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Co-inoculation effect of plant-growth-promoting rhizobacteria and rhizobium on EDDS assisted phytoremediation of Cu contaminated soils

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HIGHLIGHTS

- Rhizobacteria inoculation benefits the EDDS-enhanced phytoextraction of Cu.
- Plant tolerance was increased in the PGPR and rhizobium co-inoculated treatment.
- Microbial biomass and alpha diversity were increased in the co-inoculated soil.
- Co-inoculation can increase soil nutrients and reduce environmental risks.

GRAPHICAL ABSTRACT

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ABSTRACT

Chelants application can increase the bioavailability of metals, subsequently limiting plant growth and reducing the efficiency of phytoremediation. Plant growth-promoting rhizobacteria (PGPRs) and rhizobium have substantial potential to improve plant growth and plant tolerance to metal stress. We evaluated the effects of co-inoculation with a PGPR strain (*Paenibacillus mucilaginosus*) and a Cu-resistant rhizobium strain (*Sinorhizobium meliloti*) on the efficiency of biodegradable chelant (S,S-ethylenediaminedisuccinic acid; EDDS) assisted phytoremediation of a Cu contaminated soil using alfalfa. The highest total Cu extraction by alfalfa was observed in the EDDS-treated soil upon co-inoculation with the PGPR and rhizobium strains, which was 1.2 times higher than that without co-inoculation. Partial least squares path modeling identified plant oxidative damage and soil microbial biomass as the key variables influencing Cu uptake by alfalfa roots. Co-inoculation significantly reduced the oxidative damage to alfalfa by mitigating the accumulation of malondialdehyde and reactive oxygen species, and improving the antioxidant capacity of the plant in the presence of EDDS. EDDS application decreased microbial diversity in the rhizosphere, whereas co-inoculation increased microbial biomass carbon and nitrogen, and microbial community diversity. Increased relative abundances of *Actinobacteria* and *Bacillus* and the presence of *Firmicutes* taxa as potential biomarkers demonstrated that co-inoculation increased soil

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nutrient content, and improved plant growth. Co-inoculation with PGPR and rhizobium can be useful for altering plant–soil biochemical responses during EDDS-enhanced phytoremediation to alleviate phytotoxicity of heavy metals and improve soil biochemical activities. This study provides an effective strategy for improving phytoremediation efficiency and soil quality during chelant assisted phytoremediation of metal-contaminated soils.

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1. Introduction

Toxic heavy metals present in agricultural ecosystems are difficult to degrade, and thus, they can enter the food chain and be ingested by humans (Antoniadis et al., 2017; Shahid et al., 2017b). Chelant-enhanced phytoremediation, a technique that increases the solubility of toxic metals in soils and facilitates phytoextraction efficiency, has received increased interest in recent decades as a promising technology for the remediation of soils contaminated with toxic heavy metals (Luo et al., 2018; Tsang et al., 2012). S,S-ethylenediaminedisuccinic acid (EDDS), a strong and easily biodegradable chelating agent, is less toxic to plants and microorganisms than the more traditionally used ethylenediaminetetraacetic acid (Beiyuan et al., 2018b; Prieto et al., 2013; Tsang et al., 2009). Moreover, EDDS has the potential to release nutrients and subsequently improve phytoextraction efficiency (Fang et al., 2017). Therefore, EDDS is considered one of the most efficient chelants and has been widely used in metal-contaminated soils.

However, EDDS can increase the bioavailability of Cu in soils (Beiyuan et al., 2018a; Luo et al., 2015) and high Cu phytotoxicity can directly and indirectly induce oxidative injury to and metabolic perturbations in plant tissue (Mondaca et al., 2017). Moreover, the high phytotoxicity of metals can reduce plant biomass yield; thus, limit the efficiency of heavy metal phytoremediation (Antoniadis et al., 2017). The application of EDDS has been shown to decrease the biomass of plants grown in Pb-contaminated soils and the decrease is more pronounced at a high chelate dose (10 mmol kg⁻¹ soil) (Zhao et al., 2016). EDDS can also increase the accumulation of metals in plant tissue, which subsequently induces oxidative damage by increasing the level of reactive oxygen species (ROS) and lipid peroxidation (Cestone et al., 2012; Sidhu et al., 2018). As the main source of ROS, hydrogen peroxide (H₂O₂) and superoxide radicals (O₂^{•-}) can damage DNA, proteins, lipids, and other cellular components in plant tissues (Woodson et al., 2015). An increase in the level of malondialdehyde (MDA) generally indicates lipid peroxidation in metal-treated plants. Antioxidant enzymes can reduce oxidative damage in plants and alleviate phytotoxicity induced by excessive ROS production (Shahid et al., 2017b).

In addition, high concentrations of toxic metals in soil are detrimental to plant biomass generation, owing to a low content of nutrients (Wilson-Kokes and Skousen, 2014) and the impact on soil biological activities (Fang et al., 2017). Chelant application can result in high heavy metal concentrations in soils, which further influences soil biological activities and the microbial community composition (Asadishad et al., 2018; Beiyuan et al., 2018a). Soil enzyme activities are vital for promoting nutrient cycling and plant growth (Zhang et al., 2019) and can be indicative of heavy metal pollution (Duan et al., 2019; Yang et al., 2013). The rhizosphere, which is colonized by microbiota, is central to microbial activity, contributing to nutrient transformation, phytoremediation, and plant growth (Lundberg et al., 2012; Yergeau et al., 2014). High metal concentrations can inhibit soil enzyme activities by reacting with enzyme–substrate complexes and destroying protein activity (Belyaeva et al., 2005), which can subsequently affect specific

biological processes in soil. Previous studies have shown that the application of EDDS in Cu-contaminated soils resulted in both negative and positive impacts on the diversity of rhizosphere microbial communities and enzyme activities (Yang et al., 2013; Yoo et al., 2018). Low enzyme activity, as well as reduced rhizosphere microbial activity, can limit soil nutrient cycling and plant growth and further reduce the phytoextraction capacity. Thus, it is important for effective chelant-enhanced phytoremediation to increase plant growth, the nutrient content, and the biological activity in soil.

