Rhizobium inoculation enhanced the resistance of alfalfa and soil enzymatic activity in Cu-polluted soils

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Abstract

Although some studies have reported an important role of rhizobia in mitigating heavy metal toxicity, the regulatory mechanism of the alfalfa-rhizobium symbiosis system to resist copper (Cu) stress through biochemical reactions in the plant-soil system is still unclear. Hence, this study assessed the effects of rhizobium inoculation (i.e., *Sinorhizobium meliloti* CCNWSX0020) on the growth of alfalfa and soil enzyme activities under Cu stress. Our results showed that rhizobium inoculation markedly alleviated Cu-induced growth inhibition by increasing chlorophyll content, height and biomass and the contents of nitrogen and phosphorus in alfalfa. The content of malondialdehyde (MDA) was increased in both shoot and root of alfalfa under Cu stress. The application of rhizobium alleviated Cu-induced phytotoxicity by increasing the activity of antioxidant enzymes and soluble protein content of tissues and inhibiting the level of lipid peroxidation (i.e., MDA level). In addition, rhizobium inoculation improved soil nutrient cycling, increased soil enzyme activities (i.e., β -glucosidase activity and alkaline phosphatase) and microbial biomass nitrogen. Both Pearson correlation coefficient analysis and partial least squares path modeling (PLS-PM) identified that the interactions between soil nutrient content, enzyme activity, microbial biomass and plant antioxidant enzymes and oxidative damage could jointly regulate plant growth. This study provides comprehensive insights into the mechanism of action of the legume-rhizobium symbiosis system to mitigate Cu stress and provide an efficient strategy for phytoremediation of Cu-polluted soils.

1. Introduction

In recent decades, copper (Cu) has led to severe soil and water pollution because of industrial production, sewage irrigation and heavy use of fertilizers and pesticides (Adrees et al. 2015; Huang et al. 2018; Saxena et al. 2020). Cu contamination of both water and soil not only directly threatens plants with severe toxicity (e.g., plant growth retardation, leaf shrinkage, and interference with a variety of metabolic processes) (Chen et al. 2015), but also endangers the living environment of soil organisms and damages soil quality, which ultimately has harmful effects on human (Huang et al. 2020; Chen et al. 2021).

Cu ions are highly susceptible to absorbed by plant roots, competing with each other and transferred to other organs (Sun et al. 2015; Duan et al. 2018). Plant growth restriction, leaf shrinkage, nutrient scarcity, antioxidant enzyme activity reduction, and lipid peroxidation of cells are common phenomena of plant damage caused by Cu stress (Liu et al. 2016; Abbas et al. 2017). In response to metal-induced oxidative stress, plants have evolved integrated metal detoxification mechanisms that often regulate antioxidative enzymes and non-enzymatic antioxidants autonomously to alleviate excessive contents of reactive oxygen species (ROS) and malondialdehyde (MDA) (Chen et al. 2018; Fang et al. 2020; Zhou et al. 2020). For example, to effectively increase detoxification capacity, antioxidant enzyme activity of plants increases upon exposure to heavy metals (Mostofa et al. 2014), which favors plant growth as well as causes an enhancement in biomass and heavy metal uptake.

Soil enzymes are both mediators of most soil transformation processes and catalysts for organic matter decomposition and nutrient cycling (Ju et al. 2019; Aponte et al. 2020). Up to now, soil enzyme activities have been commonly used as an indicator of ecological health of terrestrial ecosystems under heavy metal contamination (Yang et al. 2016; Duan et al. 2018). Cu-contamination has deleterious effects on the abundance and activity of soil microorganisms, primarily in terms of soil basal respiration, microbial biomass and enzymatic activity, and even soil microbial activity (Molina-Santiago et al. 2019; Zheng et al. 2019). Nutrient deficiency and high toxicity of heavy metal contaminated soils are two dominant factors limiting plant growth. The interactions between contaminated stressed plants and soils are extremely complex and the transfer of Cu is associated with plant-soil biochemical systems. Therefore, to promote plant growth and optimize potential health risks, there is an urgent need to explore effective strategies that can mitigate metal phytotoxicity and improve soil quality.

Recently, legumes have attracted interest due to their functions in the remediation of phytotoxicity in metal-polluted soils (Naveed et al. 2015; Duan et al. 2019; Ju et al. 2019). The symbiotic relationship between legumes and soil microorganisms, such as rhizobia, can promote plant growth directly or indirectly by increasing nitrogen (N) and phosphorus (P) uptake and enhancing plant defenses in heavy metal-contaminated soils. Therefore, legume-rhizobia symbiosis is widely used in phytoremediation of heavy metals (Dary et al. 2010; Kong et al. 2015). In addition, it has been shown that rhizobia can enhance the soil quality and benefit plant growth by enhancing the activity and diversity of soil microorganisms and reducing soil pathogens (Wang et al. 2016; Ju et al. 2019). Furthermore, inoculation with rhizobia was found to be effective in reducing ROS levels and accelerating the ascorbate-glutathione cycle to protect plant seedlings from excessive Cu stress (Pajuelo et al. 2011; Chou et al. 2019). However, a detailed mitigation mechanism of legume-rhizobium mediated mitigation of Cu stress in the biochemical response of the plant-soil system are still limited.

