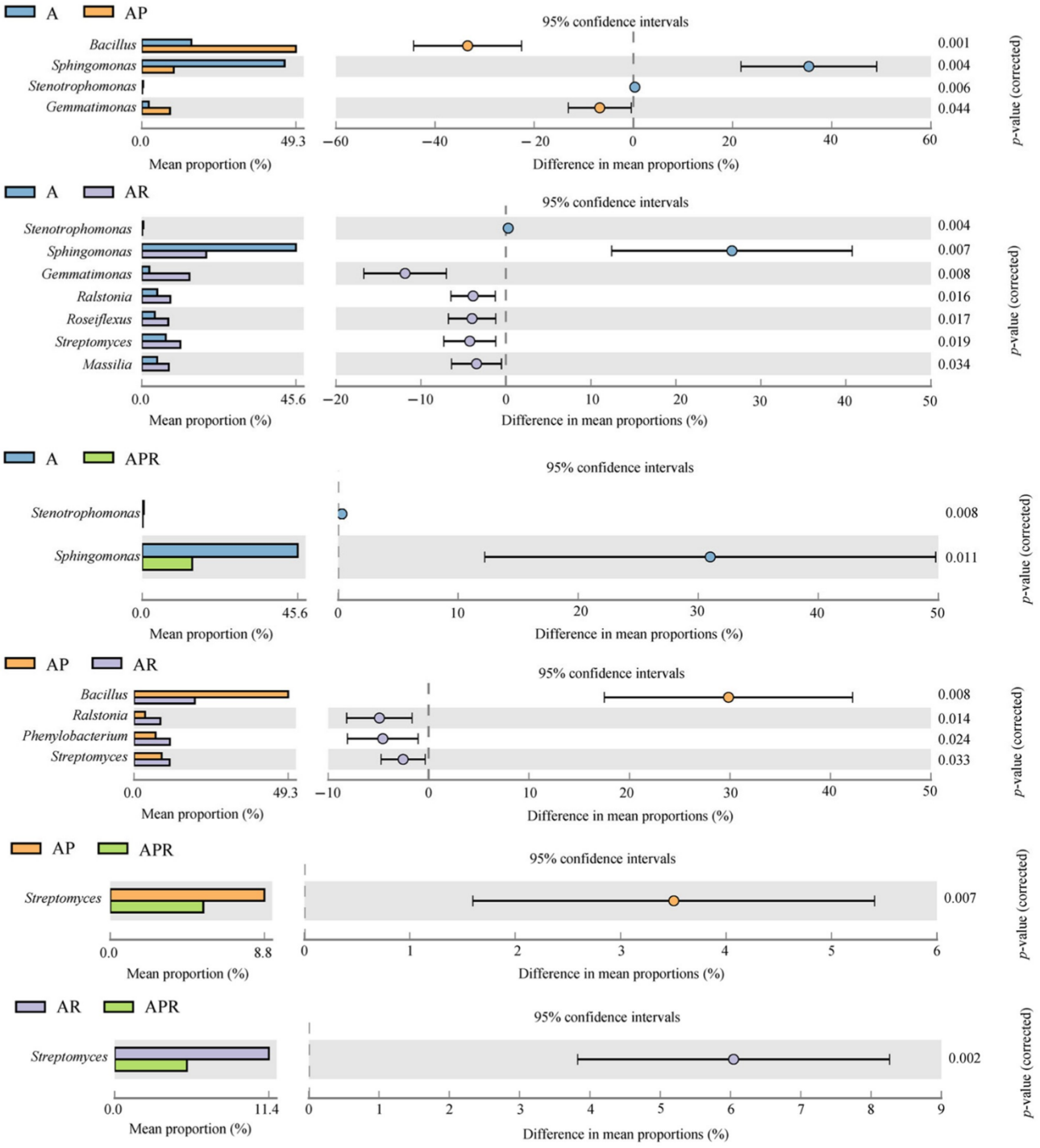

Fig. 3. A comparison between differences in the relative abundance on a bacterial genus level (top 10) among treatments based on STAMP analysis. Extended error bar plots denote statistically significant features along with the *p*-values (at a $P < 0.05$ probability level), effect sizes, and confidence intervals (95%). Treatments: A (soil + alfalfa, i.e., the control), AP (soil + alfalfa + PGPR), AR (soil + alfalfa + rhizobia), and APR (soil + alfalfa + PGPR and rhizobia).

effects, namely, in increasing soil enzyme activities.