Plant growth-promoting rhizobacteria (PGPRs) and rhizobium are used as supplements for metal phytoremediation, as they can substantially alleviate phytotoxicity and improve soil biological activities under toxic metal stress (Antoniadis et al., 2019; Sipahutar et al., 2018). PGPRs and rhizobium decrease oxidative damage to plant tissues that are exposed to heavy metal stress by enhancing antioxidant enzymatic systems (Ju et al., 2019). Additionally, PGPRs can indirectly increase plant growth by improving soil properties and biological activities (Etesami, 2018). A previous study showed that the growth-promoting phytohormone auxin (indole-3-acetic acid) alleviated the toxic effects of Pb and Zn on the growth of sunflower and increased, in combination with EDDS, the phytoextraction potential of sunflower tissue (Faessler et al., 2010). Co-application of a PGPR (*Pseudomonas* sp. DGS6) and EDDS has been shown to improve plant growth and increase Cu and Zn uptake by plants (Luo et al., 2015). Symbiotic relationships with metal-resistant rhizobium may improve the phytoremediation ability of legumes through various mechanisms, such as increasing the metal bioavailability and plant metal resistance, which increase the accumulation of heavy metals in plant tissues (Teng et al., 2015). Metal-resistant rhizobia can improve plant growth and affect the bioavailability of toxic metals in response to the addition of nitrogen (Pajuelo et al., 2011). PGPRs can increase N availability to plants by mineralizing organic N in soil (Etesami, 2018; Shahzad et al., 2014), and the degradation of EDDS can also provide a source of N (Fang et al., 2017). The exploration of synergistic plant–microbe–chelant relationships may be an effective strategy for enhancing phytoremediation efficiency (Hussain et al., 2019). Our previous studies have shown that rhizobacterial inoculation increased soil enzymatic activities and microbial diversity (Ju et al., 2019, 2020). Therefore, the combination of legume–rhizobium symbiosis and PGPRs may potentially contribute to the improvement of plant growth and soil biological activities. However, it remains unclear whether co-inoculation of PGPRs and rhizobium can regulate plant tolerance and soil quality in response to metal stress during chelant assisted phytoremediation.

Based on previous data, it is reasonable to expect that co-inoculation with PGPRs and rhizobium will alleviate Cu phytotoxicity, alter soil biological parameters, and affect the phytoremediation process in chelant-supplemented metal-contaminated soils. Alfalfa (*Medicago sativa*), a legume, has been used in phytoremediation studies because it is a fast-growing perennial plant, which is able to develop an extensive root system that serves as a niche for rhizosphere microorganisms (Ju et al., 2019; Pajuelo et al.,

2011). Therefore, the PGPR *Paenibacillus mucilaginosus* and rhizobium *Sinorhizobium meliloti* were co-inoculated during EDDS assisted phytoremediation of a Cu-contaminated soil using alfalfa. The aim of this study was to (1) investigate the effect of rhizobacterial inoculation on the efficiency of EDDS-enhanced phytoextraction of Cu; and (2) reveal the underlying mechanisms of the positive effects of co-inoculation on plant Cu tolerance and soil quality during EDDS-enhanced phytoremediation. We anticipate that the results will provide novel insights into the effects of rhizobacterial inoculation on plant growth and soil quality during chelant assisted phytoremediation and establish an efficient phytoremediation strategy.

2. Materials and methods

2.1. Experimental design

Soil samples were collected from the surface (0–20 cm) of a Cu smelter area in Huangshi City (30°43' N, 114°54' E), Hubei Province, China. After being air-dried, the samples were passed through a 2-mm sieve. The physicochemical properties of the samples, showing that Cu was the major contaminant, are presented in Table S1. A PGPR strain (*P. mucilaginosus* ACCC10013) and a Cu-resistant rhizobium strain (*S. meliloti* CCNWSX0020) (Ju et al., 2019) were used as experimental strains. *P. mucilaginosus* was grown at 30 °C with shaking at 180 rpm in an optimized liquid medium, containing (L^{-1}) $MgSO_4 \cdot 7H_2O$, 0.73 g; $K_2HPO_4 \cdot 3H_2O$, 0.4 g; NaCl, 0.06 g; $FeCl_3$, 0.6 mg; $CaCO_3$, 1 g; maltose, 2.5 g; tryptone, 1 g; and salicylic acid, 10 mg. *S. meliloti* was grown at 28 °C with shaking at 150 rpm in tryptone/yeast extract liquid medium, containing (L^{-1}) $CaCl_2 \cdot 2H_2O$, 0.7 g; tryptone, 5 g; and yeast extract, 3 g. Bacterial cells were then harvested by centrifugation ($8000 \times g$ at 4 °C for 10 min), and the pellet was immediately washed three times with phosphate buffer (pH 7.0). Bacterial suspensions were prepared in a 0.85% sterile NaCl solution and adjusted to an optical density of 0.8 at 600 nm using an ultraviolet spectrophotometer (Shimadzu UVmini-1240; Kyoto, Japan).

The soil samples (1.30 kg) were placed into 15 plastic pots. Seeds of alfalfa (Beijing Rytway Ecotechnology Co., Ltd.) were sterilized in a 20% sodium hypochlorite solution for 10 min, followed by washing five times with deionized water. Approximately 20 pre-germinated seedlings were subsequently grown in each pot, with soil moisture maintained at a water-holding capacity, close to field conditions (~70%), by adding deionized water on a daily basis to ensure the optimal growth conditions. For the inoculation treatments, PGPR and rhizobium suspensions (40 mL) were added once a week for 3 weeks to the soil surface in the pots after the emergence of the first leaf (Ju et al., 2019). An equivalent amount of deionized water was sprayed onto the soil for the uninoculated treatments. In accordance with previous studies (Luo et al., 2006, 2015) and our preliminary experiment, EDDS (Fluka Chemie GmbH; Buchs, Switzerland) was added to the soil surface at a dose of 5 mmol kg^{-1} soil on day 54, after the seedlings grew. The following five treatments ($n = 3$ each) were used in this study: 1) alfalfa control (A); 2) alfalfa + EDDS (AE); 3) alfalfa + *P. mucilaginosus* + EDDS (APE); 4) alfalfa + *S. meliloti* + EDDS (ASE); and 5) alfalfa + *P. mucilaginosus* + *S. meliloti* + EDDS (APSE). Ninety days after planting, plant and soil samples were collected for the analysis of various parameters, including plant nutrients, MDA and ROS content, antioxidant enzymatic activities, soil properties, enzymes,

microbial biomass, and compositions of microbial communities.