Alfalfa is a perennial forage grass that can survive in extreme environments and is widely distributed in the mining areas of northwest China (Cui et al. 2018a). Therefore, it is important to further improve the resistance of plant pastures to heavy metals, reduce their accumulation in tissues, and increase the biomass of plant for safe livestock production. In this study, a copper-resistant strain (*Sinorhizobium meliloti*) was selected as an exogenous additive. The main objectives of this study were: (1) to analyze the effects of rhizobium inoculation on the growth and physiological resistance of alfalfa; (2) to determine the regulatory mechanisms of rhizobium inoculation to alleviate Cu stress in plants through biochemical responses of the plant-soil system. These results will help us better understand the role of alfalfa-rhizobium symbiosis and the regulatory mechanisms against metallic Cu toxicity.

2. Materials And Methods

2.1. Experimental design

The soil sample utilized in this study was collected from the soil (0-20 cm) of the uncontaminated soil within the Yangling District, Shaanxi, China. The properties of the soil were: pH, 8.49, total Cu, 13.2 mg kg⁻¹

¹, soil organic carbon (SOC), 7.65 g kg⁻¹, total nitrogen (TN), 0.2 g kg⁻¹, total phosphorus (TP), 0.36 g kg⁻¹. The collected soil was air-dried, removed from impurities such as stones and plant debris and sieved. After that, CuSO₄ solution was added, mixed thoroughly and aged in the dark at room temperature for three months. Ultimately, the experiment included five Cu-addition level: 0, 200, 400, 600 and 800 mg kg⁻¹, respectively (denoted as Cu 0, Cu 200, Cu 400, Cu 600 and Cu 800, respectively).

The Cu contaminated soil (600 g) was evenly packed into a plastic pot (10 cm diameter). Alfalfa seeds were sterilized in hydrogen peroxide solution (30%, v:v), then washed several times with distilled water, placed in seedling trays containing germination paper, which was placed in an incubator to await seed germination. Twenty seedlings showing the same growth characteristics were selected and transplanted into each pot. After the alfalfa has grown its first leaves, the plant roots are injected with a rhizobia bacterial solution. The strain we used was a metal-resistant rhizobia (*Sinorhizobium meliloti* CCNWSX0020) (Fan et al. 2011; Duan et al. 2019). The source and growth of *S. meliloti* can be seen in our previous study (Duan et al. 2019). The *S. meliloti* suspensions (20 mL) were added to the plant roots once a week (three times in total). The uninoculated controls received the same amount of sterile distilled water. There were three replicates of each treatment. Plants and soils were harvested on day 60 after the first inoculation, and indicators such as plant and soil enzyme activity were measured.

2.2. Determination of plant indexes

The chlorophyll content of plant leaves was extracted with 90% acetone, and then quantified using a spectrophotometer (Sobrinho-Plata et al. 2014), the details of the method can be found in our previous study (Fang et al. 2020). Use a straightedge to measure alfalfa height and root length. The shoot and root were washed with sterilized deionized water and dried at 60 °C to constant weight. About 0.3 g of alfalfa samples were digested with 8 mL of HNO₃ and 2 mL of HClO₄ mixture, and then diluted to a certain volume with distilled water, and the Cu concentration was determined using an atomic absorption spectrophotometer (Hitachi, FAAS Z-2000, Japan). Total Cu uptake = shoot/root biomass × shoot/root Cu concentration. The shoot and root samples were digested with H₂SO₄ and H₂O₂, then diluted and fixed with distilled water, and finally N and P content of tissue was determined by flow analyzer.

Fresh shoots and roots were used to determine alfalfa MDA, soluble protein content and antioxidant enzyme activities. The MDA content of alfalfa tissues was determined using the kit provided by Suzhou Comin Biotechnology Co., Ltd (Suzhou, China). The procedure was as follows: 1 mL of the extraction was added to grind the shoot and root parts of the alfalfa. Then centrifuge at 8000 rpm for 15 min. The supernatant was aspirated and added to the color development solution in a boiling water bath for 30 min. After cooling and centrifugation, the MDA content in the supernatant was then determined. The soluble protein content was also determined according to the kit procedure.

Plant antioxidant enzyme activities (i.e., total superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX)) were also determined using the purchased kits. Fresh alfalfa tissue samples were first ground with 1 mL of the extraction and then centrifuged at 8000 rpm for 15 min.

The supernatant was determined spectrophotometrically after the addition of the appropriate chromogenic agent to the supernatant. For detailed steps, please refer to the previous studies of our group (Chen et al. 2018; Duan et al. 2018).

