Environmental Pollution 277 (2021) 116758

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Improvement of alfalfa resistance against Cd stress through rhizobia and arbuscular mycorrhiza fungi co-inoculation in Cd-contaminated soil☆

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ARTICLE INFO

Article history:
Received 11 September 2020
Received in revised form
24 January 2021
Accepted 14 February 2021
Available online 22 February 2021

Keywords:
Microbial community
Co-inoculation
Rhizosphere
Soil heavy metal
Symbiotic microbes

ABSTRACT

Rhizobia and arbuscular mycorrhiza fungi (AMF) are important symbiotic microbes that are advantageous to plants growing in metal-contaminated soil. However, it remains unclear how inoculated microbes affect rhizosphere microbial communities or whether subsequent changes in rhizosphere microbiomes contribute to improving plant resistance under metal stress. This study investigated the effects of rhizobia and AMF inoculation on alfalfa resistance to Cd stress. The response of rhizosphere microbial communities to inoculation and its role in increasing alfalfa' ability to cope with stress were further analyzed using high-throughput sequencing of 16S and ITS rRNA genes. Results showed that single rhizobia or AMF inoculation significantly improved alfalfa resistance to Cd stress, while their co-inoculation resulted in the greatest overall improvement. Improved resistance was reflected by the significant mitigation of Cd-induced lipid peroxidation and reactive oxygen species (ROS) stress caused by increases in antioxidant enzyme activities along with co-inoculation. Furthermore, co-inoculation significantly altered the rhizosphere microbial community structure by decreasing fungal community diversity and increasing bacterial community diversity. Results of partial least squares path modeling (PLS-PM) and variation partitioning analysis (VPA) showed that the rhizosphere bacterial community predominated over the fungal community with respected to improvements in resistance to Cd stress under the co-inoculation treatments. This improvement was specifically seen in the enrichment of certain key bacterial taxa (including *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Chloroflexi*) induced by the rhizobia and AMF co-inoculation, enhancing alfalfa' ability to uptake rhizosphere nutrients and reduce its release of photosynthetically-derived carbon (C) into soil. Our findings revealed that the co-inoculation of multiple symbiotic microbes can assist plants to effectively cope with Cd stress, providing a greater understanding of rhizosphere bacterial taxa in the microbe-induced phytomanagement.

1. Introduction

Heavy metal pollution in soil poses a critical threat to agriculture (Kong et al., 2019; Violante et al., 2010), which has acute adverse effects on plant biodiversity and biomass (Hernandez and Pastor, 2008; Lu et al., 2010). Plants can develop resistance to heavy metals either through "avoidance", by restricting metal

☆ This paper has been recommended for acceptance by Eddy Y. Zeng.

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https://doi.org/10.1016/j.envpol.2021.116758
0269-7491/

uptake, or through "tolerance", by coping with high levels of high internal metal contents (Baker, 1987). It is therefore of the utmost importance to develop a remediation strategy can improve plant resistance to heavy metals.

Over the past several decades, studies have suggested that symbiotic microbes are an eco-friendly and economical soil remediation approach that can mitigate plant stress caused by heavy metals (Colpaert and Assche, 1992; Glick, 2003; Wood et al., 2016). Among the microorganisms, rhizobia and arbuscular mycorrhizal fungi (AMF) are widely distributed throughout various plant systems and diverse environmental conditions (Kong et al., 2019; Fan et al., 2018). In addition, rhizobia-induced nitrogen (N) fixation is also beneficial for plant growth (Dary et al., 2010; Pajuelo et al., 2008). Rhizobia improves plant resistance to heavy metals by enhancing the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophores, and solubilization of phosphorous (P) (Ahmad et al., 2011; Zhang et al., 2011). It can also assist plant heavy metal uptakes via increasing their solubility and bioavailability (Hrynkiewicz et al., 2018; Ashraf et al., 2017). The AMF and associated their external mycelium components provide many potential sites for the uptake and accumulation of essential nutrients (Abbott and Robson, 1977; Wang, 2017; Riaz et al., 2020). Phytohormone compounds secreted by AMF, such as ethylene, gibberellic acids, cytokinin, and auxins (Chanclud and Morel, 2016), also promote plant growth and resistance in heavy metal-contaminated soils (Dary et al., 2010). Studies have shown that various processes associated with inoculation of rhizobia or AMF can assist plants in coping with heavy metal stress (Hildebrandt et al., 2007; Fagorzi et al., 2018). This suggests that a rhizobia and AMF co-inoculation may further improve the ability in plant-microbe remediation. Recent studies have also reported that a co-inoculation of two microbes can promote plant growth and improve remediation efficiency under various environments (Fuentes et al., 2016; Ju et al., 2019; Jian et al., 2019). For example, the co-inoculation of *Oudemansiella radicata* and *Serratia marcescens* can promote the bioremediation of fluoranthene and lead-contaminated soil (Li et al., 2019). However, it remains unclear whether the rhizobia and AMF co-inoculation can improve plant resistance to heavy metals, and if so, by what degree.

The use of symbiotic microbes to assist plants in coping with heavy metal stress depends upon the effectiveness and prevalence of the inoculated symbiotic microbes employed in plant-microbe remediation applications, which further depends on their adaptability to environmental stress and their effect on plant-soil interfaces, such as the rhizosphere (Kaur and Garg, 2018; Fang et al., 2020). The inoculation of foreign microbes can affect the indigenous microbe community in heavy metal-contaminated soil (Rajkumar et al., 2012; Kaur and Garg, 2018). The ability of symbiotic microbes to colonize and interact with other members of the indigenous microbial community is crucial for promoting plant growth in contaminated soil (Thijs et al., 2016). Therefore, the successful rhizobia and AMF co-inoculation may not only affect abiotic factors associated with the rhizosphere but may also strongly interact with the indigenous microbial community in the rhizosphere.

We currently only have a limited understanding of the effects of the rhizobia and AMF co-inoculation on rhizosphere microbial communities in heavy metal-contaminated soil. Furthermore, rhizobia and AMF are two of many other rhizosphere microbial taxa (including bacteria and fungi) that can benefit soil nutrient availability and mitigate the adverse effects caused by heavy metal uptake by plants (Glick, 2003; Hrynkiewicz et al., 2018; Lam and Lai, 2018; Tang et al., 2018). For example, microbiomes can assist host plants to modulate immunity response or nutrient uptake (Berendsen et al., 2012). Moreover, certain bacterial taxa can also produce siderophores and ACC-deaminase to assist plant nutrient absorption, thus promoting plant biomass under heavy metal stress (Chanclud and Morel, 2016; Ashraf et al., 2017; Zhang et al., 2019). These beneficial traits demonstrate the essential roles of indigenous microbial taxa in the rhizosphere, which may be involved in a range of biological processes that improve plant stress resistance. Therefore, the rhizobia and AMF co-inoculation may indirectly improve plant resistance to heavy metal stress by affecting the indigenous rhizosphere microbiome. Accordingly, identifying the influence of co-inoculation in rhizosphere microbial communities and determining the role of rhizosphere microbiome in plant resistance are crucial to improve remediation efficiency in heavy metal-contaminated soil.

Specifically, it is important to maintain a steady base level respective to animal husbandry production by improving heavy metal resistance in pastureland (e.g., alfalfa forage) while increasing plant biomass (Reed and Simon, 1991; Huang et al., 2019). Therefore, this study examines the effects of rhizobia and AMF co-inoculation on alfalfa growth and physiology in soil contaminated with cadmium (Cd). Additionally, we explored the response of rhizosphere microbial communities to symbiotic microbial inoculation and its role in mitigating Cd stress in alfalfa. The objectives of this study were as follows: 1) to compare non-inoculation and the single inoculation of rhizobia or AMF and their co-inoculation in assisting alfalfa resistance to Cd stress, 2) to evaluate the effects of inoculation of rhizobia or AMF separately, and their co-inoculation on microbial properties in the rhizosphere, and 3) to ascertain the role of indigenous rhizosphere microbiomes in improving plant resistance to Cd stress. This study is meant to enhance our understanding of the mechanisms associated with microbial inoculation in promoting plant resistance to heavy metal stress and providing a new strategy for the phytomanagement of heavy metal-contaminated soil.