2.2. Determination of soil properties and plant Cu and nutrient content

Soil physicochemical properties were analyzed by the method of Lu (2000). Soil pH was determined in a 1:2.5 (w/v) soil/water suspension using a pH meter (model 225; Denver Instrument, Denver, CO, USA). The soil organic carbon (SOC) content was analyzed using a potassium dichromate external heating method. Total nitrogen (TN) was determined using the Kjeldahl method. Available N (NH_4^+-N and NO_3^--N) was determined using a Seal autoanalyzer (Auto Analyzer 3-AA3; SEAL, Germany). Available potassium (AK) was extracted with a 1.0 mol L^{-1} CH_3COONH_4 solution and determined by ICP-AES (Optima 3300DV; PerkinElmer, Waltham, MA, USA). Air-dried soil samples were digested with strong acid (concentrated $HNO_3/HClO_4$, 4:1, v/v) for measuring heavy metal content by flame atomic absorption spectrophotometry (PinAAcle 900F; PerkinElmer, Germany).

Plant organs (shoots and roots) were washed five times with deionized water and then dried at 65 °C for 48 h to determine their Cu content. The total Cu content was quantified in the dried plant samples using flame atomic absorption spectrophotometry after acid digestion with 10 mL of concentrated $HClO_4/HNO_3$ (1:4, v/v) (Luo et al., 2005). Dried plant samples (~0.100 g) were digested with H_2O_2 and H_2SO_4 to determine the N, P, and K content. The N content was measured using an injection pump analyzer, as described by Duan et al. (2019). The P and K content was determined using an ammonium molybdate colorimetric method at 450 nm (UV3200; Shimadzu Corporation, Japan) and flame atomic absorption spectrophotometry, respectively.

2.3. Determination of plant biochemical properties and soil enzymes and microbial biomass

Fresh plant organs (whole shoot and root) were used to determine the plant physiological parameters including MDA, ROS content, and antioxidant enzymatic activities (Ju et al., 2019). The $O_2^{\cdot-}$ content was determined via extraction with a phosphate buffer followed by centrifugation at 12000g for 20 min at 4 °C; an $O_2^{\cdot-}$ reagent kit (Suzhou Comin Biotechnology Co., Ltd., Suzhou, China) was used thereafter. For the determination of MDA and H_2O_2 contents, plant organs were extracted by 10% trichloroacetic acid, then centrifuged at 8000 g for 10 min at 4 °C. The MDA and H_2O_2 contents were subsequently measured using MDA and H_2O_2 reagent kits (Suzhou Comin Biotechnology Co., Ltd.), respectively. Fresh shoots and roots were homogenized in an ice bath using 1 mL of extraction buffer (50 mM phosphate buffer solution containing 1 mM EDTA and 1 mM ascorbic acid) at 4 °C. The homogenate was centrifuged at 15000 g for 15 min at 4 °C, and the supernatant was used for assaying antioxidant enzymatic activities. The activities of total peroxidase (POD, EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), and catalase (CAT, EC 1.11.1.6) were correspondingly analyzed using commercial enzyme-specific reagent kits.

Soil saccharase (EC 3.2.1.26) and urease (EC 3.5.1.5) activities were analyzed by using sucrose and urea as the substrates and measuring with a spectrophotometer (UV3200; Shimadzu Corporation, Japan) at 508 and 587 nm, respectively (Guan et al., 1986). Acid phosphatase (EC 3.1.3.2) and β -glucosidase (EC 3.2.1.21) assays were quantified at 400 nm based on the release of *p*-nitrophenol.

Table 1
Plant biomass and Cu phytoextraction.

Treatments	Biomass (g per pot)		Concentration (mg kg ⁻¹)		TF		BCF		Total uptake (μg pot ⁻¹)		TE (μg per pot)
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	
Cu											
A	7.00 ± 0.19 ab	7.02 ± 0.33 a	14.02 ± 0.44 a	53.7 ± 2.21 d	0.49 ± 0.01 d	0.039 ± 0.001 c	0.039 ± 0.001 c	0.080 ± 0.002 d	182 ± 4.0 c	378 ± 25 ab	560 ± 29 d
AE	5.83 ± 0.17 c	2.99 ± 0.24 b	8.82 ± 0.29 c	85.0 ± 4.17 a	1.14 ± 0.05 a	0.132 ± 0.003 a	0.132 ± 0.003 a	0.115 ± 0.002 c	499 ± 7.4 a	241 ± 8.8 d	741 ± 15 c
APE	6.10 ± 0.46 bc	3.99 ± 0.45 b	10.1 ± 0.80 bc	69.9 ± 2.54 b	0.88 ± 0.02 b	0.108 ± 0.002 b	0.108 ± 0.002 b	0.122 ± 0.002 c	463 ± 7.1 b	355 ± 7.3 bc	819 ± 13 b
ASE	6.44 ± 0.38 abc	3.25 ± 0.46 b	9.69 ± 0.62 bc	68.5 ± 2.87 b	0.82 ± 0.03 b	0.109 ± 0.001 b	0.109 ± 0.001 b	0.132 ± 0.004 b	468 ± 2.6 b	319 ± 11 c	787 ± 9.4 bc
APSE	7.16 ± 0.14 a	4.15 ± 0.26 b	11.1 ± 0.34 b	62.6 ± 1.92 c	0.67 ± 0.02 c	0.105 ± 0.002 b	0.105 ± 0.002 b	0.156 ± 0.005 a	464 ± 8.5 b	408 ± 8.6 a	872 ± 8.9 a

Note: TF: translocation factor; BCF: bioconcentration factor; TE: total metal extraction by plants. A (soil + alfalfa), AE (soil + alfalfa + *P. mucilaginosus* + EDDS), ASE (soil + alfalfa + *S. melliloti* + EDDS), and APSE (soil + alfalfa + *P. mucilaginosus* and *S. melliloti* + EDDS). The total biomass is shoot biomass plus roots biomass. Values are the means ± standard errors (n = 3). Different letters stand for significant difference ($P < 0.05$) with the LSD test.

The method of potassium permanganate titration was used to analyze the activity of catalase (EC 1.11.1.6). The method of chloroform fumigation-extraction was used to analyze soil microbial biomass C (MBC) (Vance et al., 1987) and microbial biomass N (MBN) (Brookes et al., 1985).