2.3. Assays of soil properties

Soil pH was determined by conventional methods, and the detailed steps were referred to the previous study (Duan et al. 2019). The SOC and TN were determined using the $K_2CrO_7-H_2SO_4$ oxidation method and Kjeldahl method, respectively. Soil air-dried soils were wet digested with H_2SO_4 and $HClO_4$, and TP was determined at 880 nm using an ultraviolet spectrophotometer (UV3200, Shimador, Japan). Soil NH_4^+-N and NO_3^-N were determined using a continuous flow autoanalyzer. Dissolved organic carbon (DOC) in fresh soil samples was extracted with 50 mL of distilled water and then measured using a Liqui TOCII analyzer (Elementar, Germany). The available P (AP) was determined via sodium bicarbonate extraction. Total soil Cu content was determined using a modified USEPA Method 3051A. The procedure was as follows: the soil samples (sieved to 0.149 mm) were digested by fifteen mL of acid mixture (i.e., 8 mL HNO_3 , 2 mL $HClO_4$ and 5mL HCl), diluted and fixed with distilled water, and finally the Cu concentration was determined by atomic absorption spectrophotometer.

2.4. Assays of soil enzyme activities and microbial biomass

The methods for measuring soil enzyme activities including urease (UR), β -glucosidase (BG), alkaline phosphatase (ALP) and catalase were as previously described (Guan 1986; Duan et al. 2018). The UR, BG and ALP activities were determined using a spectrophotometer at 587, 400 and 578 nm, respectively. Among them, UR was used by the sodium phenolate colorimetric method, BG by the *p*-nitrophenol colorimetric method, and ALP by the sodium benzyl phosphate colorimetric method. Moreover, soil catalase activity was determined using the method of potassium permanganate titration.

Microbial biomass carbon (MBC) and nitrogen (MBN) were analyzed using an improved method (Vance et al. 1987; Cui et al. 2018b). Weigh 10 g of fresh soil samples were fumigated with ethanol-free chloroform in the dark for 24 hours, and then extracted with 40 mL of 0.5 M potassium sulfate solution for 30 min. The supernatant was filtered and the extractable organic carbon (EOC) and nitrogen (EON) contents were determined simultaneously by LiquiTOC II analyzer (Elementar, Germany). Similarly, 10 g of unfumigated soil samples were taken to determine the EOC and EON. MBC and MBN were calculated from the difference of EOC and EON between fumigated and non-fumigated samples, respectively.

2.5. Statistical analyses

All the data used in this study were average values of replicated experiments. Analysis of variance (ANOVA) with the Duncan's tests was conducted at a significant level of $P < 0.05$ by the SPSS 20 software. All bar graphs were plotted using Origin Pro 2021. Pearson correlation coefficient analysis and partial least squares path modeling (PLS-PM) analysis were performed in R software (version 3.6.2). The software packages "ggcorrplot" and "plsrm" were used, respectively. The correlation heat map was used

to compare and determine the relationships between different indicators, and PLS-PM was used to determine the direct and indirect factors affecting plant growth.

3. Results

3.1. *Plant growth phenotype and the N and P content of alfalfa*

Based on Fig. 1, the shoot height, root length and biomass of alfalfa were increased as the increasing of Cu concentration in treatments, but in a way, rhizobium inoculation alleviated the growth inhibition of the alfalfa. For example, compared to the non-inoculated treatment, alfalfa height, root length and shoot and root biomass in the inoculated treatment were increased by 19.15%, 6.94%, 35.29% and 33.33%, respectively, under the Cu 800 treatment (Fig. 1). The chlorophyll content increased significantly after inoculation with rhizobium, and the increase of chlorophyll by inoculation with rhizobium was more distinct at the Cu concentration of 800 mg kg⁻¹, which was 1.44 times higher than that of the non-inoculated treatment (Table 1). At Cu 800 mg kg⁻¹, the chlorophyll a and b were significantly lower than that of other treatments.

The N level of alfalfa shoot and root was superlative in the Cu 0 treatment, but did not differ significantly with increasing Cu stress (Table 1). Inoculation with rhizobium increased N content of shoot at the Cu concentrations of 0 mg kg⁻¹ and 800 mg kg⁻¹. Interestingly, the root N content was significantly increased by rhizobium inoculation. In addition, the P level in alfalfa tissues consistently decreased with the increasing Cu concentration (Table 1). Compared to the uninoculated treatment, inoculation with rhizobia significantly increased the P levels in the alfalfa tissues (Table 1), particularly for the Cu 600 and Cu 800 treatments ($P < 0.05$).

