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Impact of co-inoculation with plant-growth-promoting rhizobacteria and rhizobium on the biochemical responses of alfalfa-soil system in copper contaminated soil

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ABSTRACT

The effects and regulatory mechanisms of co-inoculation of plant-growth-promoting rhizobacteria (PGPRs) and rhizobium in plant-soil systems remain unclear, despite numerous reports that PGPRs or rhizobium can alleviate metal toxicity. We used the co-inoculation of the PGPR *Paenibacillus mucilaginosus* and the metal-resistant rhizobium *Sinorhizobium meliloti* for exploring the physiological and biochemical responses of the plant-soil system in metal-contaminated soil. The co-inoculation with the PGPR and rhizobium significantly increased the nutrient (N, P, and K) contents in plant tissues and promoted plant growth in soil contaminated with copper (Cu). Stress from Cu-induced reactive oxygen species and lipid peroxidation were largely attenuated by the co-inoculation by increasing the activities of antioxidant enzymes. The contents and uptake of Cu in plant tissues increased significantly in the co-inoculation treatment compared with the uninoculated control and individual inoculation treatment. Co-inoculation with PGPR and rhizobium significantly increased soil microbial biomass, enzymatic activities, total nitrogen, available phosphorus, and soil organic matter contents compared with the uninoculated control. Interestingly, co-inoculation also affected the composition of the rhizospheric microbial community, and slightly increased rhizospheric microbial diversity. These improvements of the soil fertility and biological activity also had a beneficial impact on plant growth under Cu stress. Our results suggested that alfalfa co-inoculated with PGPR and rhizobium could increase plant growth and Cu uptake in metal-contaminated soil by alleviating plant Cu stress and improving soil biochemical properties. These results indicate that the co-application of PGPR and rhizobium can have a positive effect on the biochemical responses of alfalfa-soil systems in soil contaminated by heavy metals and can provide an efficient strategy for the phytomanagement of metal-contaminated land.

1. Introduction

Soil contaminated by toxic metals is a major environmental problem worldwide, especially in China (Qu et al., 2016). Mining and the processing of metal ores are potential sources of soil contamination with toxic metals (Chen et al., 2018). Toxic metals accumulated in soil are difficult to degrade or remove in plant-soil systems, and further accumulation in plant tissues or human organs can threaten plant growth and human health (Wang et al., 2017). Phytoremediation is emerging as a more feasible measure for the economical and environmentally sound remediation of contaminated soils compared with traditional in situ techniques (Desjardins et al., 2018). Phytoremediation is effective, but many limitations remain that prevent its widespread application (Al Mahmud et al., 2018). Plants cannot attain sufficient biomass for appreciable rates of remediation when soil has accumulated excessive levels of heavy metals, in part due to poor nutrient content and the toxicity of the contaminated soil, especially mineral soil (Wilson-Kokes and Skousen, 2014). Healthy plant growth is often achieved by mutualistic relationships with soil microbes, but microbial populations (diversity and biomass) are often depleted in contaminated soils (Shi et al., 2002). Enhancing the biomass of plants, soil nutrient levels, and soil microbial diversity are therefore important factors in the success of phytoremediation (Al Mahmud et al., 2018; Yang et al., 2017).

Plant-growth-promoting rhizobacteria (PGPRs) and rhizobium can

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act as adjuncts in metal phytoremediation, substantially facilitating the growth of plants and improving the soil environment (i.e. nutrients and microbes) in the presence of otherwise inhibitory levels of metals (Kalam et al., 2017). PGPRs are a heterogeneous group of bacteria in the rhizosphere, on root surfaces, and in association with roots, including species of *Pseudomonas*, *Bacillus*, and *Arthrobacter* (Joseph et al., 2012). PGPRs can enhance plant growth directly and/or indirectly under heavy-metal stress by tolerating abiotic stress, stimulating root growth, producing agents for chelating heavy metals, and promoting soil microbial development and nutrient availability (Ma et al., 2016; Bhattacharyya and Jha, 2012). Some examples of PGPRs resistant to heavy metals that can enhance heavy-metal stress tolerance in plants have been reported (Etesami, 2018). A variety of symbiotic bacteria (i.e. *Rhizobium* spp.) have recently been used worldwide for improving plant growth, especially symbiotic nitrogen-fixing bacteria (Cocking, 2003). Various rhizobial strains highly resistant to heavy metals may affect plant growth and metal uptake by different mechanisms, including the enhancement of nitrogen content, the solubilisation of phosphate, the production of phytohormones, and the improvement of the soil environment (Hayat et al., 2010; Reichman, 2007).

PGPRs and rhizobium have the potential to play important roles in improving plant growth and enhancing metal uptake by plants in different ways under conditions of heavy-metal stress (Bhattacharjya and Chandra, 2013; Fatnassi et al., 2015). Attention must therefore be paid to the selection of appropriate plant-bacteria symbiotic combinations with large phytoremediation potential in the presence of excessive levels of toxic metals. Focusing on the positive effects of plant-bacteria symbiotic combinations on plant metal tolerance and soil quality is thus important. Visible symptoms of metal toxicity in plants are likely due to effects at the structural and ultrastructural levels (Fatnassi et al., 2015). Toxic heavy metals can interact with membrane proteins, leading to lipid peroxidation and oxidative stress by increasing the level of reactive oxygen species (ROS) in sub-cellular compartments (Chen et al., 2018; Kong et al., 2015a). Antioxidative enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), though, can preserve cellular stability and play an essential role in scavenging ROS and preventing oxidative damage (Duan et al., 2018; Mittler, 2002).

Heavy-metal pollution can also decrease the metabolic activity, biomass, and diversity of microorganisms in the rhizosphere, which can also limit the effectiveness of PGPRs or rhizobium (Wang et al., 2015). Soil enzymes and microbial biomass are vital for promoting the decomposition of organic matter and nutrient cycling and can be used as indicators of heavy-metal pollution (Ajwa et al., 1999). Microorganisms play critical roles in soil-nutrient cycling, soil structural formation, and soil-plant interactions (Harris, 2009). Changes in the activity of enzymes, microbial biomass, and microbial activity in soil could therefore alter the availability of nutrients for plant uptake and are potentially sensitive indicators of soil quality (Shi et al., 2002). Previous studies have identified the regulatory mechanisms of PGPR or rhizobium inoculation in alleviating Cu stress in plants by biochemical responses of plant systems (Pramanik et al., 2018; Rizvi and Khan, 2018). However, few studies identified the regulatory mechanisms of PGPR and rhizobium co-inoculation in alleviating Cu stress in plants through the biochemical response of plant-soil system.