2.4. DNA extraction and high-throughput sequencing

DNA was extracted from 0.5 g of soil using the Fast DNA SPIN Kit (MP Biomedicals, Cleveland, USA). The concentration and purity of the extracted DNA were assessed using an automatic microplate reader (BioTek ELX 800, USA). The universal primer set 515F (5'-GTGCCAGCMGCCGCGTAA-3') and 907R (5'-CCGTCATTCTTTGAGTTT-3') were used to amplify the V4–V5 region of the 16S rRNA genes (Kleinstuber et al., 2006), and a 12-bp barcode was attached to the reverse primer. Each PCR mixture (30 μL) comprised 15 μL of Phusion High-Fidelity PCR Master Mix with GC Buffer (New England Biolabs), 3 μL of 2-μM primers, 10 μL of 10 ng μL⁻¹ gDNA, and 2 μL of sterile ultrapure water. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR amplification was verified in triplicate and the products were mixed in equal proportions for each subsample. The GeneJET Gel Extraction Kit (Thermo Scientific, USA) was used to purify the composite DNA, which was sequenced using Illumina MiSeq platform (Illumina Corporation, USA) (Caporaso et al., 2012).

2.5. Statistical analysis

Bioconcentration factor (BCF), translocation factor (TF), and total metal extraction by plants (TE) were used in this study to evaluate the metal accumulation, transfer, and extraction capacity (Luo et al., 2018, 2019; Zhang et al., 2019) of the alfalfa as follow:

BCF = metal concentration in the plant/metal concentration in the soil;

TF = metal concentration in the shoot/metal concentration in the root;

TE = (metal concentration in the shoot × shoot biomass) + (metal concentration in the root × root biomass).

All statistical analyses were performed using R software v.3.5.2. Values are expressed as means ± standard errors (n = 3). A one-way analysis of variation (ANOVA) and a least significant difference multiple comparison ($P < 0.05$) were used to assess the significance of differences amongst the treatments. Chord plots showing the composition and abundance of microbial communities were constructed using the “circlize” package. Principal coordinates analysis (PCoA) based on Bray-Curtis distances was used to reveal the beta-diversity of the microbial communities. A canonical correspondence analysis (CCA) was used to identify the key factors affecting the microbial community structure. The PCoA and CCA used the “Vegan” package. Indicator species analysis was used to reveal different taxonomic levels (from phylum to family levels), which was conducted using the “multipatt” function of the “indicspecies” package. Linear discriminant analysis (LDA) effect size (LEfSe) was performed to investigate potential biomarkers (across five taxonomic levels, from phylum to genus for bacterial community) within soil microbiomes, based on $P < 0.05$ and an LDA threshold score of 3.5 using the “MASS” package. To identify the most important variables explaining the variation of Cu uptake in shoots and roots, we determined the relative importance of predictor variables in linear models using the “relaimpo” package. Additionally, we used the “innerplot” function of the “plspm” package to build the partial least squares path modeling

Table 2
Soil physicochemical properties, enzyme activities and microbial biomasses.

Treatments	pH	NH ₄ ⁺ -N (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	TN (g kg ⁻¹)	AK (mg kg ⁻¹)	SOC (g kg ⁻¹)	Total Cu (mg kg ⁻¹)	Urease (mg NH ₄ ⁺ -N g ⁻¹ 24h ⁻¹)	Saccharase (mg glucose g ⁻¹ 24h ⁻¹)	Catalase (mg K ₂ MnO 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	5.83 ± 0.08	3.99 ± 0.06	4.89 ± 0.09	1.38 ± 0.02	80.8 ± 3.06	16.9 ± 0.10	669 ± 3.1	0.16 ± 0.01	6.08 ± 0.48	3.63 ± 0.25	111 ± 3.3	77.1 ± 1.7	165 ± 4.5	2.10 ± 0.33
AE	6.55 ± 0.03	10.8 ± 0.18	5.79 ± 0.19	1.35 ± 0.03	72.8 ± 3.26	15.9 ± 1.50	645 ± 7.6	0.16 ± 0.00	7.91 ± 1.54	6.71 ± 1.40	99.1 ± 4.7	88.9 ± 6.2	161 ± 4.2	5.65 ± 1.34
APE	6.51 ± 0.06	11.3 ± 0.81	5.67 ± 0.28	1.39 ± 0.01	78.7 ± 3.42	16.3 ± 0.78	650 ± 6.7	0.15 ± 0.01	7.47 ± 0.91	4.63 ± 0.37	102 ± 1.7	89.4 ± 5.6	180 ± 7.4	5.99 ± 0.52
ASE	6.46 ± 0.07	18.4 ± 0.38	6.49 ± 0.12	1.38 ± 0.01	76.6 ± 4.26	16.6 ± 0.47	625 ± 5.6	0.17 ± 0.01	8.87 ± 1.31	4.38 ± 0.23	100 ± 2.1	99.4 ± 8.4	194 ± 5.1	7.35 ± 1.05
APSE	6.50 ± 0.09	19.3 ± 0.22	7.75 ± 0.24	1.40 ± 0.01	91.4 ± 6.24	17.1 ± 0.89	604 ± 3.1	0.17 ± 0.01	8.88 ± 1.06	3.79 ± 0.15	117 ± 2.5	107 ± 9.8	214 ± 7.6	11.4 ± 1.03

Note: TN, total nitrogen; AK, available potassium; SOC, soil organic carbon. A (soil + alfalfa), AE (soil + alfalfa + *P. mucilaginosus* + EDDS), APE (soil + alfalfa + *S. meliloti* + EDDS), and APSE (soil + alfalfa + *P. mucilaginosus* and *S. meliloti* + EDDS). Values are the means ± standard errors (n = 3). Different letters stand for significant difference ($P < 0.05$) with the LSD test.

(PLS-PM) to reveal the possible pathways by which predictor variables controlled the Cu uptake.

3. Results

3.1. Plant growth and soil physicochemical properties

The shoot and root biomass was 16.7% and 57.5% lower in the AE treatment than in the control (A), respectively (Table 1). However, the shoot, root, and total biomass was 22.8%, 38.8%, and 25.9% higher in the APSE treatment than in the AE treatment, respectively. The nutrient content in the shoots and roots was also higher in the APSE treatment than in the AE treatment (Table S2). The soil pH and available N were significantly higher and the total Cu content was significantly lower in the AE treatment than in the control ($P < 0.05$; Table 2). Inoculation with *P. mucilaginosus* did not significantly affect the physicochemical properties of the soil, whereas inoculation with *S. meliloti* increased the available N content. The highest NH₄⁺-N (19.3 mg kg⁻¹), NO₃⁻-N (7.75 mg kg⁻¹), TN (1.40 g kg⁻¹), AK (91.4 mg kg⁻¹), and SOC (17.1 g kg⁻¹) content and the lowest total Cu content (604 mg kg⁻¹) were found in the APSE treatment.