3.2. *Differences Cu and Cu uptake by alfalfa*

It is noteworthy that the effect of rhizobia on copper concentration in difference parts of alfalfa was not uniform (Table 2). Specifically, exogenous addition of rhizobia significantly reduced the Cu concentration in the shoot but noticeably increased the Cu concentration in the root ($P < 0.05$). In the shoots, the Cu uptake by the inoculated plants was noticeably lower than that of the non-inoculated plants in the Cu 0, Cu 400 and Cu 600 treatments ($P < 0.05$). Interestingly, rhizobium inoculation decreased the Cu uptake of root in treatments with low Cu concentrations (0 or 200 mg kg⁻¹). However, under high Cu concentrations, the Cu uptake of root was increased with by rhizobium inoculation. Except for Cu 0 treatment, the transfer coefficients of Cu were considerably less than 1.0 (Table 2). And the rhizobium inoculation increased Cu transfer coefficients in treatments with low Cu concentrations (0 or 200 mg kg⁻¹). However, under high Cu concentrations, the Cu transfer coefficients were decreased with rhizobium inoculation.

3.3. *Plant oxidative damage, soluble protein and antioxidant enzyme activity*

Exploring the effect of heavy metals on lipid peroxidation damage by measuring the MDA content of lipid peroxidation reaction (Fig. 2a). Based on Fig. 2a, the MDA content was increased in both shoots and

roots as the increasing of Cu concentration in treatments, but in a way, rhizobium inoculation reduced the accumulation of MDA in plant tissues. The content of MDA in the shoot and root parts of Cu 800 mg kg⁻¹ increased by 29.2% and 41.4%, respectively, compared with that of Cu 0 mg kg⁻¹ (Fig. 2a). In the Cu 400, Cu 600 and Cu 800 treatment, the reduction of shoot after inoculation of rhizobium was 13.8%, 17.7% and 17.8%, respectively; and reduction in the root was 17.6%, 18.4% and 24.6%, respectively. This indicating that inoculation of rhizobium had a greater relieving effect on roots than aboveground parts. Additionally, MDA content in shoots and roots was noticeably negatively correlated to N and P content ($P < 0.001$) content (Fig. 5).

In non-inoculated plants, the highest shoot and root soluble protein contents were detected in the Cu 800 treatment, which was 1.83 and 1.21 times higher compared to the Cu 0 treatments, respectively (Fig. 2b). Compared with the non-inoculated treatment, the rhizobium-inoculated plants exhibited higher soluble protein content in the alfalfa tissue (Fig. 2b). In the Cu 400, Cu 600 and Cu 800 treatment, the reduction of shoot after inoculation of rhizobium was 47.9%, 29.8% and 20.8%, respectively; and reduction in the root was 19.5%, 8.8% and 16.1%, respectively. In addition, soluble protein content in roots was significantly positively correlated to N content ($P < 0.05$) (Fig. 5b). The SOD activity in the shoots of non-inoculated plants showed no obvious change, while the SOD activity in the roots of alfalfa showed a downward trend under copper stress (Fig. 3a). Rhizobium inoculation significantly increased SOD activity of the shoot under Cu 600 mg kg⁻¹ ($P < 0.05$). Plants inoculated with rhizobium exhibited higher SOD activity in the roots compared to uninoculated plants (Fig. 3a). For example, in the Cu 600 treatment, the increases of shoot and root after inoculation of rhizobium was 22.1% and 65.3%, respectively (Fig. 3b). The highest root POD activity was exhibited in the Cu 600 treatment. Rhizobium inoculation significantly increased the POD activity in the shoots and roots ($P < 0.05$). Compared with the uninoculated alfalfa, application of rhizobium reduced the CAT activity in shoot by 31.44%, 62.40%, 61.87% and 19.92% for the Cu 0, Cu 200, Cu 400 and Cu 800 treatments, respectively ($P < 0.05$); while application of rhizobia markedly enhanced the CAT activity in the Cu 600 treatment ($P < 0.05$) (Fig. 3c). The inoculation of rhizobium had no obvious effect on CAT activity in the alfalfa root. Differences in alfalfa tissue APX activity between uninoculated and inoculated plants were not consistent and were influenced by specific Cu treatments (Fig. 3d). And the application of rhizobium increased the alfalfa APX activity in the low Cu concentrations (0 or 200 mg kg⁻¹). SOD activity of shoot was significantly and negatively correlated to the content of N and P ($P < 0.01$). However, the APX activity of alfalfa (shoot and root) was significantly and positively correlated with the content of N and P, respectively ($P < 0.05$) (Fig. 5).

3.4. Soil nutrient, enzyme activities and microbial biomass

The soil Cu concentration decreased significantly under rhizobium inoculation ($P < 0.05$) (Table 3). Rhizobium inoculation significantly increased NO₃⁻-N and NH₄⁺-N content in Cu 400 and Cu 800 treatments ($P < 0.05$). In the inoculated treatment, the highest AP content was detected in the Cu 600 mg kg⁻¹, which was higher of 42.19% and 27.93% compared to the Cu 0 and the Cu 800 treatments, respectively (Table 3). However, the effect of rhizobium inoculation on AP was not obvious. And the

rhizobium inoculation increased the DOC content in the low Cu concentrations (200 or 400 mg kg⁻¹), on the contrary, application of rhizobia significantly reduced the DOC at the Cu concentration of 600 mg kg⁻¹ ($P < 0.05$).