We can thus assume that PGPR and rhizobium co-inoculation could improve plant growth and soil quality more than an uninoculated control and individual inoculations in soil contaminated with heavy metals. Various mechanisms, such as the alleviation of copper (Cu) stress in plants by increasing the activities of plant antioxidative enzymes and improving soil biochemical properties by increasing soil microbial biomass and enzymatic activities and improving the structure of microbial communities. We therefore selected the PGPR *Paenibacillus mucilaginosus* (strain ACCC10013) and the Cu-resistant rhizobium *Sinorhizobium meliloti* (strain CCNWSX0020) as experimental subjects. The aims of this study were to: (1) study the effect of PGPR and rhizobium co-inoculation on alfalfa growth and Cu uptake, and (2) identify the regulatory mechanisms of PGPR and rhizobium co-inoculation in alleviating Cu stress in plants by biochemical responses of the plant-soil system. Our results will increase our understanding of the regulatory mechanisms of PGPR and rhizobium co-inoculation in counteracting metal toxicity and provide an efficient strategy for the phytoremediation of metal-contaminated soil.

2. Materials and methods

2.1. Pot experiment

Surface soil (0–20 cm) was collected from an area surrounding a Cu smelter in the city of Huangshi (30°42′N, 114°53′E), Hubei province, China (Fig. S1). The samples were air-dried and then passed through a 2-mm sieve. Selected physical and chemical properties of the soils were determined (Table S1). A strain of *S. meliloti* resistant to heavy metals isolated from the root nodules of *M. lupulina* plants growing in the tailings of a lead-zinc mine in China was provided by Northwest A & F University, China. This strain has been deposited in the Agricultural Culture Collection of China (ACCC19736). The complete genome sequence of *S. meliloti* has been reported and contains numbers of protein-coding genes putatively involved in Cu resistance (Li et al., 2012). The PGPR *P. mucilaginosus* (ACCC10013, provided by the Agricultural Culture Collection of China) was used as the co-inoculant with *S. meliloti* in our experiment. The rhizobium *S. meliloti* and the PGPR *P. mucilaginosus* were able to endure higher concentrations of Cu in this study (Fig. S2). *S. meliloti* was grown in a tryptone/yeast liquid medium (5 g tryptone, 3 g yeast extract, and 0.7 g $CaCl_2{\cdot}2H_2O$ L^{-1}; pH 7.2) at 28 °C with shaking at 150 rpm (Kong et al., 2015b). *P. mucilaginosus* was grown in an optimised liquid medium (2.5 g maltose, 1 g tryptone, 10 mg salicylic acid, 0.73 g $MgSO_4{\cdot}7H_2O$, 0.4 g $K_2HPO_4{\cdot}3H_2O$, 0.06 g NaCl, 0.6 mg $FeCl_3$, and 1 g $CaCO_3$ L^{-1}; pH 7.2) at 30 °C with shaking at 180 rpm.

The collected soil was packed into plastic pots (1.30 kg per pot), and the moisture content was maintained at ~70% of the maximum water-holding capacity. Alfalfa (*Medicago sativa*) seeds (Beijing Rytway Ecotechnology Co., LTD) were sterilised in 20% sodium hypochlorite for 10 min, washed three times with tap water, and rinsed with deionised water (DIW). Approximately 20 pre-germinated seedlings were transplanted into each pot, and the moisture content was maintained at ~70% of the water-holding capacity by adding DIW to ensure optimal growth conditions for the alfalfa. We tested four treatments: soil + alfalfa (SA, the control), soil + alfalfa + *P. mucilaginosus* (SAP), soil + alfalfa + *S. meliloti* (SAS), and soil + alfalfa + *P. mucilaginosus* + *S. meliloti* (SAPS). Each treatment had three replicates. After the plants had grown their first leaves, bacterial suspensions were sprayed once a week for three weeks onto the soil of the pots of the inoculated treatments. The same volume of DIW was used for the uninoculated treatment (SA). The plants were harvested after 90 d, and both bulk and rhizospheric soil were sampled from all pots for further investigation.

2.2. Measurement of soil physicochemical properties and metals content

Soil pH, soil organic matter (SOM), total nitrogen (TN), available nitrogen, phosphorus, and potassium concentration were determined as described previously (Lu, 2000). The pH of air-dried soil samples (sieved to 1 mm) was determined using a pH meter (Model 225, Denver Instrument, USA) in a suspension of 1:2.5 soil: water (w/v). The SOM content was determined by titration using the potassium dichromate external-heating method. The Kjeldahl method was used to determine the soil TN content. Ammonium N (NH_4^+-N) and nitrate N (NO_3^--N) contents were measured using a Seal Auto Analyser. Available phosphorus (AP) was extracted with 0.5 mol L^{-1} $NaHCO_3$, and its content was determined using the molybdenum-blue method at 710 nm. Available potassium (AK) was extracted by 1.0 mol L^{-1} CH_3COONH_4,

and its content was determined by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) (Perkin-Elmer Optima 3300DV, USA).

The soil samples were digested using a modified USEPA Method 3051 A for measuring total heavy-metal contents. Specifically, 0.200 g of soil was digested in 15 mL of a tri-acidic mixture (HCl, HNO_3, $HClO_4$) with a volume ratio of 1:3:1 (Duan et al., 2018). The contents of Cu in the digested samples were determined using atomic absorption spectrophotometry (Hitachi, FAAS Z-2000, Japan).

2.3. Measurement of plant biomass and Cu and nutrient contents

Shoot and root biomasses were recorded at the end of the experiment after oven-drying at 70 °C for 3 d. The shoots and roots were washed three times with DIW and further dried at 65 °C for 48 h, and the plant samples were then separated into two portions. One portion was digested with a 10-mL acid mixture (1:4 concentrated $HClO_4$:HNO_3, v/v) for quantifying total Cu content by atomic absorption spectrophotometry (Hitachi, FAAS Z-2000, Japan). The other portion was digested with H_2SO_4 and H_2O_2 for quantifying N, P, and K contents using a flow analyser (Duan et al., 2018).