3.2. Cu content and phytoextraction

The Cu content in the shoots and roots was 3.3 and 1.4 times higher after the AE treatment than in the control, respectively (Table 1). The Cu content in the roots was 7.67%, 14.1%, and 33.9% higher and that in the shoots was 17.8%, 19.4%, and 26.4% lower in the APE, ASE, and APSE treatments than in the AE treatment, respectively. The translocation factor (TF) values for the AE, APE, ASE, and APSE treatments were 2.3, 1.8, 1.7, and 1.4 times higher than those for the control, respectively. The highest bio-concentration factor (BCF) values in the shoots and roots were found in the AE and APSE treatments. The total Cu uptake to the shoots and roots was 2.7 times higher and 1.6 times lower in the AE treatment than in the control, respectively. The total Cu uptake to the roots was 47.3%, 32.4%, and 69.3% higher and that to the shoots was 7.21%, 6.21%, and 7.01% lower in the APE, ASE, and APSE treatments than in the AE treatment, respectively. The highest total metal extraction by plants (TE) value was observed in the APSE treatment (872 μg per pot), which was 1.6, 1.2, 1.1, and 1.1 times higher than that in the A, AE, APE, and ASE treatments, respectively.

3.3. Plant MDA, O₂^{•-}, and H₂O₂ content and antioxidant enzymatic activities

The application of EDDS increased the MDA, O₂^{•-}, and H₂O₂ content in plant organs (Fig. 1a–c). The MDA content in the APSE treatment was significantly lower (by 27.3% in the shoots and 33.3% in roots) than that in the AE treatment ($P < 0.05$; Fig. 1a). The O₂^{•-} content in the shoots and roots was significantly lower in the APSE treatment (by 23.7% and 27.1%, respectively; Fig. 1b) than in the AE treatment. The H₂O₂ content in the shoots was 13.7%, 23.6%, and 39.5% lower and that in the roots was 11.8%, 15.1%, and 29.5% lower in the APE, ASE, and APSE treatments than in the AE treatment, respectively (Fig. 1c). Notably, the lowest MDA, O₂^{•-}, and H₂O₂ content was observed in the APSE treatment.

The SOD activity in the shoots and roots was not significantly affected by the application of EDDS (Fig. 1d). However, compared with that in the AE treatment, the SOD activity in the shoots was 9.80%, 67.7%, and 98.3% higher and that in the roots was 56.3%, 111%, and 357% higher in the APE, ASE, and APSE treatments, respectively. The application of EDDS significantly lowered the

Fig. 1. Plant oxidative damage indicators and antioxidant enzymatic activities. Oxidative damage indicators including: MDA, malondialdehyde (a); O₂⁻, superoxide radicals (b); and H₂O₂, hydrogen peroxide (c). Antioxidant enzymatic activities including: SOD, superoxide dismutase (d); CAT, catalase (e); APX, ascorbate peroxidase (f); and POD, peroxidase (g). A (soil + alfalfa), AE (soil + alfalfa + EDDS), APE (soil + alfalfa + *P. mucilaginosus* + EDDS), ASE (soil + alfalfa + *S. meliloti* + EDDS), and APSE (soil + alfalfa + *P. mucilaginosus* and *S. meliloti* + EDDS). FW: fresh weight. Values are the means ± standard errors (n = 3). Different letters stand for significant difference ($P < 0.05$) with the LSD test.

Fig. 2. Composition and abundance of the rhizosphere at top 10 phylum (a) and genus (b) levels. A (soil + alfalfa), AE (soil + alfalfa + EDDS), APE (soil + alfalfa + *P. mucilaginosus* + EDDS), ASE (soil + alfalfa + *S. meliloti* + EDDS), and APSE (soil + alfalfa + *P. mucilaginosus* and *S. meliloti* + EDDS).

activity of CAT in plant organs compared to that of the control (Fig. 1e). Additionally, the CAT activity in the shoots was significantly lower in the APE, ASE, and APSE treatments than in the AE treatment (by 65.8%, 48.7%, and 44.7%, respectively). However, the CAT activity in the roots was significantly higher in the APE, ASE,

and APSE treatments than in the AE treatment (by 124%, 259%, and 210%, respectively). The APX activity in the shoots and roots did not significantly differ among the five treatments (Fig. 1f). The application of EDDS significantly decreased the activity of POD in the shoots compared to that of the control (Fig. 1g). Furthermore, the

Fig. 3. Linear discriminant analysis effect size (LEfSe) results revealed protist biomarkers (from phylum level to genus level) of bacterial communities. A (soil + alfalfa), AE (soil + alfalfa + EDDS), APE (soil + alfalfa + *P. mucilaginosus* + EDDS), ASE (soil + alfalfa + *S. meliloti* + EDDS), and APSE (soil + alfalfa + *P. mucilaginosus* and *S. meliloti* + EDDS).

Fig. 4. The structures of the rhizosphere bacterial communities. Principal coordinate analysis (PCoA) of changes in bacterial community composition in different treatments (a). Venn diagram of the number of shared and unique OTUs among the AE, APE, ASE, and APSE treatments (b). OTUs were defined at 97% sequence similarity. A (soil + alfalfa), AE (soil + alfalfa + EDDS), APE (soil + alfalfa + *P. mucilaginosus* + EDDS), ASE (soil + alfalfa + *S. meliloti* + EDDS), and APSE (soil + alfalfa + *P. mucilaginosus* and *S. meliloti* + EDDS).

highest POD activities in the plant organs were observed in the ASE treatment, whereas the lowest POD activities were found in the APSE treatment.

3.4. Soil enzyme activities and microbial biomass

The urease and saccharase activities did not significantly differ among the five treatments ($P > 0.05$; Table 2). The highest catalase

Fig. 5. The canonical correspondence analysis (CCA) used to identify the relationships between soil bacterial community composition and soil properties. A (soil + alfalfa), AE (soil + alfalfa + EDDS), APE (soil + alfalfa + *P. mucilaginosus* + EDDS), ASE (soil + alfalfa + *S. meliloti* + EDDS), and APSE (soil + alfalfa + *P. mucilaginosus* and *S. meliloti* + EDDS). TN: total nitrogen, AK: available potassium, SOC: soil organic carbon, Cu: soil Cu^{2+} . *** significance at the 1‰ level, ** significance at the 1% level, * significance at the 5% level.

activity was observed in the AE treatment, which was 1.9, 1.5, 1.5, and 1.8 times higher than that in the A, APE, ASE, and APSE treatments, respectively. The acid phosphatase activity was 1.1 and 1.2 times higher in the A and APSE treatments than in the AE treatment, respectively. The highest β -glucosidase activity was observed in the APSE treatment, which was 1.4 and 1.2 times higher than that in the control and AE treatment, respectively. Thus, the results indicated that the urease, saccharase, and acid phosphatase activities were the highest in the PGPR- and rhizobium-co-inoculated soil. The highest MBC (214 mg kg^{-1}) and MBN (11.4 mg kg^{-1}) content was observed in the APSE treatment (Table 2), which were significantly higher than those in the A, AE, APE, and ASE treatments (MBC: 1.3, 1.3, 1.2, and 1.1 times higher; MBN: 5.4, 2.0, 1.9, and 1.6 times higher, respectively).