The changes of soil enzymes (i.e., catalase, UR, BG and ALP) were shown in Fig. 4. In the non-inoculated treatment, the catalase activity in Cu 0 was significantly higher than that in other treatments, the increasing Cu concentration strongly inhibited catalase activity. Rhizobium inoculation remarkably increased catalase activity in Cu 400 and Cu 600 treatments ($P < 0.05$). The inoculation of rhizobia had a notable effect on the BG and ALP activity. However, the inoculation of rhizobium only enhanced the UR activity in the low Cu concentrations (0 or 200 mg kg⁻¹). Rhizobium inoculation significantly increased MBN in Cu 200, Cu 400 and Cu 600 treatments ($P < 0.05$) (Fig. S1).

3.5. Driving factors affecting plant growth

The N content of shoot and root showed the strongest and positive correlations with DOC, β -glucosidase, alkaline phosphatase, urease and SOC, but the strongest and negative correlations with MBN, $\text{NH}_4^+\text{-N}$, AP and Cu concentrations ($P < 0.05$) (Fig. S2). In addition, the plant P showed the strongest positive correlation with MBC, soil enzyme activities, and SOC; and strongest negative correlation with MBN, $\text{NH}_4^+\text{-N}$, TN, AP and Cu concentrations ($P < 0.05$) (Fig. S2). We used PLS-PM to further reveal the effects of plant physiological indicators, soil physicochemical properties and soil biochemical indicators on alfalfa growth (Fig. 6). The PLS-PM method showed that plant antioxidant enzyme activities, soil nutrient content and soil enzyme activities had a significant positive direct effect (0.35, 0.28 and 0.42, respectively; $P < 0.01$) on plant growth (Fig. 6a). The application of *S. meliloti* (0.34), soil microbial biomass (0.61), soil enzyme activities (0.82), soil nutrient (0.28), and plant antioxidant enzyme activities (0.35) had positive total effects on the plant growth, whereas the plant oxidative damage (-0.13) induced negative total effects (Fig. 6b).

4. Discussion

Plants grown in Cu-contaminated soils could suffer from physiological dysfunction, growth inhibition, reduced biomass, and metal accumulation, among other phenomena (Duan et al. 2019; Saeid and Aboalfazl 2019; Fang et al. 2020). In this study, inoculation of rhizobium (i.e., *S. meliloti*) not only increased the plant height and biomass of alfalfa, but also increased its chlorophyll content (Table 1; Fig. 1), indicating that alfalfa-rhizobium symbiosis plays an important role in soil Cu bioremediation. Moreover, our results showed that the inoculation of rhizobia significantly promoted Cu accumulation in alfalfa roots (Table 2). Stambulska and Bayliak (2019) found that the pathway that reduces the toxic effects of heavy metals on legumes may be due to their preferential accumulation in the root nodules of legume-rhizobium symbiosis. Chlorophyll is an important substance for photosynthesis in plants, and chlorophyll content has a direct impact on photosynthetic efficiency (Tang et al. 2021). Inoculation with rhizobium significantly increased chlorophyll content (Chou et al. 2019), which helped to promote photosynthetic reaction rate and alleviate the toxic effects of Cu stress. Copper stress reduces the total N

content in plant tissues and affects the N fixation of alfalfa, but inoculation with rhizobium significantly increases the N content of alfalfa roots (Table 1), providing sufficient source of N for plant growth and alleviating alfalfa toxicity. In addition, the effect of rhizobium inoculation on N content of the shoots was remarkably lower than that of the roots (Table 1). The nodules probably function in the root system and are the main nitrogen-fixing organ. Moreover, this indicates that the Cu-resistant strain (i.e., *S. meliloti*) is able to survive at 0–800 mg kg⁻¹ Cu concentrations and promote alfalfa nitrogen fixation. Heavy metal-tolerant rhizobia affect the biological effectiveness of metals in soil, mainly because rhizobia promote plant growth by regulating biological nitrogen fixation (Duan et al. 2019; Shen et al. 2019; Shen et al. 2020). The P content in alfalfa increased significantly after application with rhizobia at 600 and 800 mg kg⁻¹ Cu concentration (Table 1). These results demonstrate that alfalfa-rhizobium symbiosis alleviates Cu stress in soils by increasing biomass, N and P content, and chlorophyll content of alfalfa.

Oxidative damage and antioxidant enzyme activities can effectively reflect the intensity of toxicity suffered by plant cells, and are important for understanding the metal-specific toxicity mechanisms in plants (Chen et al. 2018; Romero-Puertas et al. 2018). Excessive accumulation of Cu led to the production of large amounts of MDA in alfalfa seedlings, which intensified oxidative stress in cells (Fig. 2). Moreover, the MDA contents were significantly and negatively correlated to both plant N and P content (Fig. 5). The high level of MDA caused severe damage to plant cell membrane and inhibited plant uptake of N, P and other nutrients. Under Cu stress, plants inoculated with rhizobia significantly reduced the MDA content in the aboveground and roots (Fig. 2). The results of PLS-PM further supported that the rhizobium inoculation had a strong negative effect on MDA (Fig. 6). This indicates that plants inoculated with rhizobium suffered less oxidative damage and had a higher antioxidant capacity under Cu stress. These results suggest that inoculation with rhizobium can effectively reduce the damage to alfalfa by reactive oxygen species under Cu stress.