2.4. Measurement of plant MDA, H_2O_2, and $O_2^{\bullet -}$ contents and the activities of antioxidant enzymes

The level of lipid peroxidation was evaluated by malondialdehyde (MDA) content (Heath and Packer, 1968). Plant oxidative damage was assessed by measuring the amounts of superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) (Fan et al., 2015). The MDA content and H_2O_2 content were measured using an MDA reagent kit (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China) and an H_2O_2 reagent kit (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China), respectively. The production of $O_2^{\bullet -}$ was determined using an $O_2^{\bullet -}$ reagent kit (Suzhou Comin Biotechnology Co., Ltd., Suzhou, China) (Chen et al., 2018).

Fresh shoots and roots were homogenised in an ice bath with 1 mL of extraction buffer (50 mM phosphate buffer containing 1 mM ascorbic acid and 1 mM EDTA) at 4 °C. The homogenate was centrifuged at 15 000*g* at 4 °C for 15 min, and the supernatant was used for assaying the activities of antioxidant enzymes. The activities of superoxide dismutase (SOD, ExPASy entry EC 1.15.1.1), catalase (CAT, ExPASy entry EC 1.11.1.6), ascorbate peroxidase (APX, ExPASy entry EC 1.11.1.11), and peroxidase (POD, ExPASy entry EC 1.11.1.7) were assessed using enzyme-specific commercial reagent kits (Suzhou Comin Biotechnology Co., Ltd.) (Duan et al., 2018).

2.5. Measurement of soil microbial biomass and the activities of soil enzymes

Soil microbial biomass carbon (MBC) and microbial biomass N (MBN) were estimated by chloroform fumigation-extraction (Ajwa et al., 1999). Urease (ExPASy entry EC 3.5.1.5) and saccharase (ExPASy entry EC 3.2.1.26) activities were determined as described by Guan et al. (1986) using urea and sucrose as the substrates and quantified with a spectrophotometer (UV-2450, SHIMADZU) at 587 and 508 nm, respectively. Catalase (ExPASy entry EC 1.11.1.6) activity was determined by potassium permanganate titration (Guan et al., 1986) and was expressed as milliliters of 0.02 mol L^{-1} $KMnO_4$ per gram soil per 20 min. Assays for determining the activities of acid phosphatase (ExPASy entry EC 3.1.3.2) and β-glucosidase (ExPASy entry EC 3.2.1.21) were based on the release of *p*-nitrophenol as described by Garciagil et al. (2000), and the activities were quantified at 400 nm.

2.6. DNA extraction, PCR, and analysis of 16 S rRNA gene sequences

DNA was extracted from 0.5 g of soil using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, USA) following the manufacturer's instructions. The 515 F/926 R universal-primer set was used to amplify the V4-V5 region of the 16 S rRNA gene, with a 12-bp bar code on the reverse primer. Each 30-μL PCR mixture contained 15 μL of Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs), 3 μL of 2 μM primer, 10 μL of 10 ng μL^{-1} DNA, and sterile ultrapure water to 30 μL. Thermal cycling consisted of an initial denaturation at 98 °C for 1 min followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR amplification was conducted in triplicate, and the products were pooled for each subsample. A composite DNA sample for sequencing was created by combining equimolar ratios of the amplification products from the individual subsamples. The composite DNA was gel-purified with a GeneJET Gel Extraction Kit (Thermo Scientific) and sequenced using an Illumina MiSeq platform (Quince et al., 2009; Zhou et al., 2011).

2.7. Statistical analysis

All statistical calculations, such as correlations and determinations of significant differences, were carried out using SPSS 20.0 (SPSS Inc, Chicago, USA). A one-way ANOVA and LSD multiple comparisons ($p < 0.05$) were used to assess the significant difference among different treatments (uptake of Cu, soil properties, enzyme activities, and so on). The Pearson correlation analysis was performed to measure the pairwise relationship between different variables (i.e. soil properties, soil bacterial groups, and others). The beta diversity of microbial communities was analysed by principal coordinates analysis (PCoA) based on Bray-Curtis distances. The most important factors affecting the structures of the microbial communities were identified by a canonical correspondence analysis (CCA) using the Vegan package in R 3.5.0. All graphs were created by Origin Pro 2018b (OriginLab, USA).

3. Results

3.1. Soil physicochemical properties

The individual inoculation with PGPR and rhizobium significantly affected soil TN, AP, and SOM contents, and inoculation with only rhizobium significantly affected soil NH_4^+-N and NO_3^--N contents (Table S2, $p < 0.05$). NH_4^+-N, NO_3^--N, TN, AP, and SOM contents were 95.9%, 266.7%, 7.2%, 63.2%, and 4.4% higher in SAS than SA, respectively, and TN, AP, and SOM contents were 4.0%, 78.9%, and 2.9% higher in SAP than SA, respectively. In addition, the highest NH_4^+-N, NO_3^--N, TN, AP, and SOM contents were observed in SAPS, which were 2.8-, 5.7-, 1.1-, 2.1-, and 1.1-fold higher than SA, respectively. The individual inoculation and co-inoculation did not significantly affect pH or AK content compared with the uninoculated control. Overall, co-inoculation with PGPR and rhizobium improved the soil properties and fertility greatly.

3.2. Plant biomass and nutrient contents

Shoot and root biomasses were significantly higher in SAS and SAPS ($p < 0.05$), but only slightly higher in SAP, than SA (Table 1). The highest biomass was observed in SAPS, at 8.56 g pot^{-1} and 10.07 g pot^{-1} in shoots and roots, respectively. Meanwhile, shoot and root biomasses were 22.3% and 43.5% higher in SAPS than SA, respectively. Inoculation with either PGPR or rhizobium did not significantly affect the shoot or root N content, but N content was dramatically higher for the co-inoculation than the uninoculated control (Table S3). Shoot and root N, P, and K contents were all the highest in SAPS. N, P, and K contents were higher in shoots than roots in all treatments. In terms of plant phenotype, co-inoculation with PGPR and rhizobium improved plant growth compared with the uninoculated control and individual inoculations (Fig. S3).

Table 1
Biomass, Cu concentrations, and uptake of Cu in plant tissues.