2. Materials and methods

2.1. Experimental design and pot experiment

The soil samples were collected from aeolian sandy soil (0–40 cm) within an undisturbed field in the Jungar Banner, Inner Mongolia, China (39°46′23.7 N, 110°38′39.1 E), air-dried for two weeks, and passed through a 2-mm sieve. Visible plant residue was carefully removed manually. The AMF (*Glomus mosseae*) was obtained from the Chinese Bank of Glomeromycota (BGC XJ01), and the rhizobia (*Sinorhizobium meliloti*) was obtained from the Agricultural Culture Collection of China (ACCC 17501).

The experiment comprised of eight treatments, including a control soil treatment (CK) and a spiked Cd soil treatment (Cd) which was spiked by adding $CdCl_2$ at a dosage of 5.0 mg kg^{-1} in the soil samples and incubated for two months prior to inoculation treatments. Each soil treatment (CK, Cd) comprised of four different microbial inoculation treatments (i.e., non-inoculation (A); inoculation with *Sinorhizobium meliloti* (*S. meliloti*) alone (AR); inoculation with *Glomus mosseae* (*G. mosseae*) alone (AM); co-inoculation applying both *S. meliloti* and *G. mosseae* (ARM). Each PVC pot (10 cm diameter, 15 cm height) contained 1.5 kg of soil. Fertilizer containing 150 mg kg^{-1} KCl, 20 mg kg^{-1} KH_2PO_4, and 50 mg kg^{-1} NH_4NO_3 was added to each pot. Furthermore, 15 g of the *Glomus mosseae* matrix was added 1 cm below the soil surface. The same amount of the inoculum was filtered (11 μm, Whatman, UK), after which the obtained filtrate (15 ml) along with the microbial communities (excluding AMF) and the remaining sterilized mycorrhizal inoculum matrix were both added to the soil of the other pots. Alfalfa seeds were sterilized for 5 min in a 30% (v/v) hydrogen peroxide (H_2O_2) solution one day prior to sowing (Kereszt et al.,

2007). Cold sterile water was then used to rinse the seeds three times, subsequently soaked in pure water overnight. For the rhizobia inoculation treatment (AR and ARM), rhizobia (i.e., *S. meliloti*) specimens were grown in a tryptone-yeast liquid medium (Ju et al., 2019). After the first plant leaves emerged, bacterial suspensions were sprayed onto the AR and ARM treatments soil once a week for 3 times. Each pot contained twenty seeds. Two weeks after sowing, seedlings were thinned to ten plants per pot. Soil moisture of all pots was maintained by addition of deionized water (every 1–3 days) at 70% of the soil water holding capacity to ensure optimal alfalfa growth conditions. Three replicates were produced for each treatment and irrigated daily. Rhizosphere soil was physically brushed away from the root surface using a sterile soft bristle paintbrush after 120 days of plant growth. Each soil sample included two subsamples. One subsample was passed through a 2-mm sieve after air drying for physicochemical analyses. The other subsample was immediately stored at −80 °C to extract genomic DNA.

2.2. Soil physiochemical analysis

Soil organic carbon (SOC) content was measured using the dichromate oxidation method (Jones and Willett, 2006; Fang et al., 2017). Soil pH was measured using a meter with a glass electrode (IS126 pH meter, InsMark, Inc., Shanghai, China) in a 1:5 soil to water (w/v) suspension. Soil available phosphorus (AP) and total phosphorus (TP) were extracted using $NaHCO_3$ and H_2SO_4–$HClO_4$ respectively, and then measured by the molybdenum blue method (710 nm) using an ultraviolet spectrophotometer (Hitachi UV2300) (Olsen and Sommers, 1982). Soil nitrate nitrogen (NO_3^--N) and ammonium nitrogen (NH_4^+-N) concentrations were measured in a 1 M potassium chloride (KCl) solution [soil: solution = 1:10 (w/v)] using a segmented-flow analyzer after extraction (Kachurina et al., 2000). Total nitrogen (TN) in soil was determined using the Kjeldahl method (Bremner and Mulvaney, 1982). The Cd content in soil were analyzed according to the modified United States of America Environmental Protection Agency (USEPA) Method 3051A (USEPA, 1998). In detail, a tri-acidic mixture ($HClO_4$, HNO_3, and HCl) at a 1:3:1 vol ratio (15 ml) was used to digest soil samples (0.2 g). Soil available Cd (A-Cd) was extracted with 0.1 M $CaCl_2$ at a 1:5 (w/v) soil ratio after shaking for 2 h at 25 °C (Houba et al., 1996; Smilde et al., 1992). Atomic absorption spectrometry (Hitachi, FAAS Z-2000, Japan) was used to determine all heavy metal concentrations.

2.3. Plant physiochemical analysis

The collected plant samples were washed with deionized (DI) water, dried to a constant weight, crushed, and then digested using a 10 ml mixture of $HClO_4$ and HNO_3 at a 4:1 vol ratio. Malondialdehyde (MDA) was measured to assay the level of membrane damage (De Vos et al., 1991). The amount of H_2O_2 and superoxide radicals (O_2^-) was evaluated to assay the plant oxidative damage (Fan et al., 2015). The reagent kits provided by Suzhou Comin Biotechnology Co., Ltd. (Suzhou City, Jiangsu Province, China) were used to measure the H_2O_2, O_2^-, and MDA content (Ju et al., 2019). Activities of catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) were determined using enzyme-specific commercial reagent kits provided by Suzhou Comin Biotechnology Co., Ltd. (Ju et al., 2019). At the end of the experiment, both shoot and root biomass were recorded after being oven-dried for 3 d at 70 °C. The total heavy metal concentrations in alfalfa shoots and roots were analyzed using the atomic absorption spectrometry.

2.4. DNA extraction, HiSeq sequencing and bioinformatics analysis

Total bacterial DNA was extracted from samples using the PowerSoil DNA Isolation Kit at the MO BIO Laboratories. The quantity and quality of DNA were evaluated using absorbance ratios at 280 nm/260 nm and 230 nm/260 nm, respectively. The samples were stored at −80 °C for further processing. The primers ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) and ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) were used to amplify the ITS1 region of fungi (Gardes and Bruns, 1993). The V3–V4 region of the bacterial 16S rRNA gene was amplified using a common primer pair (reverse primer, 5′-GGACTACHVGGGTWTC TAAT-3'; forward primer, 5′-ACTCCTACGGGAGGCAGCA-3′) combined with barcode sequences and adapter sequences (Huse et al., 2008). PCR amplification, which contained 0.2 μl Q5 high-fidelity DNA polymerase, 1 μl dNTP, 10 μl bμffer, 10 μl high GC enhancer, 10 μM of each primer, and 60 ng genome DNA, was conducted in a total volume of 50 μl, applying a total of five cycling procedures. All PCR products were quantified using the Quant-iT dsDNA HS Reagent and pooled together. High-throughput sequencing analysis of bacterial rRNA genes was performed using the Illumina HiSeq 2500 platform (Biomarker Technologies Corporation, Beijing, China).

Operational taxonomic units (OTUs) were determined using the UNITE reference database (http://unite.ut.ee/index.php) for ITS and the SILVA reference database (http://www.arb-silva.de) for 16S rRNA genes as described by Cui et al. (2019). Alpha diversity was calculated using the Simpson's and Shannon-Wiener diversity indices applying the "diversity" function (Vegan package). The relative abundance of microbes was determined in percentages.