The soluble protein content of plants can indicate the physiological and biochemical reactions in plants, and the higher the content, the more vigorous the metabolic activity, which is conducive to better resistance of plants to external stresses (Yang et al. 2019). Plants inoculated with rhizobium exhibited higher soluble protein content in alfalfa tissue compared to uninoculated plants (Fig. 2b). This finding indicates that the addition of rhizobia improved the symbiotic performance of alfalfa and promoted an increase in soluble protein content, especially under non-optimal conditions. Meanwhile, alfalfa has high antioxidant enzyme activities that protect their cells from oxidative damage caused by external environmental stresses (i.e., higher MDA levels) (Filis et al. 2016; Hojati et al. 2017). Cu stress reduced the SOD activity in alfalfa tissue (Fig. 3a). Cu-contamination induced destruction of membranes and enzyme systems results in the reduce of SOD activity, especially at high Cu concentration (600 and 800 mg kg⁻¹). Interestingly, inoculation with rhizobia had a more pronounced effect on the increase of SOD activity in alfalfa roots (Fig. 3a). This result indicates that rhizobium application enhanced superoxide dismutase activity in tissues and improved their resistance to adversity stresses. Furthermore, in comparison with uninoculated alfalfa tissue, the activities of POD and APX in shoot and root of inoculated alfalfa were increased at different levels of symbiosis development (Fig. 3). Moreover, our results showed that a

significant positive correlation between APX activities and the N and P content of alfalfa (Fig. 5). Heavy metal resistant rhizobacteria in soil can directly affect soil Cu concentration through chelation, precipitation and biosorption. In addition, the alfalfa-rhizobium symbiotic system can promote plant nitrogen fixation, stimulate the release of phytohormones and antioxidant enzyme activities, and ultimately reduce the toxicity in alfalfa and promote alfalfa growth (Hao et al. 2014; Fang et al. 2020). Nonetheless, addition of rhizobia reduced the Cu-induced CAT activities in the shoots, except for the Cu 600 treatment (Fig. 3c). It suggests that rhizobium inoculated in the shoots inhibit the damage caused by excess hydrogen peroxide and therefore did not need to induce more CAT activities to eliminate the production of hydrogen peroxide caused by Cu concentration in the alfalfa tissues (Cao et al. 2017; Bei yuan et al. 2021). Hence, it is hypothesized that rhizobia addition mitigates Cu-toxicity in alfalfa by increasing antioxidant enzyme activities and soluble protein content.

Soil enzyme activity was an important indicator to characterize the fertility and health of soils contaminated with toxic metals. Rhizobium inoculation significantly increased catalase activity in Cu 400 and Cu 600 treatments (Fig. 4a). Some studies showed that the soil catalase could achieve detoxification by changing the valence reference of heavy metal ions (Yang et al. 2016; Fang et al. 2017). Meanwhile, inoculation with rhizobium significantly promoted alkaline phosphatase activity (Fig. 4d). Phosphatase in soils was mainly produced by microbial activity and was critical for mobilizing the organic forms of P (Duan et al. 2019; Wei et al. 2019). This suggests that soil catalase and phosphatase activities were positively influenced directly and indirectly by the legume-rhizobia symbiosis. There was a significant negative correlation between urease, phosphatase and catalase activities and Cu content (Fig. S2), it was shown that the legume-rhizobia symbiosis regulated the rate of enzyme synthesis by Cu or altered the interference of enzyme-producing microbial community toxicity, thus reducing metal toxicity in soil (Yu et al. 2019; Fang et al. 2020). Moreover, N and P contents of alfalfa were significantly positively correlated with activities of four soil enzymes (Fig. S2). Further, PLS-PM results showed a significant positive total effect of soil enzymes and nutrient content on plant growth following *S. meliloti* application (Fig. 6). Based on the above information, we found that the legume-rhizobium symbiosis system promoted alfalfa growth in Cu-contaminated soil by regulating plant physiological and biochemical properties, soil nutrients, and soil enzyme activities.

5. Conclusions

Our results showed that excessive accumulation of Cu in alfalfa exhibited varied toxicity symptoms, including decreased biomass, chlorotic leaves, and inhibition of antioxidant enzyme activities. Rhizobium inoculation significantly improved the growth of alfalfa under Cu stress due to effectively improving detoxification ability. Legume-rhizobium symbiosis system resists Cu-induced oxidative stress in alfalfa tissues by enhancing antioxidant enzyme activities to scavenge superfluous MDA. Moreover, soil catalase, phosphatase, and β -glucosidase, as well as MBN, were higher in the rhizobium inoculated treatment compared to the non-inoculated control. These results can improve our understanding of the mechanism of microbial inoculation to mitigate Cu toxicity and provide guidance for pasture cultivation in heavy metal contaminated areas.