Metal	Test	Biomass (g pot^{-1})		Concentration (mg kg^{-1})		Total uptake (μg pot^{-1})		*Transfer coefficient*
		Shoot	Root	Shoot	Root	Shoot	Root	
Cu	SA	7.01 ± 0.19c	7.02 ± 0.33c	26.1 ± 1.94 d	53.7 ± 2.21c	182.3 ± 3.97 d	389.5 ± 7.01c	0.49 ± 0.01c
	SAP	7.50 ± 0.17 bc	8.29 ± 0.41 bc	35.6 ± 1.79c	74.8 ± 4.30 b	258.2 ± 4.07c	627.6 ± 10.06 b	0.48 ± 0.01c
	SAS	8.11 ± 0.11 ab	8.58 ± 0.56 ab	45.4 ± 2.31 b	76.6 ± 3.70 b	354.6 ± 11.18 b	654.5 ± 12.16 b	0.59 ± 0.03 b
	SAPS	8.56 ± 0.18 a	10.07 ± 0.57 a	56.5 ± 3.40 a	86.7 ± 5.79 a	487.5 ± 9.41 a	824.4 ± 17.45 a	0.65 ± 0.01 a

Note: SA (soil + alfalfa), SAP (soil + alfalfa + *P. mucilaginosus*), SAS (soil + alfalfa + *S. meliloti*), and SAPS (soil + alfalfa + *P. mucilaginosus* and *S. meliloti*). The transport ability of Cu from roots to shoots in the plant is given by the *Transfer coefficient* (*Cu shoot / Cu root*), where *Cu shoot* and *Cu root* are Cu concentrations in shoots and roots, respectively. Data are means ± SEs of three independent replicates. Different letters indicate significant differences ($p < 0.05$) with the LSD test.

3.3. The contents and uptake of Cu in plant tissues

Cu content was considerably higher in roots than shoots for both inoculated and uninoculated plants (Table 1). Cu content was 36.4%, 73.9%, and 116.5% higher in shoots and 39.3%, 42.6%, and 61.5% higher in roots in SAP, SAS, and SAPS than SA, respectively. Shoot and root Cu contents were the highest in the PGPR and rhizobium co-inoculated plants, at 56.6 and 86.5 mg kg^{-1}, respectively, and were 2.2-, 1.6-, and 1.2-fold higher in shoots and 1.6-, 1.2-, and 1.1-fold higher in roots in SAPS than SA, SAP, and SAS, respectively. The total uptakes of Cu in shoots and roots were significantly higher for PGPR and rhizobium individual inoculation and co-inoculation compared with the uninoculated control ($p < 0.05$), similar to the shoot and root Cu contents. Total Cu uptakes were 2.7-, 1.9-, and 1.4-fold higher in shoots and 2.1-, 1.3-, and 1.3-fold higher in roots in SAPS than SA, SAP, and SAS, respectively. The translocation of Cu from roots to shoots was generally higher in SAPS, indicated by the highest transfer coefficient. The efficiency of Cu phytoextraction thus higher in the PGPR and rhizobium co-inoculated plants.

3.4. Plant MDA, H_2O_2, and $O_2^{\bullet -}$ contents and antioxidant enzymatic activities

Shoot and root MDA, H_2O_2, and $O_2^{\bullet -}$ contents were all the lowest in SAPS (Fig. 1). MDA, H_2O_2, and $O_2^{\bullet -}$ contents were 26.1%, 29.5%, and 31.3% lower in shoots and 33.3%, 35.1%, and 42.2% lower in roots, respectively, for the co-inoculated than the uninoculated treatments. Root MDA content was dramatically lower in SAP and SAS than SA by 22.22% and 27.78%, respectively (Fig. 1A, $p < 0.05$). In contrast, shoot MDA was only slightly lower in SAP and SAS than SA· H_2O_2 content was significantly lower in SAS than SA by 14.3% in shoots and 24.4% in roots, respectively (Fig. 1B, $p < 0.05$). Inoculation with PGPR, however, did not significantly affect shoot or root H_2O_2 content. Shoot and root $O_2^{\bullet -}$ contents were significantly lower in SAS than SA ($p < 0.05$), similar to H_2O_2 content, but were not significantly affected by SAP (Fig. 1C). Interestingly, MDA, H_2O_2, and $O_2^{\bullet -}$ contents were higher in shoots than roots in all treatments.

Shoot and root SOD, CAT, and APX activities were the highest in SAPS, and shoot and root POD activities were the highest in SA (Fig. 2). Shoot and root SOD activities were significantly higher in SAP, SAS, and SAPS than SA (Fig. 2A, $p < 0.05$). SOD activity was 2.5-, 1.8-, and 1.4-fold higher in shoots and 4.1-, 1.2-, and 1.2-fold higher in roots in SAPS than SA, SAP, and SAS, respectively. Shoot and root CAT activities were significantly higher in SAP, SAS, and SAPS than SA (Fig. 2B, $p < 0.05$). CAT activity was 2.8-, 1.8-, and 1.4-fold higher in shoots and 3.4-, 1.6-, and 1.4-fold higher in roots in SAPS than SA, SAP, and SAS, respectively. Shoot and root APX activity were also significantly higher in SAP, SAS, and SAPS than SA (Fig. 2C, $p < 0.05$). APX activity was 3.7-, 2.2-, and 1.7-fold higher in shoots and 3.5-, 1.6-, 1.5-fold higher in roots in SAPS than SA, SAP, and SAS, respectively. Shoot POD activity was significantly lower in SAP, SAS, and SAPS than SA by 59.8%, 54.8%, and 68.5%, respectively (Fig. 2D, $p < 0.05$), in contrast to the SOD, CAT, and APX activities. Root POD activity was significantly lower in SAPS, but not SAP and SAS, than SA. SOD, CAT, and APX activities were higher in shoots than roots in all treatments, but POD activity in SAP and SAPS was higher in roots than shoots.

3.5. Soil microbial biomass and enzymatic activities

MBC content was significantly higher in SAP, SAS, and SAPS than SA by 10.1%, 36.2%, and 65.7%, respectively (Table S4, $p < 0.05$). MBN content was also significantly higher in SAP, SAS, and SAPS than SA, by 1.8-, 3.3-, and 3.7-fold, respectively ($p < 0.05$). MBC and MBN contents were both the highest in SAPS, at 259.15 and 7.05 mg kg^{-1}, respectively.

The relative activities of the soil enzymes (i.e. urease, saccharase, catalase, acid phosphatase, and β-glucosidase) are shown in Table 2. Urease and catalase activities were significantly higher in SAP and SAS than SA ($p < 0.05$). Inoculation with either PGPR or rhizobium, however, did not significantly affect the activities of saccharase, acid phosphatase, or β-glucosidase. The activities of urease, saccharase, catalase, acid phosphatase, and β-glucosidase were 25.0%, 28.3%, 48.7%, 28.5%, and 47.5% higher in SAPS than SA, respectively.