Urease activity reflects the soil N status and associative changes, and it is important for N cycling (Raiesi and Salek-Gilani, 2018). Soil urease activity, being a key variable of soil NH_4^+-N and NO_3^--N concentrations (Fig. 7B and C), was significantly positively correlated to available N concentrations (Fig. 1), suggesting that PGPR and rhizobia co-inoculation can promote N cycling by stimulating urease activity. Soil saccharase activity is associated with soluble nutrients, such as SOM, and reflects soil C cycling and fertility (Ge et al., 2011). Co-inoculation significantly increased SOM concentrations in this study, and the highest saccharase activity was observed in the co-inoculated treatment, indicating that PGPR and rhizobia co-inoculation can promote C cycling and enhance soil fertility. Soil β-glucosidase is a rate-limiting enzyme in the microbial degradation of cellulose to glucose and C cycling in soil, providing a higher C requirement for microorganisms to repair soil contaminated with metals (Tica et al., 2011). This was confirmed by the fact that the PGPR and rhizobia co-inoculation treatment significantly increased soil β-glucosidase activity. Soil enzyme activities explained greater variation in soil nutrient cycling than microbial biomass and bacterial composition (Fig. 8).

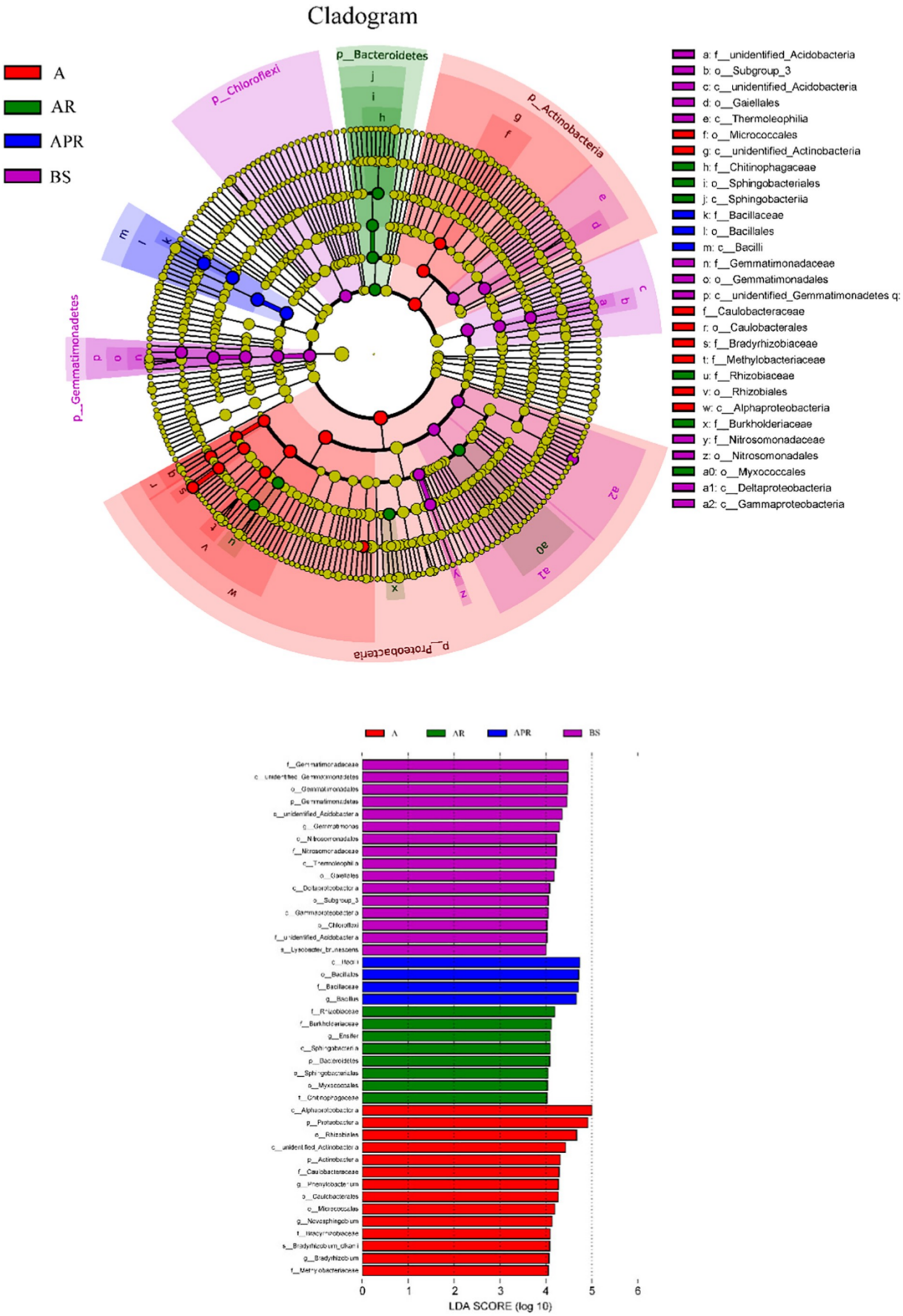

Fig. 4. Biomarker analysis of the bacterial community in the rhizosphere based on LEfSe results. BS (blank soil, i.e., only soil), A (soil + alfalfa, i.e., the control), AP (soil + alfalfa + PGPR), AR (soil + alfalfa + rhizobia), and APR (soil + alfalfa + PGPR and rhizobia).

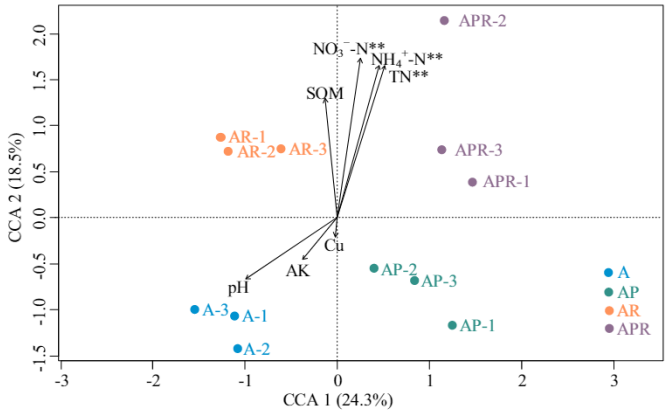

Fig. 5. Canonical correspondence analysis (CCA) used to identify relationships between rhizosphere bacterial community composition and soil physicochemical properties. Treatments: A (soil + alfalfa, i.e., the control), AP (soil + alfalfa + PGPR), AR (soil + alfalfa + rhizobia), and APR (soil + alfalfa + PGPR and rhizobia). * denotes significance at a $P < 0.05$ probability level; ** denotes significance at a $P < 0.01$ probability level; *** denotes significance at a $P < 0.001$ probability level.