3.5. Rhizosphere microbial community composition and structure

Proteobacteria, *Firmicutes*, *Actinobacteria*, and *Acidobacteria* were the most dominant phyla in the A, APE, and APSE treatments, accounting for >80% of the rhizobacterial relative abundance (Fig. 2a). However, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* were the most dominant phyla in the AE and ASE treatments. The relative abundances of *Proteobacteria*, *Bacteroidetes*, and *Chloroflexi* were significantly higher in the AE treatment than in the control. Moreover, the abundances of *Firmicutes*, *Acidobacteria*, *Planctomycetes*, and *Gemmatimonadetes* were significantly lower in the AE treatment than in the control ($P < 0.05$; Table S3). The relative abundances of *Firmicutes* and *Actinobacteria* in the APSE treatment were higher than those of the AE treatment. The APSE treatment had the highest relative abundance of *Bacillus* (Fig. 2b). Indicator species analysis showed that *Actinobacteria* and *Firmicutes* taxa (from the phylum to the family levels) were the main bacterial indicators in the A and APSE treatments, respectively (Table S4). The LefSe analysis also revealed that *Firmicutes* taxa were a potential biomarker for the APSE treatment (Fig. 3).

The Shannon, Simpson, Chao1, abundance-based coverage estimator, and phylogenetic diversity indices (Table S5) were used

Fig. 6. Linear models reveal the relative importance (%) of environmental drivers for Cu uptake in shoots (a) and roots (b). PVEM represents the proportion of variance explained by the model. Soil physicochemical properties including pH, Cu, available N ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$), SOC (soil organic carbon), AK (available potassium); Microbial alpha diversity indexes including Shannon and Simpson; Microbial biomass including MBC (microbial biomass C) and MBN (microbial biomass N); Plant oxidative damage including H_2O_2 (hydrogen peroxide), $\text{O}_2^{\cdot-}$ (superoxide radicals), and MDA (malondialdehyde).

to evaluate and compare the diversity and richness of rhizosphere microbial communities. The values of the indices were significantly lower for the AE treatment than for the control, which was also supported by the rarefaction curve analysis (Fig. S1). Furthermore, the values of all indices were higher for the APSE treatment than for the AE treatment. The PCoA plot (Fig. 4a) clearly shows the differences in the rhizosphere microbial community structure between the control and the other treatments. The rhizosphere microbial community structure was not significantly different among the AE, APE, ASE, and APSE treatments, which was also confirmed by the Venn diagram (Fig. 4b), generated based on the operational taxonomic units. The CCA results indicated that soil properties accounted for the largest variation (52.56%) in the rhizosphere microbial community compositions (Fig. 5) and suggested that pH and $\text{NO}_3^-\text{-N}$, Cu, $\text{NH}_4^+\text{-N}$, and AK content significantly contributed to the variation in the rhizosphere microbial community structure

Fig. 7. Cascading relationships of Cu uptake with soil physicochemical properties, microbial diversity, microbial biomass, and plant oxidative damage. Partial least squares path modeling (PLS-PM) disentangling major pathways of the influences of soil physicochemical properties, microbial diversity, microbial biomass, and plant oxidative damage on Cu uptake in shoots (a) and roots (c). Blue and orange arrows indicate positive and negative flows of causality, respectively. Numbers on the arrow indicate significant standardized path coefficients. Significant levels are denoted * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. R^2 indicates the variance of dependent variable explained by the model. Standardized total effects (direct plus indirect effects) calculated by the PLS-PMs (a and c) are displayed in b and d, respectively. GOF, goodness-of-fit index. Soil physicochemical properties are categorized into soil pH, Cu, and nutrient. Plant oxidative damage is categorized into shoot oxidative damage (represented in a) and root oxidative damage (represented in c). Microbial diversity including alpha diversity indexes—Shannon and Simpson; microbial biomass including MBC and MBN; soil nutrient including $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, SOC, and AK; oxidative damage including H_2O_2 , $\text{O}_2^{\bullet-}$, and MDA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

($P < 0.05$).

3.6. Effects of environmental variables on the Cu uptake

Soil physicochemical properties, microbial diversity, microbial biomass, and plant oxidative damage, as environmental variables, explained 99.24% of the variation in the Cu uptake by the shoots (Fig. 6a), with soil pH identified as the most important variable (relative influence: 22.8%). Additionally, these variables explained 99.87% of the variation in the Cu uptake by the roots (Fig. 6b), and MBC was identified as the most important variable (relative influence: 20.5%). Moreover, oxidative damage (H_2O_2 , MDA, and $\text{O}_2^{\bullet-}$ content; relative influence: 18.5%, 10.9%, and 8.1%, respectively) was another key variable for the Cu uptake by the roots. Partial least squares path modeling (PLS-PM) was used to evaluate the effects of soil physicochemical properties, microbial diversity, microbial biomass, and plant oxidative damage on the Cu uptake by shoots and roots (Fig. 7). Soil pH (0.973), nutrients (0.313), and plant oxidative damage (0.178) had positive total effects on the Cu uptake by the shoots, whereas soil Cu (−0.039), microbial diversity

(−0.099), and microbial biomass (−0.015) showed negative total effects (Fig. 7b). In contrast, microbial diversity (0.479) and microbial biomass (0.986) had positive total effects on the Cu uptake by the roots, whereas plant oxidative damage (−0.880) showed the most negative total effect (Fig. 7d).