3.6. Structure of the rhizospheric microbial community

Proteobacteria, *Firmicutes*, *Actinobacteria*, and *Acidobacteria* dominated all rhizospheres in the treatments (Fig. 3). Rhizospheric relative abundances differed strongly amongst the treatments. *Proteobacteria* relative abundance was the highest in all treatments. *Acidobacteria*, *Gemmatimonadetes*, and *Chloroflexi* relative abundances were significantly higher in SAP and SAS than SA ($p < 0.05$). *Firmicutes*, *Acidobacteria*, *Gemmatimonadetes*, *Chloroflexi*, and *Cyanobacteria* were significantly more abundant in SAPS than SA, by 188.3%, 39.8%, 163.6%, 30%, and 340.5%, respectively ($p < 0.05$); while *Proteobacteria*, *Actinobacteria*, and *Armatimonadetes* were significantly less abundant in SAPS than SA, by 25.3%, 24%, and 28.8%, respectively ($p < 0.05$). *Rhizobiaceae* relative abundance was the highest in SAS, and *Bacillaceae* relative abundance was the highest in SAPS (Fig. S4A). *Brandyrhizobium*, *Rhizobium*, and *Mesorhizobium* relative abundances were all the highest in SA (Fig. S4B). In contrast, *Ensifer* relative abundance was the highest in SAS. Interestingly, the PD and Shannon indices were significantly higher in SAS and SAP than SA ($p < 0.05$), while were only slightly higher in SAPS than SA (Table S5). Alpha diversity was slightly higher in the inoculated treatments than the uninoculated control. The structures of the rhizospheric microbial communities across all soil samples were identified using PCoA analysis (Fig. S5). The rhizospheric microbial communities were distinctly differentiated between SA and the other treatments (SAP, SAS, and SAPS). The CCA indicated that variations in the compositions of the communities were due mostly (46.27%) to the soil properties (Fig. 4). The CCA also indicated that soil TN ($p < 0.001$), NH_4^+-N ($p < 0.001$), AP ($p < 0.01$), SOM ($p < 0.01$), pH ($p < 0.05$), and NO_3^--N ($p < 0.05$) were the major contributors to the variation in the rhizosphere

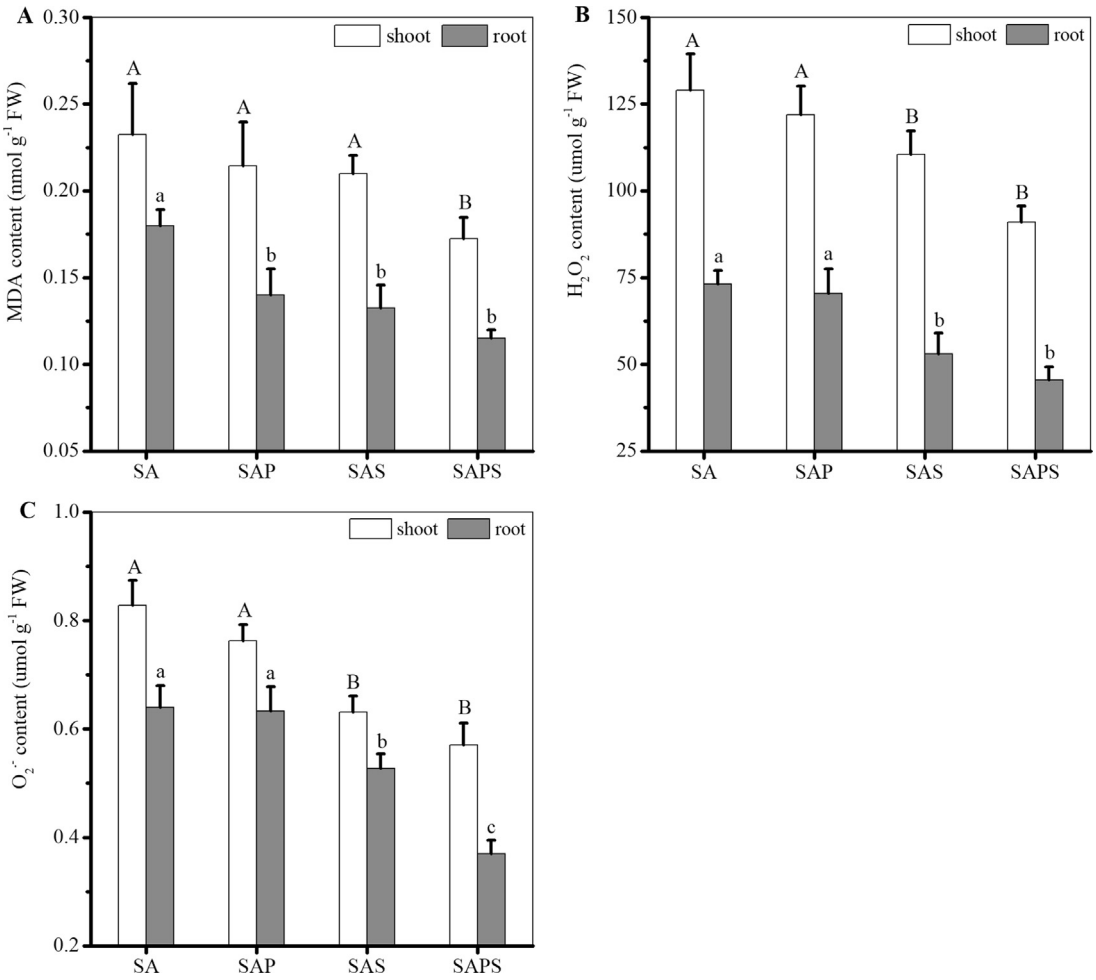

Fig. 1. The MDA (A), H_2O_2 (B) and $O_2^{\bullet -}$ (C) contents of plant shoots and roots in different treatments. SA (soil + alfalfa), SAP (soil + alfalfa + *P. mucilaginosus*), SAS (soil + alfalfa + *S. meliloti*), and SAPS (soil + alfalfa + *P. mucilaginosus* and *S. meliloti*). FW, fresh weight. Data are means ± SEs of three independent replicates. Different letters indicate significant differences ($p < 0.05$) with the LSD test.

microbial communities based on an ANOVA ($p < 0.05$).