Moreover, PLS-PM also revealed that soil enzyme activities had a positive total effect on soil nutrient concentrations. These results demonstrated that PGPR and rhizobia co-inoculation can promote soil C and N cycling by stimulating enzyme activities in metal contaminated soil while subsequently providing higher C and N requirements for microbial activity.

Changes in soil microbial biomass reflect modifications in SOM stocks and other soil properties, while also playing an important role in nutrient cycling, C dynamics, and the development and function of soil systems (Raiesi and Salek-Gilani, 2018). The rhizobia inoculation treatment had the lowest MBC content (Table 3), suggesting that this treatment had a significant effect on soil microbial biomass C. This is consistent with findings from Zhang et al. (2010), who reported that *Rhizobium* inoculation decreased microbial biomass C in the rhizosphere of faba (fava) beans. PGPR and rhizobia inoculation decreased soil MBC, and this was likely due to the inhibition of indigenous microbes by inoculated bacteria through spatial and nutritional competition. Soil MBC content, being the most important SOM variable (Fig. 7), was significantly negatively correlated to SOM (Fig. 1), which was attributed to the potential short-term conversion of C biomass to soil organic C (Zhang et al., 2019). MBC was also significantly negatively correlated to TN and NO_3^--N concentrations, which could be attributable to excess N, effectuating greater competition for the limited amount of available C; consequently inhibiting microbial growth (Song et al., 2014). Additionally, soil MBN was a key variable for total N and available N in soil (Fig. 7B-D). In contrast to MBC content, soil MBN content significantly increased in the rhizobia inoculation treatment, while it only slightly increased in the PGPR inoculation treatment. A significant increase in soil MBN could have resulted from the higher N provision provided by the rhizobia inoculation treatment for microbial activity (Table 1 and Fig. 1), which was confirmed by findings from Zhou et al. (2017) who reported that the addition of N significantly increased microbial biomass N. PLS-PM further revealed that microbial biomass had a significant and direct effect on soil nutrients while also having a detectable negative total effect (Fig. 8). Our results also indicated that nutrient cycling feedback loops for microbial resource acquisition can be regulated by PGPR and rhizobia co-inoculation treatments.