2.5. Statistical analysis

Pearson correlation analysis was used to assess associations between environmental factors and microbial alpha diversity. Two-way analysis of variance (ANOVA) was used to analyze the effects of inoculation, the addition of Cd, and their combined interaction on microbial alpha diversities, plant biomass, Cd uptake, and soil properties. A value of $P < 0.05$ was considered significant in this study. When necessary the original data were normalized by standardization or log-transformation before analysis, and the heterogeneity of variance was tested. Non-metric multidimensional scaling (NMDS) was used to visualize bacterial and fungal community structure based on Bray-Curtis dissimilarity matrices (Vegan package). Analysis of similarities (ANOSIM) was used to identify the difference in bacterial or fungi community structure among all treatments. Two-way permutational multivariate analysis of variance (PERMANOVA) was used to test the effects of inoculation, the addition of Cd, and their combined interaction on microbial Bray–Curtis dissimilarity using the "adonis" function (Vegan package). The environmental matrix was constructed by the Mantel test, which was used to conduct variation-partitioning analysis (VPA) to identify the relative importance of microbial-community composition to subsequently explain variation in soil properties and soil Cd determined by redundancy analysis (RDA) using the "varpart" function (Vegan package). The most significant factors that shaped the microbial community structure were identified by RDA or canonical correspondence analysis (CCA) and a Monte Carlo permutation test via Hellinger transformation data of microbial species and soil variable data standardized using the Vegan package. Partial least squares path modeling (PLS-PM) was used to further identify potential pathways that affect plant biomass and Cd uptake. The model was constructed using the "innerplot" function from the plspm package. A partial Mantel test was used to control covarying effects of each treatment (Vegan package). Correlations among microbial communities and

inoculation and additive Cd treatments were evaluated using Mantel test in Vegan package. Indicator species analysis was conducted using the "multipatt" function in indicspecies package (version 1.7.4). All statistical analyses were performed using the R software package (version 3.3.2).

3. Results

3.1. Soil physicochemical properties in rhizosphere

The NO_3^--N, NH_4^+-N, SOC, and AP contents were significantly affected by Cd, inoculation treatments, and their interaction ($P < 0.05$) according to the results from two-way ANOVA. For all Cd treatments, the SOC content, and SOC:TN and SOC:TP ratios were higher than that of the control, except for soil pH. Additionally, the co-inoculation significantly increased both NH_4^+-N and AP contents (Table S1).

3.2. Plant physicochemical properties and Cd uptake in plant tissues

Both the addition of Cd and inoculation significantly affected alfalfa biomass, including shoots and roots ($P < 0.01$; Table 1). Moreover, inoculation treatments greatly increased the shoot and root biomass of alfalfa, while the addition of Cd significantly reduced the shoot and root biomass of alfalfa ($P < 0.01$). For example, the highest alfalfa biomass (3.35 ± 0.116 g pot^{-1}) was observed in the rhizobia and AMF co-inoculation treatment with the addition of Cd. Additionally, the concentration of Cd in alfalfa shoots and roots was significantly affected by the addition of Cd, inoculation, and their interaction. The inoculation and Cd treatments greatly increased the Cd uptake content in alfalfa shoots and roots (Table 1). The Cd content in roots was higher than that in shoots under all inoculation treatments compare to the control (non-inoculation) ($P < 0.05$). Moreover, the Cd concentrations in inoculation treatments (i. e., AR, AM, and ARM) were all 1.2-fold higher in shoots relative to the control (A) with the addition of Cd. The highest total Cd uptake in shoots (30.6 ± 1.81 μg pot^{-1}) was observed in the co-inoculation treatments with the addition of Cd. The total Cd uptake was significantly higher (by factors of 1.4 and 1.6) in shoots of the single inoculation and co-inoculation treatments compare to the control (non-inoculation) with the addition of Cd, respectively ($P < 0.05$). The rhizobia and AMF co-inoculation treatment had the highest total Cd uptake among the single inoculation and non-inoculation treatments.

After inoculation, the antioxidant capacity of shoots and roots notably increased, for which SOD, CAT, and POD activities were all significantly higher in the AR, AM, and ARM treatments compared to the A treatments ($P < 0.05$; Table 2 and Table S2). The highest SOD, CAT, and POD activities of alfalfa were observed in the rhizobia and AMF co-inoculation treatment compared with non-inoculation and single inoculation treatments. For example, SOD activity of shoots was greater by the factor of 1.4 in the ARM treatments compare to the A treatment. The MDA content of shoots was notably lower in the AR, AM and ARM treatments than in A treatment (i.e., by 27.28%, 21.81%, and 34.09%, respectively) ($P < 0.05$; Table 2). In addition, MDA, H_2O_2, and O_2^- contents were the lowest in the co-inoculation treatment, compared with control non-inoculated plants.

3.3. Alpha diversity of the rhizosphere microbial community

All soil samples identified 4,847,280 high-quality microbial sequences, including 2,593,280 fungal and 2,254,000 bacterial sequences, which can be clustered into 904 and 197 OTUs, respectively, at the 97% similarity cut-off level. For bacteria communities, the OTUs, ACE, Chao1 indicators, Simpson, and Shannon-Wiener indices were all significantly affected by the addition of Cd, inoculation, and their interaction ($P < 0.05$; Table S3 and Fig. 1). In particular, the addition of Cd greatly decreased the bacteria Simpson's diversity index under inoculation treatments. Inoculation significantly decreased bacteria Simpson's diversity index with the addition of Cd, while it increased bacteria Simpson's diversity index in the control treatment. For the fungal communities, both Chao1 indicators and Simpson's diversity indices were notably affected by the addition of Cd, inoculation, and their interaction. In particular, the addition of Cd significantly increased the Simpson's diversity index of fungi under all inoculation treatments ($P < 0.05$; Fig. 1). Inoculation significantly increased the Simpson's diversity index of fungi along with both the additive Cd treatments and the control treatment.

Table 1
Biomass, Cd content, and uptake of Cd in plant tissues.

Treatment		Biomass (g pot^{-1})		Content (mg kg^{-1})		Total uptake (μg pot^{-1})	
		Shoot	Root	Shoot	Root	Shoot	Root
A	CK	3.26 ± 0.234 Ac	2.47 ± 0.071 Ab	0.170 ± 0.016 Ba	1.18 ± 0.131 Bb	0.55 ± 0.044 Bc	2.90 ± 0.306 Bb
	Cd	2.45 ± 0.021 Bc	2.17 ± 0.028 Bb	7.61 ± 0.789 Aa	24.0 ± 3.10 Aa	18.6 ± 1.80 Ac	52.2 ± 7.30 Ab
AR	CK	4.15 ± 0.169 Ab	2.96 ± 0.281 Aa	0.202 ± 0.022 Ba	1.57 ± 0.168 Ba	0.84 ± 0.054 Bab	4.67 ± 0.881Ba
	Cd	3.01 ± 0.169 Bb	2.50 ± 0.146 Aab	8.89 ± 0.702 Aa	25.6 ± 2.50 Aa	26.7 ± 2.38 Aab	64.2 ± 10.1 Aab
AM	CK	3.99 ± 0.182 Ab	2.83 ± 0.176 Aab	0.180 ± 0.007 Ba	1.23 ± 0.144 Bab	0.72 ± 0.062 Bb	3.51 ± 0.607 Bab
	Cd	2.87 ± 0.094 Bb	2.35 ± 0.127 Bb	8.87 ± 0.482 Aa	24.3 ± 0.717 Aa	25.4 ± 0.579 Ab	57.1 ± 2.03 Aab
ARM	CK	4.63 ± 0.103 Aa	3.16 ± 0.091 Aa	0.194 ± 0.008 Ba	1.20 ± 0.127 Bab	0.90 ± 0.056 Ba	3.79 ± 0.353 Bab
	Cd	3.35 ± 0.116 Ba	2.74 ± 0.176 Ba	9.12 ± 0.248 Aa	26.6 ± 1.93 Aa	30.6 ± 1.81 Aa	73.0 ± 9.22 Aa

Factors	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Cd	319	***	43.2	***	2421	***	1354	***	2322	***	653	***
Inoculation	59.5	***	17.4	***	4.12	*	0.926	0.451	25.2	***	4.43	*
Cd * Inoculation	2.67	0.082	0.401	0.754	3.86	*	0.789	0.518	22.4	***	3.65	*

Note: A (alfalfa), AR (alfalfa + *S. meliloti*), AM (alfalfa + *G. mosseae*), and ARM (alfalfa + *S. meliloti* + *G. mosseae*). CK and Cd represent control check soil and adding Cd soil, respectively. Values are the means (±standard errors) of three replicate soil cores. Different uppercase letters indicate significant differences ($P < 0.05$) within a column between the CK and Cd at different inoculation, and different lowercase letters indicate significant differences ($P < 0.05$) within a column amongst the different inoculation in the CK and Cd with the Tukey test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Table 2
The malondialdehyde (MDA), reactive oxygen species (O_2^-, H_2O_2), and antioxidant enzymes contents (superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD)) of plant shoots in different treatments.