4. Discussion

4.1. Role of inoculation with PGPR and rhizobium in alfalfa growth, Cu content and uptake

Plant biomass was higher in the co-inoculation with PGPR and rhizobium than in the uninoculated control and individual inoculations (Table 1). Shoot and root N, P, and K contents were all the highest in SAPS (Table S3). Inoculation with either PGPR or rhizobium had beneficial effects on plant growth in the presence of heavy metals, because PGPR and rhizobium supported rich nutritional sources (Dary et al., 2010; Kong et al., 2017). The effect on plant growth was greater with co-inoculation than with the individual inoculations, perhaps due to the increase in plant growth by a variety of mechanisms, i.e. PGPR can induce rhizobium occupancy in legume nodules (Tilak et al., 2006), or PGPR and rhizobium can provide balanced nutrition to the plants and improve the absorption of N, P, and mineral nutrients (Korir et al., 2017). Our results indicated that inoculation, especially co-inoculation with PGPR and rhizobium, significantly improved the growth of plants in soil contaminated with heavy metals by enhancing plant biomass and nutrition.

Our results also indicated that inoculation with either *P. mucilaginosus* or *S. meliloti* significantly increased the contents and total uptake of Cu in plant shoots and roots (Table 1). This result was consistent with previous reports that inoculation with PGPR and rhizobium had a beneficial effect on the uptake and accumulation of excessive levels of toxic metals by plants (Kong et al., 2015b; Xu et al., 2015). Interestingly, the effect on increasing the total uptake of Cu was greater for the inoculation with rhizobium than the inoculation with PGPR. Pajuelo et al. (2011) concluded that, in addition to N fixation, metal-resistant rhizobium demonstrated the production of plant growth-regulating substances or effects on metal solubility and bioavailability, both of which affect metal uptake. Metal-tolerant PGPRs, however, only assist metal phytoremediation (Ma et al., 2016). Our results also indicated that co-inoculation with PGPR and rhizobium increased total Cu uptake the most, followed by the individual inoculations and the uninoculated control. This result may be attributed to the higher plant biomass and better nutrition in the co-inoculated treatment than the other treatments and to the increased tolerance of co-inoculated plants to Cu stress (Fatnassi et al., 2015). The largest transfer coefficient was considerably less than 1 in the co-inoculation treatment, suggesting that Cu accumulated mainly in the roots and that a very low level of Cu was translocated to shoots, which was beneficial for the phytostabilisation of heavy metals. Metal cations such as Cu^{2+}, though, can bind quite tightly to organic ligands within root cell walls (Kong et al., 2017). In brief, the above results indicated that the alfalfa-rhizobium-PGPR symbiosis could potentially be used for Cu phytostabilisation, which would play an important role in avoiding the leaching and transfer of toxic metals into the food chain and ultimately affecting human health.

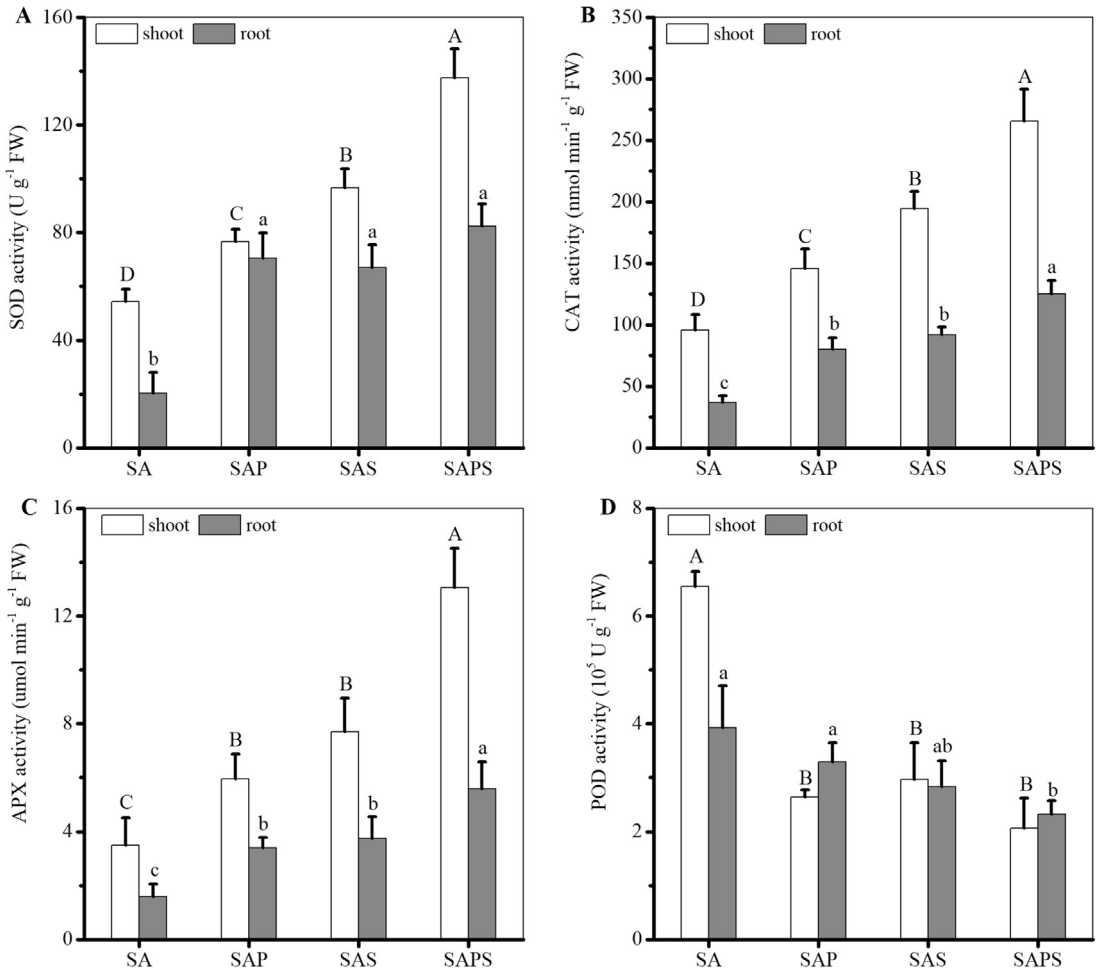

Fig. 2. The antioxidant enzyme of plant shoots and roots in different treatments. SOD (superoxide dismutase) (A), CAT (catalase) (B), APX (ascorbate peroxidase) (C) and POD (peroxidase) (D). SA (soil + alfalfa), SAP (soil + alfalfa + *P. mucilaginosus*), SAS (soil + alfalfa + *S. meliloti*), and SAPS (soil + alfalfa + *P. mucilaginosus* and *S. meliloti*). Data are means ± SEs of three independent replicates. Different letters indicate significant differences ($p < 0.05$) with the LSD test.