Rhizosphere microbial communities not only act as primary agents of nutrient cycling (Kuzyakov and Razavi, 2019) but are also regarded as sensitive indicators in the progress of heavy metal remediation (Yu et al., 2019). In this study, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and

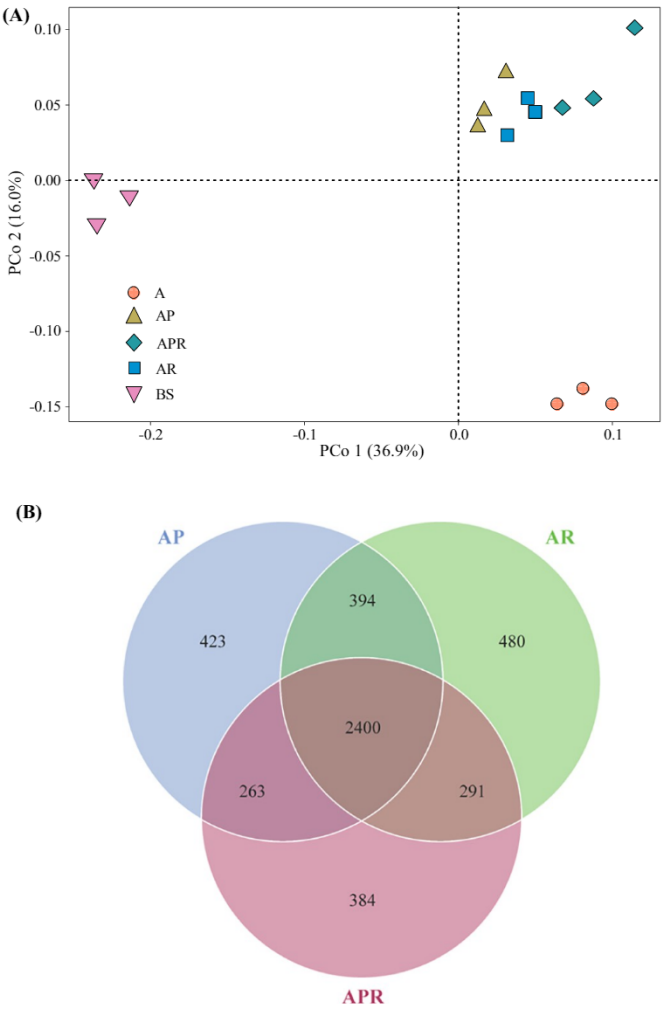

Fig. 6. Rhizosphere bacterial community structure. Principal coordinates analysis (PCoA) of changes in bacterial community composition for the different treatments (A). Venn diagram of the number of shared and unique operational taxonomic units (OTUs) among the AP, AR, and APR treatments (B). OTUs were defined at a 97% sequence similarity. BS (blank soil, i.e., only soil), A (soil + alfalfa, i.e., the control), AP (soil + alfalfa + PGPR), AR (soil + alfalfa + rhizobia), and APR (soil + alfalfa + PGPR and rhizobia).