Treatment		MDA (nmol g^{-1} FW)	O_2^- (nmol g^{-1} FW)	H_2O_2 (μmol g^{-1} FW)	SOD (U g^{-1} FW)	CAT (nmol min^{-1} g^{-1} FW)	POD (U g^{-1} FW)
A	CK	18.5 ± 0.851 Ba	167 ± 17.3 Ba	0.853 ± 0.048 Ba	234 ± 11.0 Bc	20.3 ± 2.14 Ab	134 ± 9.53 Bc
	Cd	22.0 ± 2.56 Aa	201 ± 17.6 Aa	0.975 ± 0.040 Aa	279 ± 15.6 Ab	22.3 ± 1.18 Ac	194 ± 30.9 Ac
AR	CK	15.3 ± 0.875 Aab	136 ± 1.21 Bb	0.680 ± 0.119 Aab	254 ± 19.2 Abc	23.4 ± 2.82 Ab	194 ± 16.0 Ab
	Cd	16.0 ± 0.601 Ab	164 ± 10.3 Ab	0.710 ± 0.034 Abc	299 ± 32.6 Ab	25.5 ± 0.916 Abc	235 ± 33.1 Abc
AM	CK	16.0 ± 0.436 Abc	133 ± 7.28 Bb	0.611 ± 0.074 Bab	299 ± 12.9 Aab	24.1 ± 2.15 Ab	227 ± 12.1 Bb
	Cd	17.2 ± 1.58 Ab	162 ± 12.1 Ab	0.799 ± 0.035 Ab	319 ± 17.9 Ab	26.8 ± 2.24 Ab	266 ± 3.38 Ab
ARM	CK	13.0 ± 1.83 Ac	115 ± 7.50 Bb	0.665 ± 0.084 Aa	333 ± 23.7 Ba	30.6 ± 0.586 Ba	285 ± 12.0 Ba
	Cd	14.5 ± 1.37 Ab	135 ± 6.08 Ab	0.723 ± 0.015 Ac	389 ± 15.9 Aa	36.6 ± 1.50 Aa	346 ± 19.5 Aa

Factors	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Cd	19.9	***	44.5	***	14.2	**	26.3	**	17.9	**	39.5	***
Inoculation	33.8	***	29.8	***	16.9	***	32.2	***	48.6	***	62.7	***
Cd*Inoculation	1.88	0.174	0.71	0.527	1.81	0.185	0.867	0.478	1.55	0.240	0.529	0.669

Note: A (alfalfa), AR (alfalfa + *S. meliloti*), AM (alfalfa + *G. mosseae*), and ARM (alfalfa + *S. meliloti* + *G. mosseae*). CK and Cd represent control check soil and adding Cd soil, respectively. FW, fresh weight. Values are the means (±standard errors) of three replicate soil cores. Different uppercase letters indicate significant differences ($P < 0.05$) within a column between the CK and Cd at different inoculation, and different lowercase letters indicate significant differences ($P < 0.05$) within a column amongst the different inoculation in the CK and Cd with the Tukey test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

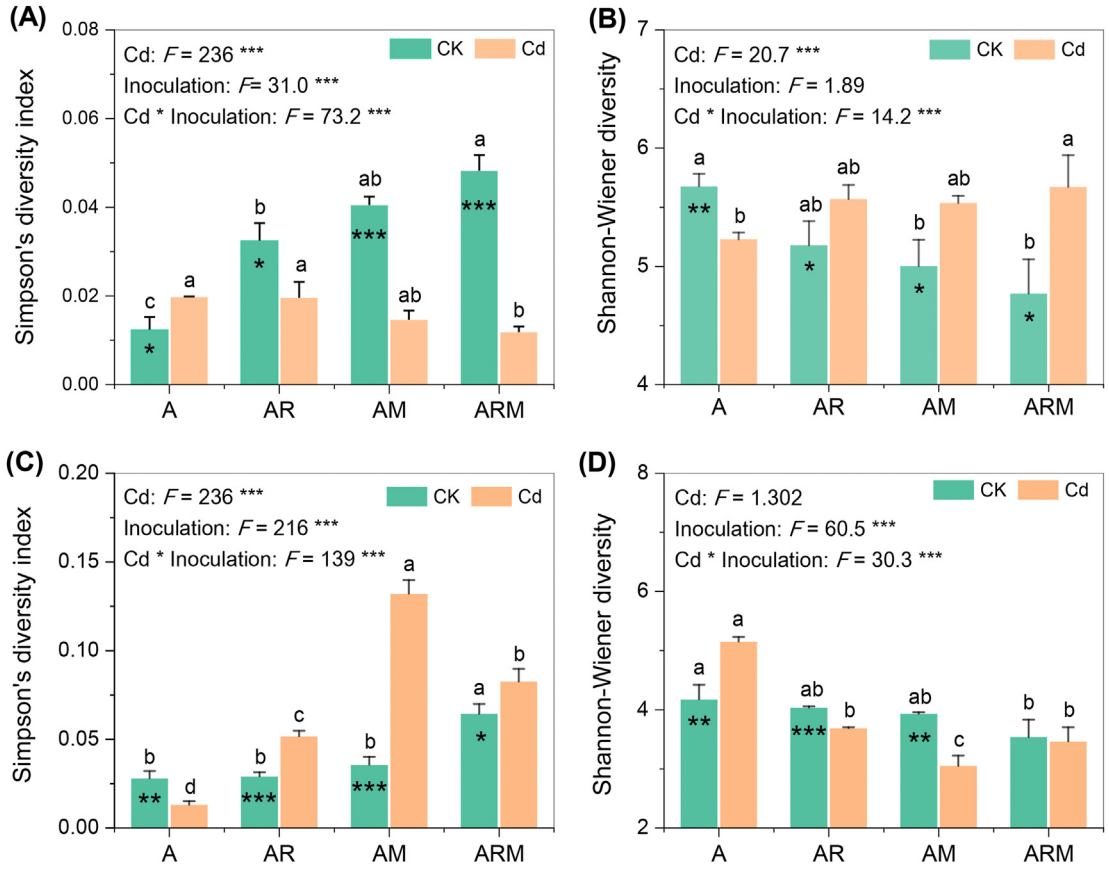

Fig. 1. Differences in bacterial (A, B), fungal (C, D) alpha diversity among the treatments. (The data are presented as the mean ± standard error of three replicate soil. A (alfalfa), AR (alfalfa + *S. meliloti*), AM (alfalfa + *G. mosseae*), and ARM (alfalfa + *S. meliloti* + *G. mosseae*). CK and Cd represent control check soil and adding Cd soil, respectively. Asterisk indicates significant differences ($P < 0.05$) between treats (Cd and CK) at each microbe based on T test; Lowercase letters indicate that means are significantly different ($P < 0.05$) among different microbe within Cd and CK.).