4.2. Regulatory mechanisms of PGPR and rhizobium co-inoculation in alleviating Cu stress in alfalfa

Recent studies have reported that plant tolerance was associated with lipid peroxidation and antioxidant enzymatic activity (Chen et al., 2018; Duan et al., 2018). Cu-induced ROS accumulation in the shoots and roots was significantly lower in the treatment with rhizobium inoculation than the uninoculated control (Fig. 1), and PGPR inoculation did not significantly affect ROS accumulation, suggesting that rhizobium inoculation could alleviate the oxidative stress caused by excessive Cu levels through controlling $O_2^{\bullet -}$ and H_2O_2 levels. Earlier studies indicated that the application of exogenous N could relieve oxidative stress in plants induced by heavy metals (Hu et al., 2015; Zhang et al., 2014). A high MDA level from the generation of ROS and subsequent oxidative stress indicated membrane damage due to the peroxidation of polyunsaturated fatty acids (Wen et al., 2011).

MDA content and ROS accumulation in both shoots and roots were the lowest in the co-inoculation with PGPR and rhizobium (Fig. 1), suggesting that plant tissues in the co-inoculation treatment had less damage and higher antioxidant activities. High antioxidant enzymatic activity in plants protects cells from the damage from excessive ROS caused by toxic metal stress (Hojati et al., 2017). The activities of antioxidant enzymes (SOD, CAT, and APX) were significantly higher in the plants inoculated with either PGPR or rhizobium than in uninoculated plants under Cu stress (Fig. 2), thus confirming that plants inoculated with PGPR or rhizobium were adapted to Cu stress by eliminating ROS by altering SOD, CAT, and APX activities. An increase in SOD activity has been attributed to an increase in the concentration of superoxide radicals, because PGPR or rhizobium inoculation can promote the de-novo synthesis of enzymes (Fatima and Ahmad, 2005).

Table 2
Different enzyme activities in soil with different treatments.

Treatments	Urease (mg NH_4^+ -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})
SA	0.16 ± 0.01 b	6.08 ± 0.48 b	2.63 ± 0.06 c	107.5 ± 1.95 b	77.1 ± 0.13 b
SAP	0.20 ± 0.01 a	5.62 ± 0.54 b	3.12 ± 0.05 b	109.4 ± 3.62 b	85.9 ± 4.65 b
SAS	0.19 ± 0.01 a	7.03 ± 0.45 ab	3.23 ± 0.08 b	108.9 ± 2.28 b	91.1 ± 6.14 b
SAPS	0.20 ± 0.01 a	7.80 ± 0.47 a	3.91 ± 0.10 a	138.1 ± 1.64 a	113.7 ± 4.68 a

Note: SA (soil + alfalfa), SAP (soil + alfalfa + *P. mucilaginosus*), SAS (soil + alfalfa + *S. meliloti*), and SAPS (soil + alfalfa + *P. mucilaginosus* and *S. meliloti*). Data are means ± SEs of three independent replicates. Different letters indicate significant differences ($p < 0.05$) with the LSD test.

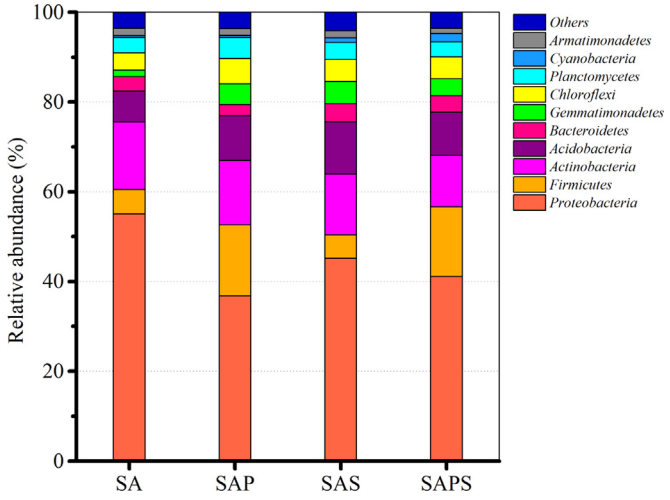

Fig. 3. Relative abundance of the rhizosphere at top 10 phylum level. SA (soil + alfalfa), SAP (soil + alfalfa + *P. mucilaginosus*), SAS (soil + alfalfa + *S. meliloti*), and SAPS (soil + alfalfa + *P. mucilaginosus* and *S. meliloti*).

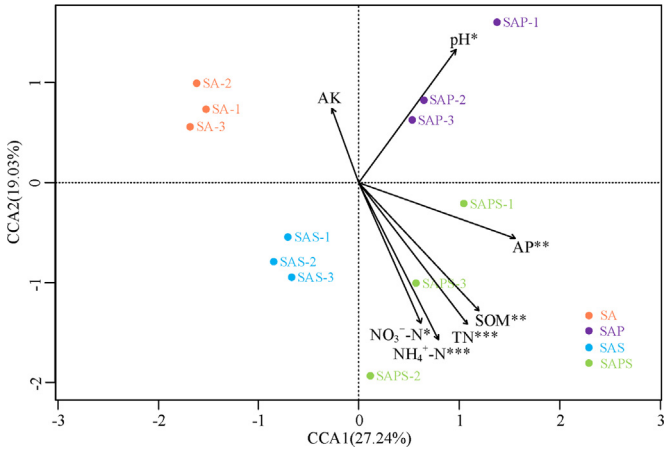

Fig. 4. Canonical correspondence analysis (CCA) used to identify the relationships between soil properties and the composition of the soil bacterial community. SA (soil + alfalfa), SAP (soil + alfalfa + *P. mucilaginosus*), SAS (soil + alfalfa + *S. meliloti*), and SAPS (soil + alfalfa + *P. mucilaginosus* and *S. meliloti*). TN, total nitrogen; AP, available phosphorus; AK, available potassium; SOM, soil organic matter. * **, significant at $p < 0.001$; **, significant at $p < 0.01$; *, significant at $p < 0.05$.