Acidobacteria were the dominant phyla in rhizosphere bacterial communities for all treatments (Fig. 2). The relative abundances of *Proteobacteria* and *Actinobacteria* were greater in a previous long-term N addition experiment (Fierer et al., 2012) associated with N cycling. The relative abundances of *Proteobacteria* and *Actinobacteria* (Table S2) significantly decreased in the inoculated treatments (AP, AR, and APR) compared to the control (A), suggesting that limitations of symbiotic N-fixing bacteria (Remigi et al., 2016) are induced by short-term PGPR and rhizobia inoculation. These results potentially confirmed that PGPR and rhizobia co-inoculation treatments provide sufficient N (Table 1) for plant growth while limiting the activity of indigenous N-fixing bacteria. This was confirmed by a decrease in the relative abundances of *Bradyrhizobium* and *Mesorhizobium* resulting from an increase in soil TN concentrations following the inoculation treatments (Table S3). The PGPR and rhizobia co-inoculation treatment significantly increased the relative abundances of the phyla *Firmicutes* and *Acidobacteria*. *Firmicutes* are known for their tolerance to extreme conditions and low substrate availability (Hortal et al., 2013) as well as producing multiple enzyme activities (Kumar et al., 2012). *Acidobacteria* plays a key role in the degradation of organic matter and the recovery of nutrients under a nutrient-deficient environment, such as copper tailings mines (Sun

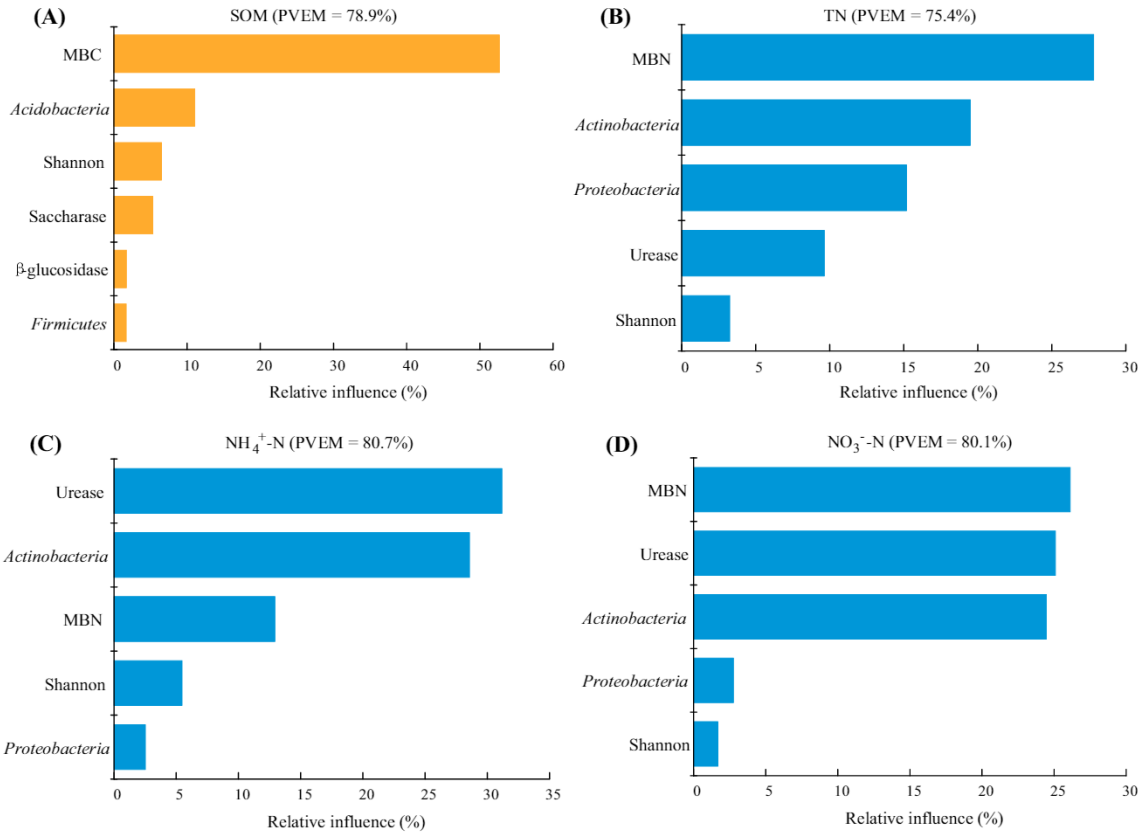

Fig. 7. Relative influence (%) of environmental drivers associated with soil carbon (A) and nitrogen (B, C, and D) concentration. Environmental drivers include the microbial diversity index (the Shannon index) and biomass (MBC and MBN) as well as soil enzyme activity and bacteria associated with soil carbon and nitrogen cycling, respectively. PVEM represents the proportion of variance explained by the model.