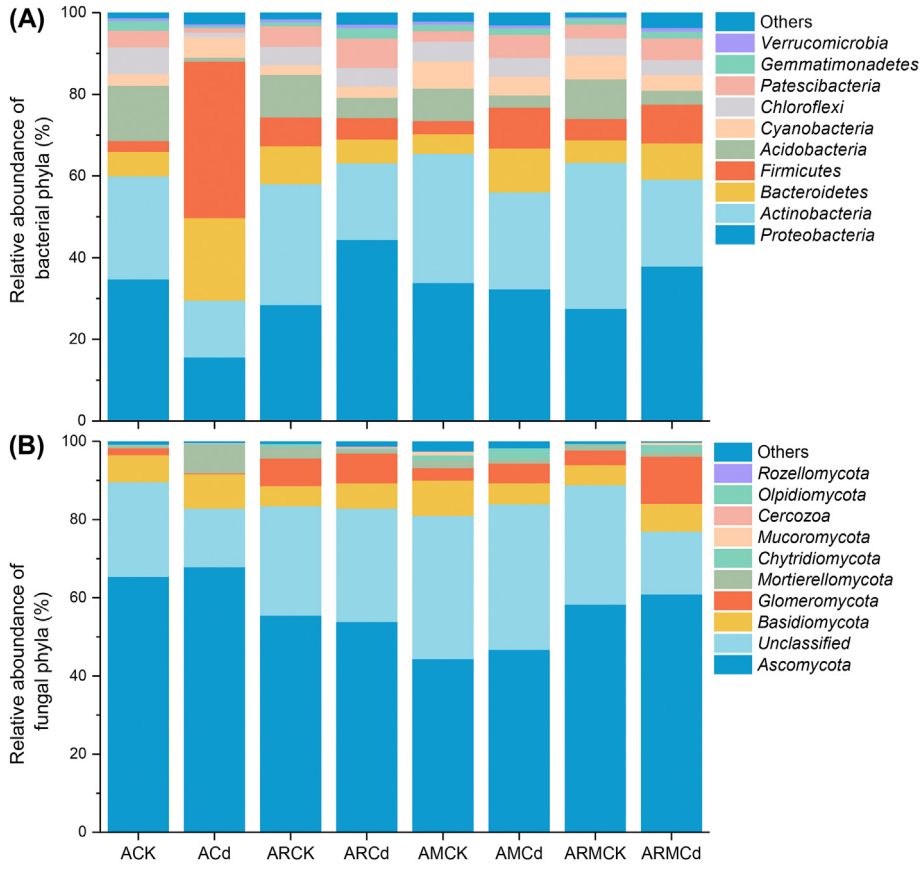

Fig. 2. Relative abundance of the dominant bacterial (A) and fungal (B) taxa in different treatments. (ACK (alfalfa), ACd (alfalfa + Cd), ARCK (alfalfa + *S. meliloti*), ARCd (alfalfa + *S. meliloti* + Cd), AMCK (alfalfa + *G. mosseae*), AMCd (alfalfa + *G. mosseae* + Cd), ARMCK (alfalfa + *S. meliloti* + *G. mosseae*), and ARMCd (alfalfa + *S. meliloti* + *G. mosseae* + Cd)).

3.4. Structures of the rhizospheric microbial community

Our study detected 10 bacterial phyla throughout the different treatments (with a relative abundance > 1%), namely, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, *Cyanobacteria*, *Chloroflexi*, *Patescibacteria*, *Gemmatimonadetes*, and *Verrucomicrobia* (Fig. 2A). Other phyla with a relative abundance <1% were clustered as "Others". *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Firmicutes* were the dominant bacterial phyla in all treatments. The addition of Cd, inoculation, and their interaction significantly affected the relative abundances of *Firmicutes*, *Bacteroidetes*, and *Acidobacteria* (Table S5). In particular, the addition of Cd notably decreased the relative abundance of *Actinobacteria* in all inoculation (AR, AM, and ARM) treatments, while it increased the relative abundance of *Firmicutes* in two inoculation (AM and ARM) treatments.

Our study detected 10 fungal phyla throughout the different treatments (with a relative abundance > 1%), namely, *Ascomycota*, *Unclassified*, *Basidiomycota*, *Glomeromycota*, *Mortierellomycota*, *Chytridiomycota*, *Mucoromycota*, *Cercozoa*, *Olpidiomycota*, and *Rozellomycota* (Fig. 2B). The dominant fungal phyla in all treatments were *Ascomycota* (44.3–65.7%) and *Unclassified* (14.9–36.6%). The addition of Cd significantly affected the relative abundance of *Cercozoa* and *Rozellomycota*, and inoculation greatly affected the relative abundances of *Glomeromycota*, *Mortierellomycota*, and *Rozellomycota* ($P < 0.05$; Table S5). Other phyla accounted for a minute fraction of the fungal-community composition, such as *Basidiomycota* and *Glomeromycota*. Also, analysis of indicator species showed that these bacterial taxa (i.e., *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, *Bacteroidetes*, and *Firmicutes*) and fungal taxa (i.e., *Glomeromycota*, *Mortierellomycota*, and *Rozellomycota*) from a phylum to family level were the main indicators of rhizosphere microbial communities after co-inoculation with rhizobia and AMF (Table 3, S6, and S7).

Two-way ANOVAs analysis indicated that the addition of Cd, inoculation, and their interaction remarkably affected the community structures of both bacteria and fungi ($P < 0.001$; Table S5). Moreover, NMDS and ANOSIM analysis showed that both the fungal and bacterial community structures significantly differed in the Cd and inoculation treatments ($P < 0.001$; Table S8 and Fig. 3). The partial Mantel test further confirmed that additive Cd and inoculation treatments were both greatly correlated to dissimilarity within the fungal and bacterial communities (Table S9).

3.5. Relationships of microbial community with soil properties

Pearson correlation analysis identified significant correlations between Cd, OTUs, and alpha diversity indicators (i.e., Shannon, Simpson, Chao1, and ACE) of the bacterial community ($P < 0.05$; Table S4). Significant correlations were observed among SOC, TN, SOC:TN, and SOC:TP with OTUs and these alpha diversity indicators in the bacterial community except for Shannon indicators. However, no significant correlation was found between alpha diversity indicators of the fungal community and these soil properties except for OTUs and Shannon indicators in the fungal community with SOC and SOC:TN.

The results of VPA showed that soil properties and Cd content generally explained most variation (62.8%) in the bacterial

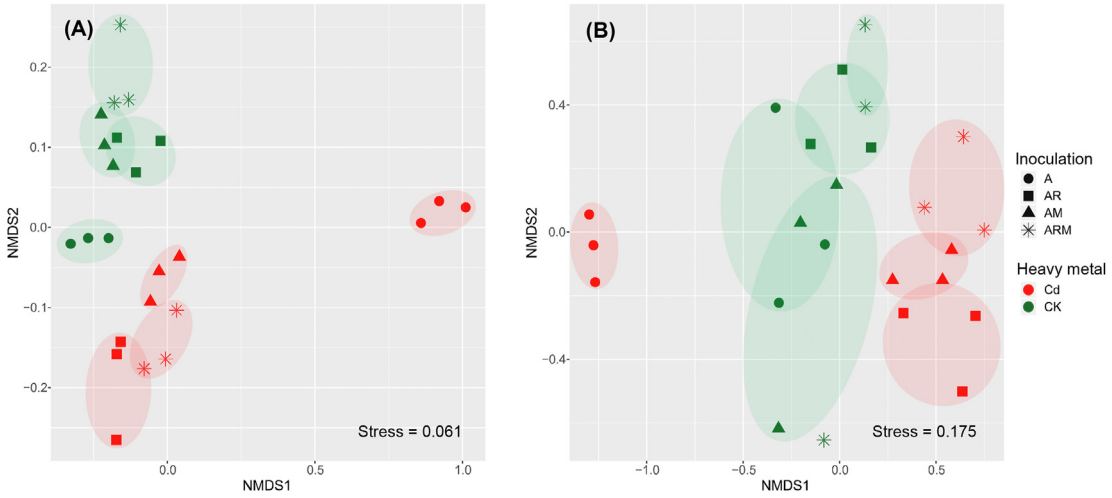

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination plots derived from the Bray-Curtis distance matrix. (A (alfalfa), AR (alfalfa + *S. meliloti*), AM (alfalfa + *G. mosseae*), and ARM (alfalfa + *S. meliloti* + *G. mosseae*). CK and Cd represent control check soil and adding Cd soil, respectively. The distance matrices were based on the relative abundances of the microbial OTUs. The configuration stresses were (a) 0.061 and (b) 0.175. Samples are clustered together by different treatments.).

community, while they only explained 12.9% of variation in the fungal community (Fig. 4A and B). The Mantel test and correlation analysis indicated that all soil variables were significantly correlated to the bacterial community except for TP and SOC:TP, while only Olsen-P and pH have significantly affected the fungal community (Fig. 4C). In addition, the RDA also confirmed that soil variables had greater apparent influence on the bacterial communities of the different treatments compared to the fungal communities of the different treatments (Fig. S3). According to the correlation heat map and RDA or CCA, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, and *Chloroflexi* in bacterial communities significantly correlated to soil variables except for TP ($P < 0.05$; Fig. 4D and Fig. S4A). In the fungal community, however, only *Chytridiomycota*, *Olpidiomycota*, and *Rozellomycota* showed a notable correlated to most soil variables ($P < 0.05$; Fig. 4E and Fig. S4B).