Increases in CAT and APX activities are probably due to the stimulation of the synthesis of these enzymes by bacteria, especially rhizobium (Wang et al., 2010). POD activity, though, was lower in the plants inoculated with PGPR or rhizobium (Fig. 2D). The contrasting responses of POD activity under Cu stress in plant tissues inoculated with PGPR or rhizobium indicated that different mechanisms may be involved in the defence against oxidative stress (Duan et al., 2018). SOD, CAT, and APX activities were all the highest in the treatment with *P. mucilaginosus* and *S. meliloti* co-inoculation, suggesting that co-inoculation could substantially alleviate the adverse effects of metal stress on plant growth by increasing the activities of antioxidant enzymes. SOD, CAT, and APX activities were higher in the co-inoculation than the individual inoculations, likely because *P. mucilaginosus* and *S. meliloti* can establish an effective symbiotic system in legumes (Kong et al., 2017). Our results were also supported by Fatnassi et al. (2015), who reported increased activities of various ROS-scavenging enzymes in *Vicia faba* under Cu stress inoculated with the PGPRs *Enterobacter cloacae*, *Pseudomonas* sp., and *Rhizobium*.

The tolerance of plants under stress environments is also affected by soil biological activity, which in turn is influenced by soil microbial biomass, soil enzymatic activities, and microbial communities (Elleuch et al., 2013; Islam et al., 2016). Our results indicated a significant increase in soil MBC and MBN contents for inoculation with either *P. mucilaginosus* or *S. meliloti* relative to the uninoculated control (Table S4), suggesting that inoculation with PGPR or rhizobium could improve soil quality by increasing soil MBC and MBN contents. MBC and MBN contents were the highest for the co-inoculation with PGPR and rhizobium, perhaps due to the larger crop biomass after co-inoculation, which would lead to the return of more organic residues and exudates to the soil, thereby accelerating microbial biomass accumulation and activity (Saini et al., 2004). Inoculation with either *P. mucilaginosus* or *S. meliloti* in our study slightly increased soil urease, saccharase, catalase, acid phosphatase, and β-glucosidase activities relative to the uninoculated control, but co-inoculation significantly increased the activities of all five enzymes (Table 2), suggesting that co-inoculation could increase the activities of soil enzymes, which has recently been reported (Arif et al., 2016; Islam et al., 2016). The activities of soil saccharase, catalase, acid phosphatase, and β-glucosidase were significantly positively correlated with N content and microbial biomass (Fig. S6). Soil urease activity was also significantly positively correlated with TN and SOM contents. The increase in the enzymatic activities may therefore be associated with shifts in rhizospheric microbial activity and soil physical and chemical properties from the increase in N content and mitigation of soil toxicity by PGPR and rhizobium co-inoculation (Sipahutar et al., 2018).

Our results indicated specific bacterial compositions at the phylum level. *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Firmicutes* were the four dominant phyla (Fig. 3). Rhizospheric microbial relative abundance differed strongly amongst the treatments. Co-inoculation with PGPR and rhizobium affected rhizospheric microbial community structures (Fig. S5) and slightly increased alpha diversity compared to the uninoculated control (Table S5). The relative abundances of *Rhizobiaceae* and *Bacillaceae* were significantly higher for the inoculation with *S. meliloti* and *P. mucilaginosus*, respectively, than the uninoculated soil (Fig. S4), suggesting that competition with the indigenous soil microflora did not affect the functioning of the applied bacteria. The relative abundance of *Bacillaceae* was the highest in the PGPR and rhizobium co-inoculated treatment, and the higher *Bacillaceae* abundance would contribute to the soil AP content (Li and Wong, 2012). The relative abundances of *Brandyrhizobium*, *Mesorhizobium*, and *Rhizobium* were higher (Fig. S4), perhaps due to plant-microbe interactions that could alleviate Cu toxicity in the rhizospheres (Fatnassi et al., 2015; Kong et al., 2015b). The correlation analysis between soil nutrient contents and rhizobia also indicated that the relative abundances of *Brandyrhizobium*, *Mesorhizobium*, and *Rhizobium* were significantly correlated with soil TN and SOM contents (Table S6). These results suggested that the changes of soil biochemical properties have been significantly affected by co-inoculation with PGPR and rhizobium, which may contribute to their metabolic activities, their interaction with alfalfa, or an interaction of PGPR with rhizobium (Elleuch et al., 2013; Kalam et al., 2017; Thokchom et al., 2017). Soil TN, NH_4^+-N, AP, SOM, and NO_3^--N contents and pH were the main factors determining the composition of the soil microbial communities (Fig. 4). Therefore, we could regulate soil microbial activity in Cu contaminated soil through altering the above soil properties, and the co-inoculation of PGPR and rhizobium had positive effects on improving soil properties.

The alleviation of Cu stress in alfalfa by co-inoculation with PGPR and rhizobium may have two potential mechanisms. The higher activities of the antioxidant enzymes in the treatment with PGPR and rhizobium co-inoculation may have decreased the level of tissue damage. Alternatively, co-inoculation may have changed the community structure and diversity of the soil microorganisms by improving soil properties and increasing microbial biomass and enzymatic activities. These mechanisms imply that inoculation with PGPR and rhizobium, especially co-inoculation, has a positive effect on alleviating Cu stress in

alfalfa.

5. Conclusions

Our study demonstrated a positive effect of co-inoculation with the PGPR *P. mucilaginosus* and the rhizobium *S. meliloti* on Cu uptake in alfalfa by modulating the biochemical responses of the alfalfa-soil system in soil contaminated with heavy metals. Co-inoculation with PGPR and rhizobium significantly reduced Cu-induced damage to alfalfa by mitigating ROS accumulation and lipid peroxidation. Co-inoculation also substantially improved antioxidant capacity and alfalfa growth and increased soil TN, AP, SOM, microbial biomass contents, enzymatic activities, and microbial diversity. These improvements of the properties and fertility of polluted soil may also have a potential impact on Cu uptake in alfalfa. These results will improve our understanding of the mechanism of microbial co-inoculation to alleviate Cu toxicity and suggest some innovative approaches to the phytomanagement of metal-contaminated land around the world.

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Supplemental information

The supplementary information provides additional tables and figures showing the results of soil physicochemical properties and soil microbial biomass, the nutrients content in plant tissues, soil rhizospheric microbial alpha diversity indexes and beta diversity among different treatments, Pearson correlation coefficients relating soil physicochemical properties to relative abundance of bacterial groups and soil enzyme activities, the map of sampling area, and plant growth phenotype.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.10.016.

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