et al., 2018). As it pertains to biomarkers in the PGPR and rhizobia co-inoculation treatment, these included members of the *Bacillus* genus (Fig. 4), belonging to phylum *Firmicutes*, which has previously been associated with a lower degree of metal pollution (Tipayno et al., 2012), a higher soil nutrient turnover rate, and the promotion of plant growth (Molina-Santiago et al., 2019). Additionally, the relative abundances of bacteria associated with soil C and N cycling had an obvious positive total effect on soil nutrient concentrations (Fig. 8B). It is interesting to note that the PGPR and rhizobia co-inoculation treatment increased the soil α-diversity index (Table S4), and co-inoculation can also affect soil microbial community structure (Fig. 6) and regulate soil microbial activity in Cu contaminated soil through the alteration in N concentrations (Fig. 5). Our results indicated that short-term PGPR and rhizobia co-inoculation could improve nutrition levels of Cu contaminated soil and promote plant growth by regulating microorganisms associated with nutrient cycling.

This study investigated relationships between soil nutrients, soil enzymes, and microbial activities following PGPR and rhizobia inoculation. The higher amount of soil nutrients observed in the PGPR and rhizobia co-inoculation treatment was due to the ability of co-inoculation to regulate soil enzyme and microbial activity associated with nutrient cycling. There are specific compatibility relationships between PGPR and rhizobium, underscoring the importance of microorganisms and symbiotic system selection in maximizing plant growth and soil nutrients under specific environmental conditions (Hidri et al., 2019). These mechanisms imply a significant potential in PGPR and rhizobia co-inoculation to provide a favorable nutrient environment for metal contaminated soil phytoremediation initiatives.

5. Conclusions

Inoculation, particularly PGPR (*P. mucilaginosus*) and rhizobia (*S. meliloti*) co-inoculation, increased the phytostabilization of Cu and prevented toxic metals from entering the food chain in this study. Co-inoculation increased the C and N concentration of metal contaminated soil and subsequently improved plant growth. Soil enzyme and microbial activities associated with nutrient cycling had significant positive effects on soil nutrient availability. Soil urease, saccharase, and β-glucosidase, as well as microbial biomass N, were higher in the co-inoculated treatment compared to the uninoculated control. Additionally, the co-inoculation treatment improved soil nutrient levels by regulating microbial activity and community structure. These results will help improve our understanding of the impact of short-term PGPR and rhizobia co-inoculation on plant growth, soil enzymatic activity, and microbial community structure in metal contaminated soil globally. However, further studies are required to address the presence of nutrient cycling genes (i.e., phenotype or genotype) in microbial populations following rhizobacteria inoculation and co-inoculation treatments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