4. Discussion

4.1. Implications of co-inoculation with PGPR and rhizobium on Cu phytoextraction during EDDS assisted phytoremediation

The application of EDDS significantly decreased the plant biomass (Table 1), which could be attributed to increased toxicity and oxidative damage, resulting from high metal concentrations in the plant tissue (Fig. 1). Co-inoculation with PGPR and rhizobium increased plant biomass and improved plant growth. Additionally, the application of EDDS significantly increased the accumulation of Cu in alfalfa shoots and roots, which subsequently inhibited plant growth. The TF and BCF values were higher for the AE treatment than for the control, suggesting that EDDS significantly increased

the phytoextraction of Cu. Moreover, EDDS effectively increases the bioavailability of Cu for root uptake and subsequent translocation to aboveground organs (Luo et al., 2005). However, the solubilization of Cu by EDDS was shown to exert toxic effects on plant roots and decrease their biomass (Luo et al., 2015), resulting in a decreased Cu accumulation in alfalfa roots.

The PLS-PM results indicated that increased soil nutrients enhanced Cu uptake by the shoots, while excessive Cu accumulation resulted in a higher level of oxidative damage (Fig. 7a and b). The Cu concentrations in the shoots were lower in the APSE treatment than in the AE, APE, and ASE treatments, suggesting that co-inoculation with PGPR and rhizobium decreased Cu accumulation in plant shoots in the presence of EDDS. This phenomenon may be owing to a higher plant resistance after co-inoculation, which prevents excessive Cu accumulation in the shoots, and to the fact that rhizobacterial inoculation is more likely to affect Cu accumulation in the roots than in the shoots (Fatnassi et al., 2015; Ju et al., 2020; Sun et al., 2014). Co-inoculation of the PGPR and rhizobium not only decreased the accumulation of Cu in the shoots in the presence of EDDS but also improved the shoot growth by decreasing oxidative damage and increasing biomass. These results were inconsistent with our previous findings, which showed that co-inoculation of PGPR and rhizobia increased the accumulation of Cu in the shoots and decreased the oxidative damage to shoots in the absence of EDDS (Ju et al., 2019). The results of this study showed that soil pH was the most important variable for Cu uptake by the shoots (Fig. 6a) and had the most positive total effect on Cu uptake. The highest root Cu content was observed in the co-inoculation treatment, which could be attributable to the significant increase in root tolerance to Cu stress. The results of this study indicated that oxidative damage was the key variable for Cu uptake by the roots and that MBC was the most important factor (Fig. 6b). PLS-PM also suggested that oxidative damage had the most negative total effect on Cu uptake, whereas microbial biomass had the most positive total effect. Additionally, the total Cu uptake by the roots was the highest in the co-inoculation treatment, indicating that co-inoculation of the PGPR and rhizobium promoted the extraction of the toxic metal from the soil by plant roots. This result may be attributed to (1) siderophores produced by PGPRs, which could have increased the bioavailability of Cu (Luo et al., 2015); (2) a higher biomass of rhizobacteria-co-inoculated plants (Fatnassi et al., 2015); and (3) low oxidative damage and higher MBC in the co-inoculation treatment, which were beneficial for Cu uptake by the roots.

Beiyuan et al., 2018b found that reabsorbed Cu-EDDS complexes and destabilized Cu on the surface of the oxide after extraction with EDDS might increase the risk of Cu leaching. Furthermore, the TF values for the individually inoculated and co-inoculated treatments were <1, and the highest BCF value in the roots was observed in the co-inoculation treatment (Table 1). These results indicated that the rhizobacterial (PGPR and rhizobium) inoculation was beneficial for the accumulation of Cu in the roots and was not conducive to Cu transfer to shoots (Ju et al., 2019), eventually reducing the risk of residual Cu entering the groundwater (Luo et al., 2018). The TE values of Cu were 1.1, 1.1, and 1.2 times higher in the APE, ASE, and APSE treatments than in the AE treatment, respectively. These findings suggested that inoculation, especially PGPR and rhizobium co-inoculation, further stimulated the total Cu extraction owing to the increased biomass of the plants, in particular the shoots, and also improved the phytoaccumulation of Cu in the shoots and roots (Luo et al., 2019; Zhang et al., 2019). A previous study also concluded that microbe–chelant co-application was an effective strategy to further improve the efficiency of phytoremediation (Hussain et al., 2019). Our results further imply that co-inoculation of PGPR and rhizobium stimulates plant growth and the

phytoextraction process (total Cu extraction) during EDDS assisted phytoremediation.

4.2. Regulatory mechanisms involved in the improvement of plant Cu tolerance and soil quality by rhizobacterial inoculation

The application of EDDS increased the Cu-induced accumulation of MDA, $O_2^{\bullet-}$, and H_2O_2 in plant organs (Fig. 1), resulting in severe oxidative damage and limitation of plant growth. Increases in MDA and ROS levels in plant organs exposed to metal stress, caused by EDDS application (Sidhu et al., 2018), are owing to the alteration of the lipoxygenase pathway by the accumulated chelant and metal–chelate complexes (Yin et al., 2015). The application of EDDS resulted in a negative impact on the antioxidant enzyme system (SOD, CAT, APX, and POD) of alfalfa, confirming that EDDS application increases oxidative damage to the test plant by reducing its ability to scavenge ROS. The decreases in the SOD, CAT, and APX activities can be attributed to the reduced synthesis and/or inactivation of the enzymes, caused by high concentrations of heavy metals, resulting from EDDS application (Cestone et al., 2012). A decrease in the POD activity, owing to EDDS application, has been previously indicated that the POD activity decreased when the Cu content in the soil spiked to 600 mg kg^{-1} after EDDS application (Wang et al., 2018).

Co-inoculation with *P. mucilaginosus* and *S. meliloti* could alleviate the oxidative damage to EDDS-treated plants by decreasing the MDA and ROS accumulation. Our results indicated that co-inoculation of the PGPR and rhizobium increased the available N content in the EDDS-treated soil because of the increased soil urease activity and *Actinobacteria* abundance (Table S3), known to improve N cycling in soil (Samaddar et al., 2019). Additionally, increases in organic matter and microbial biomass N in the soil, caused by rhizobacterial inoculation, can increase the N availability via mineralization of organic N (Etesami, 2018; Shahzad et al., 2014). Hu et al. (2015) showed that exogenous application of N could decrease oxidative damage to plant tissue under metal stress. The soil N content in this study was significantly correlated with the levels of H_2O_2 and $O_2^{\bullet-}$ in shoots and roots (Fig. S2). The SOD, CAT, and APX activities were higher in the plants inoculated with the rhizobacteria than in the uninoculated plants, indicating that plant adaptation to Cu oxidative stress occurs via the alteration of SOD, CAT, and APX activities. The SOD and CAT activities were significantly correlated with the soil available N content. Additionally, co-inoculation was associated with higher SOD, CAT, and APX activities than were individual inoculations, which was because of the effective symbiotic system of the PGPR and rhizobia strains, providing a nutrient environment that was highly conducive to plant growth (Fatnassi et al., 2015; Ju et al., 2019). Thus, our results indicated that EDDS application led to severe Cu-induced oxidative damage to plants, whereas co-inoculation of PGPR and rhizobium strains increased antioxidant enzyme activities, which subsequently decreased the level of induced oxidative damage.