3.6. Cascading relationships among inoculation and additive Cd treatments with microbial communities, soil properties, plant biomass, and Cd uptake

PLS-PM indicated that inoculation of rhizobia and ARM directly affected fungi and bacterial diversity, and indirectly affected antioxidant enzyme activity, lipid peroxidation, and alfalfa biomass. Inoculation had positive effects on bacterial diversity (OTU, ACE, Chao 1, Simpson and Shannon indicators) but not for fungal diversity. Inoculation positively affected antioxidant enzyme activity and alfalfa biomass, and negatively affected lipid peroxidation (Fig. 5D). Moreover, inoculation had the greatest total effects on antioxidant enzyme activity (0.60) and plant biomass (0.55). In addition, bacterial community diversity had a significant positive total effect on antioxidant enzyme activity (0.45) and alfalfa biomass (0.38) compared to fungal community diversity.

4. Discussion

4.1. Implications of rhizobium and AMF co-inoculation in plant resistance on Cd stress

This study found an obvious increase in alfalfa biomass after four-month growth, and the highest biomass was observed in the co-inoculation of the rhizobia and AMF treatment (Table 1 and S1). Compare to the non-inoculated treatments, the single inoculation treatments with either rhizobia or AMF was accompanied by an increase in biomass production and improved nutrient acquisition (Ghnaya et al., 2015; Mnasri et al., 2017). Gao et al. (2018) showed that AMF inoculation increased the nodule numbers of legumes, which suggested that mycorrhiza formation provided adequate phosphorus (P) to support the development of nodules and bacteroid for nodulation. Among all inoculation treatments, the rhizobia and AMF co-inoculation treatment had the greatest overall effect on alleviating Cd stress in alfalfa.

The feasibility of a remediation strategy largely depends on the bioactivity of the residual heavy metals in soil and their availability in plants (Wood et al., 2016; Nagajyoti et al., 2010). The total Cd uptake in alfalfa shoots and roots was significantly higher in the rhizobia and AMF co-inoculation treatment than in the single inoculation or the non-inoculation treatments (Table 1). The concentration of Cd in alfalfa shoots remained stable, which indicated that inoculation could prevent excessive Cd accumulation in plant tissues and promote plant biomass at the same time (Table 1). Moreover, Cd primarily accumulated in the roots with an extremely low amount of Cd translocated to shoots, which hindered the occurrence of Cd-induced toxicity symptoms that appeared in shoots and subsequently benefited phytostabilization (Ghnaya et al., 2015; Ju et al., 2019).

Inoculation can increase alfalfa growth and improve its resistance to Cd stress. A significant increase in the Cd content of roots after inoculation was observed ($P < 0.05$; Table 1). However, the increased Cd content in roots did not lead to increased content in shoots nor did it inhibiting alfalfa growth, which further indicated that inoculation could improve alfalfa resistance to Cd stress. Our results showed that ROS accumulation in shoots and roots significantly decreased after inoculation (Table 2 and S2). The generation of ROS and the subsequent oxidative stress resulted in high MDA content, which indicated that the peroxidation of polyunsaturated fatty acids cause membrane damage (Duan et al., 2018). This implied that among all the inoculation treatments cell regulatory properties of H_2O_2 and O_2^- alleviated Cd-induced stress in alfalfa. The significant increase in antioxidant enzyme activities (POD, CAT, and SOD) in both roots and shoots of alfalfa after inoculation further indicated an alleviation in Cd stress (Table 2 and S2). The elevated SOD activity could be attributed to increased superoxide radicals, and this is because rhizobia and AMF can enhance the plant enzyme

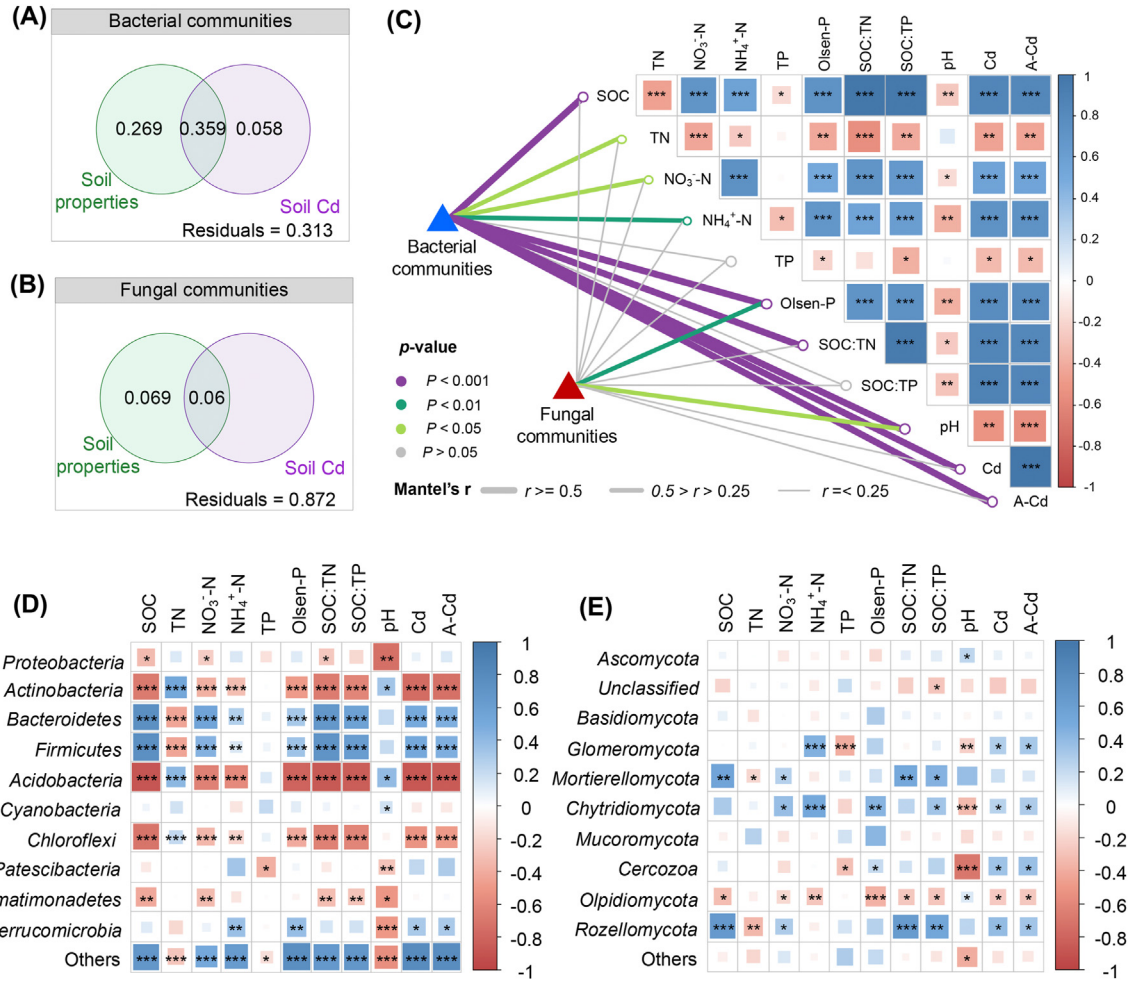

Fig. 4. Variation-partitioning analysis showing the variance percentages in the soil properties and soil Cd explained by the bacterial (A) and fungal (B) communities. Relationships between soil variables and microbial community structures (C). Correlation heat map of bacterial taxa (phylum level, D) and fungal taxa (phylum level, E) with soil properties. (Soil properties include SOC, soil organic carbon content; TN, total nitrogen content; TP, total phosphorus content; NO_3^-- N content; NH_4^+-N content; Olsen-P content; SOC: TN; SOC: TP; pH. Soil Cd includes soil total Cd and available Cd contents. Available Cd were represented by $CaCl_2$-extracted contents. Pairwise comparisons of environmental factors were displayed with color gradient denoting Spearman's correlation coefficients. Taxonomic groups were related to each soil variables by mantel test. SOC, soil organic-carbon content; TN, total nitrogen content; TP, total phosphorus content; NO_3^-- N content; NH_4^+-N content; Olsen-P content; SOC: TN; SOC: TP; pH; Cd, soil total Cd; A-Cd were represented by $CaCl_2$-extracted contents. * Correlation is significant at $P < 0.05$ (two-tailed); ** Correlation is significant at $P < 0.01$ (two-tailed); *** Correlation is significant at $P < 0.001$ (two-tailed).). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