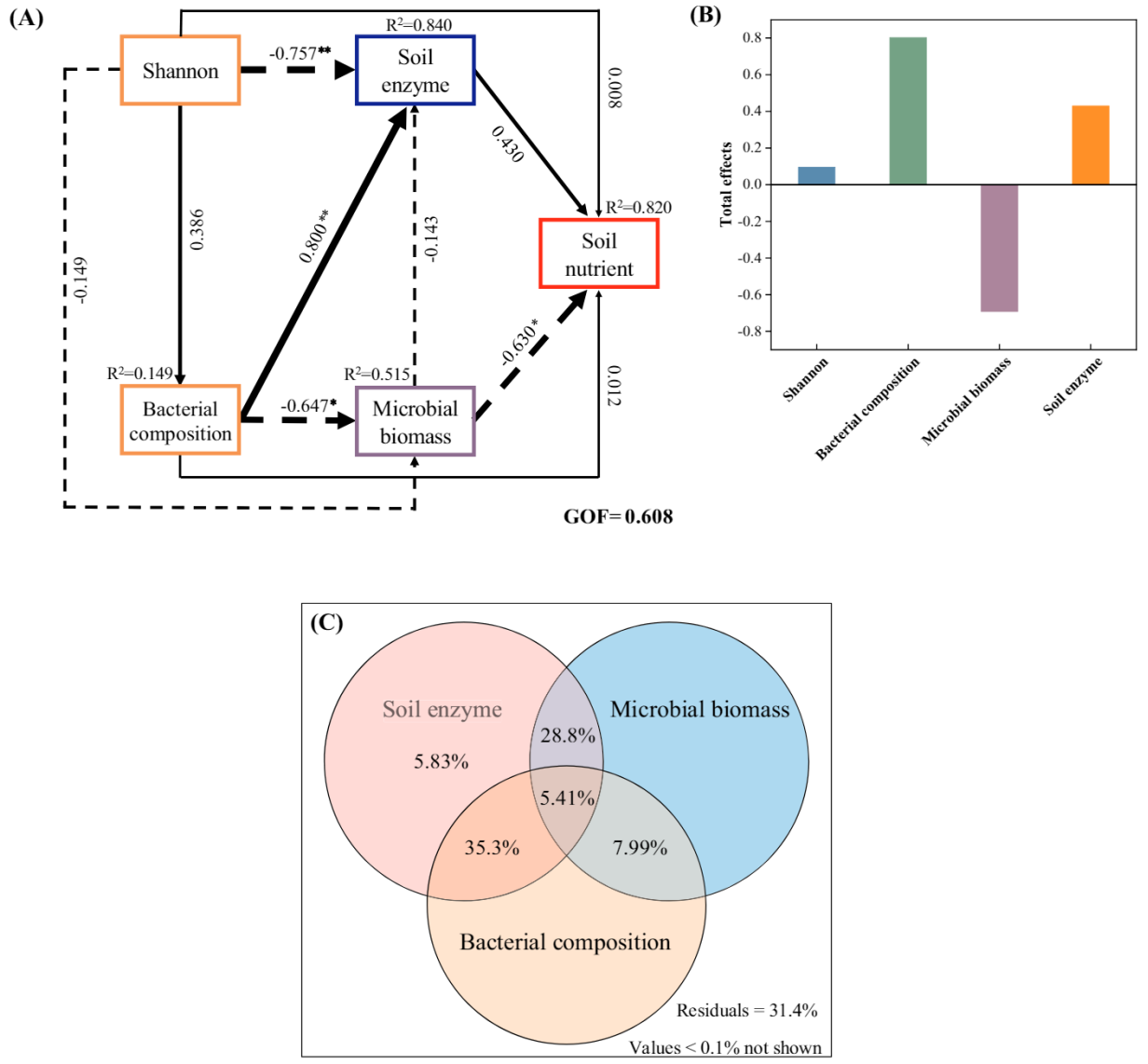

Fig. 8. Cascading relationships between soil nutrient cycling with the microbial diversity index, biomass and composition, and soil enzyme activity. Partial least squares path modeling (PLS-PM) results explaining major pathways of influence of the Shannon index, bacterial relative abundance (at a phylum level), microbial biomass, and soil enzyme activity on the soil nutrient cycle (A). Standardized total effects (i.e., direct plus indirect effects) were calculated using PLS-PM (B). Variation partitioning analysis illustrated the contribution of soil enzyme activity, microbial biomass, and bacterial composition to soil nutrient cycling (C). Bacterial composition includes the relative abundance of bacteria associated with soil carbon (*Firmicutes* and *Acidobacteria*) and nitrogen (*Proteobacteria* and *Actinobacteria*) cycles; microbial biomass includes microbial biomass carbon and nitrogen; soil enzyme activity includes urease, saccharase, and β-glucosidase activities associated with carbon and nitrogen cycling; soil nutrients include SOM, TN, NH_4^+-N, and NO_3^--N. Solid and dashed lines denote positive and negative flows of causality, respectively. Numbers on arrow lines denote significant standardized path coefficients. * significance at $P < 0.05$ probability level; ** significance at $P < 0.01$ probability level; *** significance at $P < 0.001$ probability level. R^2 values represent the variance of dependent variables explained by the inner model. GOF denotes the goodness of fit index.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2019.103450.

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