Declarations

Author contribution

Chengjiao Duan: writing - original draft, writing - review & editing, visualization; **Yuxia Mei:** Methodology, Writing - review & editing; **Qiang Wang:** Methodology, Writing - review & editing; **Yuhan Wang:** Methodology, Writing - review & editing; **Qi Li:** Investigation, Methodology; **Maojun Hong:** Investigation; **Sheng Hu:** Investigation; **Shiqing Li:** Writing - review & editing; **Linchuan Fang:** Funding acquisition, Methodology, Resources, Supervision, Writing - review & editing.

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Data availability

All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate: Not applicable.

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Competing interests: The authors declare no competing interests.

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Tables

Table 1 The chlorophyll, nitrogen and phosphorus content in alfalfa tissue.

Treatments		Chlorophyll content (mg g ⁻¹)			Nitrogen (g kg ⁻¹)		Phosphorus (g kg ⁻¹)	
		a	b	Total	Shoot	Root	Shoot	Root
Cu 0	Control	0.62 ± 0.01 Ab	0.42 ± 0.02 Aa	1.04 ± 0.02 ABb	21.1 ± 0.37 Ab	17.5 ± 0.17 Aa	1.25 ± 0.01 Ab	0.81 ± 0.01 Ab
	<i>S.meliloti</i>	0.66 ± 0.02 BCa	0.47 ± 0.03 Aa	1.13 ± 0.03 Ba	23.1 ± 0.41 Aa	17.2 ± 0.18 Aa	1.51 ± 0.03 Aa	1.33 ± 0.02 Aa
Cu 200	Control	0.63 ± 0.01 Ab	0.43 ± 0.01 Aa	1.06 ± 0.01 Ab	20.0 ± 0.39 Ba	15.9 ± 0.29 Bb	1.2 ± 0.01 Ba	0.76 ± 0.01 Ba
	<i>S.meliloti</i>	0.74 ± 0.02 Aa	0.48 ± 0.02 Aa	1.22 ± 0.03 Aa	21.4 ± 0.93 Ba	17.2 ± 0.23 Aa	1.24 ± 0.01 Ba	0.74 ± 0.02 Ba
Cu 400	Control	0.56 ± 0.02 Aa	0.41 ± 0.01 Aa	0.97 ± 0.01 Bb	17.1 ± 0.11 Ca	14.3 ± 0.07 Cb	1.09 ± 0.03 Ca	0.60 ± 0.01 Ca
	<i>S.meliloti</i>	0.62 ± 0.01 Ca	0.42 ± 0.01 ABa	1.04 ± 0.01 Ca	17.8 ± 0.30 Ca	15.2 ± 0.09 Ca	0.97 ± 0.02Db	0.63 ± 0.01 Ca
Cu 600	Control	0.61 ± 0.03 Aa	0.40 ± 0.02 Aa	1.01 ± 0.04 ABb	17.7 ± 0.32 Ca	14.4 ± 0.12 Cb	0.90 ± 0.01 Db	0.59 ± 0.01 Cb
	<i>S.meliloti</i>	0.71 ± 0.01 ABa	0.45 ± 0.01 Aa	1.16 ± 0.01 ABa	18.4 ± 0.05 Ca	15.6 ± 0.02 Ca	0.97 ± 0.02 Da	0.64 ± 0.02 Ca
Cu 800	Control	0.38 ± 0.04 Bb	0.22 ± 0.01 Bb	0.61 ± 0.02 Cb	17.1 ± 0.18 Cb	14.7 ± 0.15 Cb	0.74 ± 0.01 Eb	0.51 ± 0.01 Db
	<i>S.meliloti</i>	0.52 ± 0.03 Da	0.37 ± 0.01 Ba	0.88 ± 0.02 Da	18.9 ± 0.38 Ca	16.2 ± 0.07 Ba	1.05 ± 0.02 Ca	0.55 ± 0.02 Da

Note: The capitalized letters indicate significant differences between different Cu concentrations, whereas the lower-case letters indicate significant differences between non-inoculated and inoculated alfalfa under the same Cu concentration condition ($P < 0.05$). Each value represents the mean ± SE (n = 3).

Table 2 Cu concentrations and the total uptake of Cu in alfalfa tissue.