Soil biochemical properties, which are influenced by the soil nutrient content and biological activity (Islam et al., 2016; Ju et al., 2020), play a key role in improving plant tolerance to stressful environments. The results of this study suggested that the application of EDDS significantly decreased the acid phosphatase activity (Table 2), likely owing to a significant increase in the pH, caused by EDDS (Yoo et al., 2018). However, EDDS application did not significantly alter the activities of saccharase, urease, and β -glucosidase, indicating weak effects of the biodegradation of EDDS on enzymatic activities (Yang et al., 2013). In contrast, soil enzyme activities were significantly increased in the co-inoculation treatment with rhizobacteria, which could be attributed to a shift in soil physicochemical properties, such as increased soil fertility and

decreased soil toxicity (Ju et al., 2020; Sipahutar et al., 2018). The soil MBC content did not significantly change, whereas the MBN content significantly increased in the AE treatment relative to that in the control. These results may be attributed to no change in the SOC content and an increase in the available N content after EDDS application. Our recent study has found that the application of EDDS promoted N cycling in soils and plant tissue and also improved microbial metabolism (Fang et al., 2017). PGPR and rhizobium inoculation increased the soil MBC and MBN content relative to that in the uninoculated control, indicating an increased nutrient content and decreased metal toxicity in the soil (Abdu et al., 2017). Additionally, PLS-PM revealed that soil microbial biomass plays a key role in improving plant Cu tolerance.

EDDS application increased the relative abundance of *Firmicutes* and decreased that of *Proteobacteria* (Fig. 2a). *Firmicutes* taxa were found to be potential biomarkers for the APSE treatment (Fig. 3), as co-inoculation with the PGPR and rhizobium increased the *Firmicutes* abundance. *Firmicutes* likely decrease in highly metal-contaminated soils (Tipayno et al., 2012) and increase in soils with a high nutrient content (Ramirez et al., 2012), which demonstrates that EDDS has negative effects on soil health and nutrient cycling, whereas co-inoculation decreases soil toxicity and increases soil nutrients. The co-inoculated soil had a greater abundance of *Actinobacteria* than the uninoculated soil. *Actinobacteria* can adapt to easily accessible carbon substrates and nutrient-rich environments (Ju et al., 2020). Thus, our results demonstrated that co-inoculation reduced soil metal toxicity and increased the soil nutrient content by altering the bacterial composition. Additionally, the relative abundance of *Bacillus*, which supports plant growth, was strongly enriched in the co-inoculation treatment (Fig. 2b). EDDS application significantly decreased the diversity and abundance of the rhizosphere bacterial community (Table S5). EDDS is known to significantly and readily solubilize Cu in the soil, thereby causing toxicity to soil organisms and substantially destroying the stability of soil microbial communities (Yang et al., 2013). Co-inoculation restored the abundance and diversity of the rhizosphere bacterial community by improving biological activities through the alteration of primary soil environmental factors such as pH, as well as the Cu and N content (Fig. 5). The rhizosphere bacterial community structure was distinctly different between the control and EDDS-applied treatments (Fig. 4a), thus indicating that EDDS application substantially affected the bacterial community structure. Compared with the EDDS treatment, rhizobacterial inoculation did not significantly alter the rhizosphere bacterial community structure (Fig. 4b). A stable rhizosphere bacterial community can avoid destabilizing effects of biotic or abiotic factors on community diversity (Trivedi et al., 2012).

Thus, co-inoculation with the PGPR and rhizobium improved plant tolerance in the EDDS-treated Cu-contaminated soil through two potential mechanisms. First, co-inoculation induced high antioxidant enzyme activities, which potentially alleviated oxidative damage to plant tissue. Second, a higher soil nutrient content and lower metal toxicity in the co-inoculation treatment, as indicated by higher soil enzymatic activities, abundances of beneficial bacteria, microbial biomass, and microbial diversity, potentially enhanced the tolerance of alfalfa plants to metal stress. These mechanisms therefore indicate the potential of co-inoculation for improving the efficiency of EDDS assisted phytoremediation of soils contaminated with heavy metals.

5. Conclusions

In this study, we demonstrated that rhizobacterial inoculation further stimulated Cu phytoextraction during EDDS-enhanced phytoremediation by enhancing plant Cu tolerance and soil

quality. EDDS application damaged alfalfa organs, owing to excessive Cu-induced accumulation of MDA and ROS, which subsequently inhibited the antioxidant capacity of plant organs. However, co-inoculation of PGPR and rhizobium increased the tolerance of alfalfa to Cu stress by mitigating the accumulation of MDA and ROS and increasing antioxidant enzyme activities in the presence of EDDS. Additionally, co-inoculation increased the soil nutrient content and decreased the soil toxicity by increasing enzymatic activities, as well as microbial activity and diversity, during EDDS assisted phytoremediation. These results provide fundamental information for improving phytoremediation efficiency and soil quality during chelant assisted phytoremediation of metal-contaminated soils.

Declaration of competing interest

This study does not involve human subjects. This manuscript is original and it has not been previously published or submitted in part or whole. No conflict of interest exists in the submission of this manuscript, and the manuscript is approved by all authors for publication.

CRediT authorship contribution statement

Wenliang Ju: Methodology, Investigation, Data curation, Writing - original draft. **Lei Liu:** Methodology, Investigation, Data curation, Writing - original draft. **Xiaolian Jin:** Investigation, Software, Formal analysis. **Chengjiao Duan:** Investigation, Software, Formal analysis. **Yongxing Cui:** Investigation, Software, Formal analysis. **Jie Wang:** Investigation, Software, Formal analysis. **Dengke Ma:** Investigation, Software, Formal analysis. **Wei Zhao:** Writing - review & editing. **Yunqiang Wang:** Writing - review & editing. **Linchuan Fang:** Funding acquisition, Project administration, Writing - review & editing.

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Appendix A. Supplementary data

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