de novo synthesis (Riffat and Masood, 2005). As a result, high antioxidant enzymatic activity in plants can protect cells against damage resulting from excessive ROS caused by Cd stress (Fang et al., 2020). Specifically, compared to the single inoculation treatments, the co-inoculation treatment resulted in the highest ROS content in alfalfa but the lowest corresponding MDA content (Table 2 and S2), which was consistent with the highest overall alfalfa biomass in the co-inoculation treatments. Compared to other microbial inoculation experiments (i.e., *Sinorhizobium* and *Agrobacterium*) (Jian et al., 2019), rhizobia and AMF co-inoculation, as fungi and bacteria, respectively, has advantage of effectively combining to mobilize N and P while exuding hormones to promote plant resistance (Ju et al., 2020; Lam and Lai, 2018). Therefore, rhizobia and AMF co-inoculation can assist plants in mitigating Cd stress by promoting plant SOD, CAT, and POD activities, ultimately eliminating ROS to the greatest extent. The co-inoculation of multiple symbiotic microbes can assist plants to effectively cope with environmental stress (Ben-Laouane et al., 2020).

4.2. Regulatory mechanisms involved in the improvement of plant Cd resistance through co-inoculation with rhizobium and AMF

The way in which rhizobia and AMF co-inoculating assists in increasing plant resistance is the key to revealing the mechanisms that promote growth in inoculation. Doak et al. (1998) reported that species diversity is positively correlated to stability. In our study, microbe-based inoculation significantly increased bacterial diversity and decreased fungal diversity after co-inoculation in the rhizosphere of alfalfa under Cd stress. This suggested that co-inoculation is beneficial to the restoration of bacterial community diversity while being unfavorable to fungal community diversity with the addition of Cd (Fig. 1). Community diversity reflects both community stability and functional diversity, and diversity can eventually influence process rates and state variables in ecosystems (Hillebrand and Matthiessen, 2009; Hu et al., 2016). Therefore, the potential of the bacterial community to assist alfalfa resistance to Cd stress was greater compared to the fungal community. Moreover, PLS-PM also identified significant positive effects of bacterial

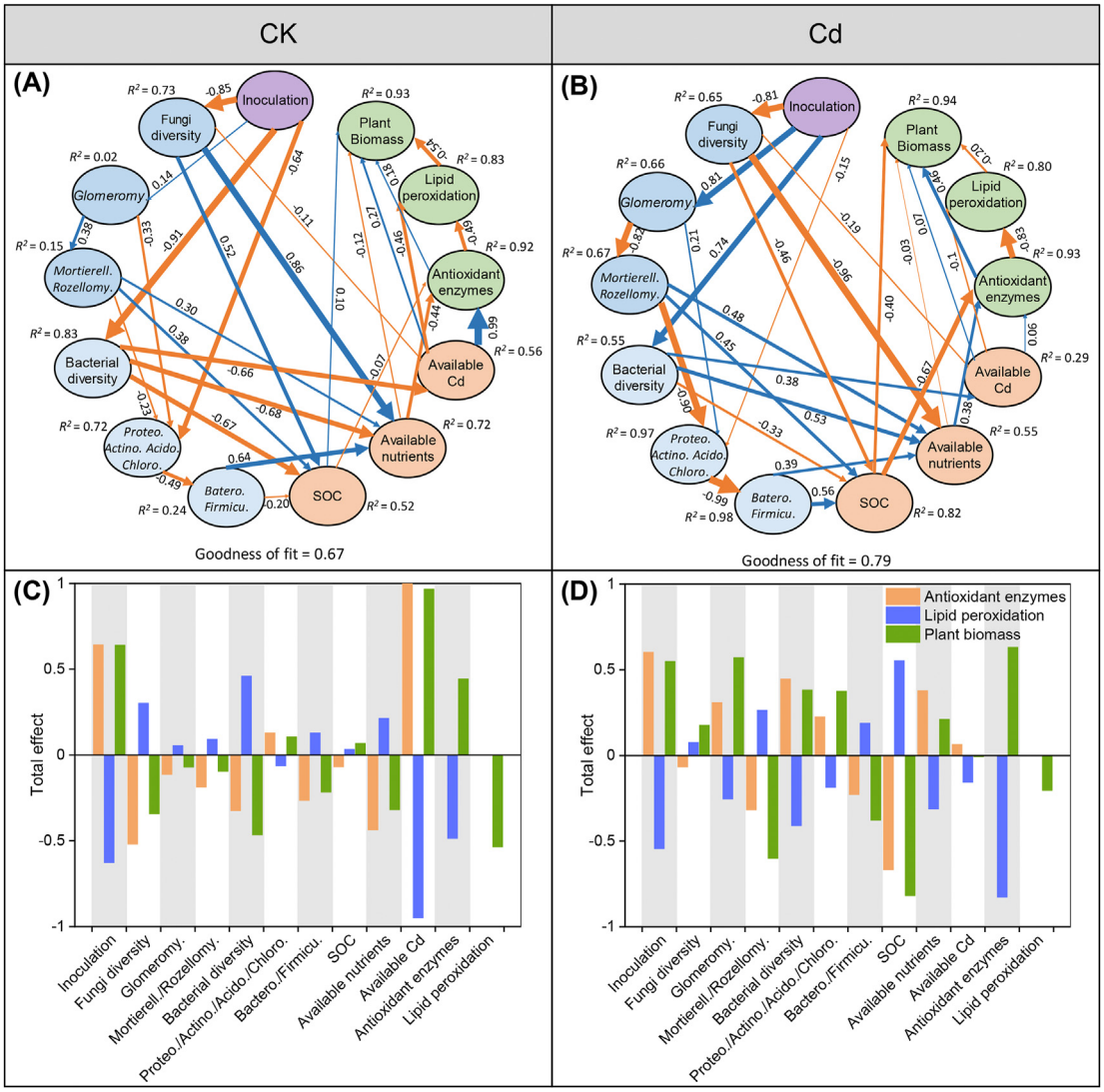

Fig. 5. Cascading relationships of treatments (Inoculation and Cd addition) with microbial communities, soil properties, and plant resistance. Partial least squares path modelling (PLS-PM) disentangling major pathways of the effects of treatments, microbial communities, and soil properties on plant resistance (A)(B); as well as the total effects of each variables on plant biomass, antioxidant enzymes, and lipid peroxidation (C)(D). (Yellow and blue arrows indicate negative and positive flows of causality ($P < 0.05$), respectively. R^2 indicates the dependent variable variance explained by the model. Numbers on the arrow indicate significant standardized path coefficients. Soil nutrient includes SOC, soil organic-carbon content; NH_4^+-N content; NO_3^--N content; Olsen-P content.). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

diversity on antioxidant enzyme activity and alfalfa biomass under Cd stress (Fig. 5).

The co-inoculation significantly affected both the bacterial and fungal community structure (Tables S5, S8, and S9, and Figs. 2 and 3). Given that microbial assemblages on a phylum level contain these microbial taxa with similar functional, metabolic, and morphological characteristics (Banerjee et al., 2018), our study specifically explored phylum changes within the microbial taxa. For bacterial taxa, the relative abundances of *Acidobacteria*, *Proteobacteria*, *Actinobacteria*, and *Chloroflexi* significantly increased, while the relative abundances of *Bacteroidetes* and *Firmicute* significantly decreased after co-inoculation (Fig. 2A and S1; Table S5). The increase in the relative abundance of *Proteobacteria* could be ascribed to rhizobia inoculation, employing rhizobia species (*S. meliloti*) that belongs to *Proteobacteria* (Table S6). *Bacteroidetes* and *Firmicutes* were the key taxa found under Cd contamination, but they were not the key taxa found after rhizosphere inoculation treatments (Table 3). Considering that many pathogens derive from *Bacteroidetes* and *Firmicutes*, the high abundance of *Proteobacteria* (such as *Pseudomonas*) thus reduces pathogen density as well as decreases disease incidences. This is caused by both intensified resource competition and interference with the pathogen (Hu et al., 2016). This supports the hypothesis that an increase in *Proteobacteria* can suppress the taxa reproduction capacity of *Bacteroidetes* and *Firmicutes* (Fig. S1A). The *Bacteroidetes* and *Firmicutes* can survive under potentially harsh conditions, such as acidic environments, pest control (i.e., pesticides) initiatives, and heavy metal pollution, while these two phyla do not appear to be competitive in healthy (non-contaminated) environments (Quadros et al., 2016; Gomez et al., 2013). These results implied that inoculation improved the rhizosphere microenvironment under Cd stress after inoculation. Furthermore, an increase in the relative abundance of *Actinobacteria*, *Acidobacteria*, and *Chloroflexi* could potentially be induced by a decrease in the relative abundance of *Bacteroidetes* and *Firmicutes* (Fig. S1A).

For fungal taxa, the relative abundance of *Glomeromycota*

Table 3
Taxa identified at the phylum level as rhizosphere potential indicators through indicator taxa analysis after inoculations at Cd addition treatments.

Microbes	Treatments	IndVal	*P*	Taxon (phylum)
Bacterial taxa	A (4)	0.976	0.013	*Firmicutes*
		0.918	0.002	*Bacteroidetes*
		0.797	0.014	*Spirochaetes*
		0.776	0.014	*Deferribacteres*
	AR (1)	0.863	0.018	*Fibrobacteres*
	AM (2)	0.802	0.041	*Actinobacteria*
		0.784	0.049	*Fusobacteria*
	ARM (4)	0.937	0.009	*Chloroflexi*
		0.875	0.008	*Proteobacteria*
		0.815	0.001	*Acidobacteria*
		0.807	0.024	*Actinobacteria*
Fungal taxa	A (2)	0.995	0.017	*Rozellomycota*
		0.923	0.017	*Mortierellomycota*
	AR (0)	–	–	–
	AM (0)	–	–	-
	ARM (1)	0.756	0.033	*Glomeromycota*

Note: The data show the top indicators based on their relative abundances with ≥70% perfect indication. A (alfalfa), AR (alfalfa + *S. meliloti*), AM (alfalfa + *G. mosseae*), and ARM (alfalfa + *S. meliloti* + *G. mosseae*).

significantly increased after AMF inoculation (AMF belongs to *Glomeromycota*) (Fig. 2B and S1B; Table S5). On the other hand, decrease in the relative abundance of *Mortierellomycota* and *Rozellomycota* could be ascribed to an increase in the relative abundance of *Glomeromycota* (Fig. S1B). As a result, after the rhizobia and AMF co-inoculation, these bacterial taxa (i.e., increased taxa include *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Chloroflexi*, while decreased taxa include *Bacteroidetes* and *Firmicutes*) and fungal taxa (i.e., increased taxa include *Glomeromycota*, while decreased taxa include *Mortierellomycota* and *Rozellomycota*) could potentially be the key rhizosphere microbiomes that improve alfalfa resistance to Cd.

The mechanisms associated with an increase in alfalfa resistance to Cd stress after symbiotic microbial inoculation could be attributed to the enhancement of the overall rhizosphere environment through means of symbiotic microbes (Hu et al., 2016; Hartman et al., 2017). Accordingly, this study further explored the relationship of the key taxa of microbes and soil properties in the improvement of alfalfa resistance to Cd. A significant increase in available rhizosphere soil nutrients after co-inoculation could assist plant nutrient acquirements and consequently improve their growth under Cd stress (Ju et al., 2020; Wood et al., 2016). However, it must be noted that the increase in key bacterial taxa negatively affected SOC and available soil nutrients, while the decrease in key bacterial taxa positively affected these variables after co-inoculation (Fig. 4). This suggested that co-inoculation reduced both SOC and available nutrient content in the rhizosphere of alfalfa. The production and secretion of organic molecules, such as organic acids via root systems, are typical ways that plants respond to stress, which can subsequently reflect the degree of environmental stress on plants (Sharma and Dietz, 2006). The content of SOC in the rhizosphere mainly derived from the production of plant root exudates, and this is because no other C sources were used in this short-term controlled pot experiment. Therefore, the decrease in SOC content in the rhizosphere could indicate alfalfa alleviation to Cd stress (Shen et al., 2020). Moreover, a subsequent increase in alfalfa biomass led to a decrease in available nutrients in the rhizosphere due to the absorption of nutrients by root systems.

Previous studies have indicated that many microbial taxa, namely, *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Chloroflexi* (e.g., *Acidobacteria* (*GP1*, *GP3*, and *GP6*), *Rhizobiales*, *Burkholderiales*, *Pseudomonadales*, and *Frankineae*) can assist plants to take up nutrients and improve plant productivity (Solans et al., 2016; Banerjee et al., 2018). For example, *S. meliloti* can induce rhizobia to occupy the nodules of legumes to assist nutrient uptake (Ju et al., 2019). One study showed that rhizobia may also provide disease protection, in that nutrient-providing rhizobia dominated root the microbiome of *Trifolium* that was enriched by the bacteria genera (Hartman et al., 2017). Additionally, many bacteria are known to alleviate iron (Fe), P, magnesium (Mg), and calcium (Ca) deficiencies, which prevent their translocation to plant roots due to heavy metal competition (Ouzounidou et al., 2006). These processes could also promote alfalfa growth by increasing the availability of microelements in the rhizosphere (Bulgarelli et al., 2013; Rajkumar et al., 2012). Therefore, the dominant process that the key bacterial taxa to improve alfalfa resistance to Cd stress could be the assistance that they provide alfalfa to uptake rhizosphere nutrients and reduce the plant photosynthetically derived carbon (C) release into soil (Fig. 5).

In contrast to the bacterial community, VPA showed that the fungal community had fewer effects on soil variables (Fig. 4A and B), which is consistent with our results that the fungal community did not strongly correlate to SOC and nutrients in the rhizosphere apart from Olsen-P and pH (Fig. 4C). Our results indicated that the effects associated with fungal communities in improving alfalfa resistance to Cd stress were lower than that of bacterial communities. These results were also supported by the weak correlations found between key fungal taxa and rhizosphere variables (Fig. 4E). However, variations in these key fungal taxa caused by AMF inoculation strongly affected key bacterial taxa (Fig. S2), suggesting these key fungal taxa could play indirect roles in assisting alfalfa growth under Cd stress through means of affecting the bacterial community. These results also shed light on the reasons why rhizobia and AMF co-inoculation promote alfalfa resistance and growth compared to their single inoculations. The key fungal taxa had a direct effect on the key bacterial taxa investigated by analyzed PLS-PM (Fig. 5), which has an indirect beneficial influence on plant resistance and growth. In general, the rhizosphere bacterial community, rather than the corresponding fungal community, mainly improved alfalfa resistance to Cd stress under rhizobia and AMF co-inoculation treatments.

5. Conclusions

This study offers new insight into how symbiotic microbes affect rhizosphere microbial taxa to assist alfalfa against Cd stress, for which the interplay among community members in the rhizosphere or the dynamics of community assemblages following inoculation was determined to be the important factor. Compared to their single inoculation, the rhizobia and AMF co-inoculation produced the greatest overall effect on alfalfa resistance to Cd stress. The rhizobia and AMF co-inoculation strongly affected the key bacterial taxa (*Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Chloroflexi*), which primarily improved alfalfa resistance to Cd stress by assisting plant to uptake nutrients from the rhizosphere and reduce the allocation of plant photosynthetically derived C into the soil. These findings are important for further improve plant resistance and ensure forage and crop production.

Credit author statement

Xia Wang: Writing – original draft, Visualization; **Linchuan Fang**: Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing; **Jingzi Beiyuan**: Resources, Writing – review & editing; **Yongxing Cui**: Methodology, Writing – review & editing; **Qi Peng**: Investigation; **Shilei Zhu**: Investigation; **Man Wang**: Investigation; **Xingchang Zhang**: Writing – review & editing.

Declaration of competing interest

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

Acknowledgments

This study was financially supported by the National Natural Science Foundation of China (41977031), and the Natural Science Foundation of Shannxi Province, China (2020JC-31).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.116758.

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