Note: The capitalized letters indicate significant differences between different Cu concentrations, whereas

Treatments		Cu concentrations (mg kg ⁻¹)		Total uptake (µg plant ⁻¹)		TF
		Shoot	Root	Shoot	Root	
Cu 0	Control	8.54 ± 0.07 Da	24.9 ± 0.56 Ea	3.25 ± 0.03 Ba	9.30 ± 0.21 Da	0.34 ± 0.01 Ab
	<i>S.meliloti</i>	6.45 ± 0.12 Db	3.06 ± 0.22 Eb	2.68 ± 0.05 Bb	1.18 ± 0.08 Eb	2.13 ± 0.19 Aa
Cu 200	Control	12.3 ± 0.90 Ca	42.8 ± 0.92 Da	2.96 ± 0.22 Ba	14.3 ± 0.31 Ca	0.29 ± 0.03 Bb
	<i>S.meliloti</i>	10.4 ± 0.15 Ba	26.1 ± 0.04 Db	3.41 ± 0.05 Aa	10.3 ± 0.02 Db	0.40 ± 0.01 Ba
Cu 400	Control	15.9 ± 0.27 Ba	50.4 ± 0.33 Cb	2.45 ± 0.04 Ca	15.6 ± 0.10 Bb	0.32 ± 0.01 Ba
	<i>S.meliloti</i>	8.54 ± 0.08 Cb	64.6 ± 0.32 Ca	1.89 ± 0.02 Cb	22.4 ± 0.11 Ba	0.13 ± 0.01 Ca
Cu 600	Control	28.7 ± 0.56 Aa	93.3 ± 1.20 Ab	4.13 ± 0.08 Aa	18.8 ± 0.24 Ab	0.31 ± 0.01 Ba
	<i>S.meliloti</i>	15.7 ± 0.70 Ab	119 ± 0.75 Aa	3.16 ± 0.14 Ab	31.5 ± 0.20 Aa	0.13 ± 0.01 Cb
Cu 800	Control	13.2 ± 0.70 Ca	55.2 ± 2.89 Bb	1.49 ± 0.08 Da	9.06 ± 0.47 Db	0.24 ± 0.02 Ca
	<i>S.meliloti</i>	7.50 ± 0.70 CDb	83.1 ± 0.68 Ba	1.31 ± 0.12 Da	19.9 ± 0.16 Ca	0.09 ± 0.01 Cb

the lower-case letters indicate significant differences between non-inoculated and inoculated alfalfa under the same Cu concentration condition ($P < 0.05$). TF (translocation factor: shoot concentration/root concentration). Each value represents the mean ± SE (n = 3).

Table 3 Effect of rhizobium inoculation on soil physicochemical properties.

Treatments		Cu concentration (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)	TN (g kg ⁻¹)	AP (mg g ⁻¹)	SOC (g kg ⁻¹)	DOC (mg kg ⁻¹)
Cu 0	Control	8.23 ± 0.36 Ea	2.67 ± 0.04 ABb	0.44 ± 0.01 Da	0.77 ± 0.01 Aa	18.3 ± 0.59 Eb	6.66 ± 0.11 Aa	133 ± 3.93 ABa
	<i>S.meliloti</i>	5.34 ± 0.17 Eb	2.94 ± 0.07 Ba	0.43 ± 0.01 Ca	0.76 ± 0.01 Ca	22.8 ± 0.67 Da	6.65 ± 0.20 Aa	138 ± 2.80 ABa
Cu 200	Control	194 ± 0.75 Da	2.83 ± 0.03 Aa	0.41 ± 0.01 Da	0.77 ± 0.01 Aa	21.7 ± 0.09 Da	6.67 ± 0.14 Aa	136 ± 0.71 Ab
	<i>S.meliloti</i>	185 ± 0.48 Db	2.73 ± 0.03 Ca	0.42 ± 0.01 Ca	0.78 ± 0.01 ABa	21.8 ± 0.19 Da	6.63 ± 0.18 Aa	144 ± 2.50 Aa
Cu 400	Control	336 ± 1.09 Ca	2.71 ± 0.05 ABb	1.84 ± 0.01 Ab	0.77 ± 0.01 Aa	35.8 ± 0.86 Ba	6.54 ± 0.13 Aa	97.8 ± 2.24 Cb
	<i>S.meliloti</i>	310 ± 3.74 Cb	3.22 ± 0.05 Aa	2.31 ± 0.01 Aa	0.79 ± 0.01 Aa	35.5 ± 1.42 Ba	6.57 ± 0.09 Aa	113 ± 1.47 Da
Cu 600	Control	536 ± 7.06 Ba	2.61 ± 0.08 Ba	1.57 ± 0.01 Ba	0.78 ± 0.01 Aa	38.7 ± 1.08 Aa	6.75 ± 0.02 Aa	135 ± 0.86 Aa
	<i>S.meliloti</i>	505 ± 7.29 Bb	2.71 ± 0.04 Ca	1.63 ± 0.01 Ba	0.78 ± 0.01 ABa	39.4 ± 0.34 Aa	6.45 ± 0.05 Ab	124 ± 1.92 Cb
Cu 800	Control	757 ± 8.76 Aa	2.65 ± 0.02 Bb	1.21 ± 0.01 Cb	0.77 ± 0.01 Aa	27.3 ± 1.23 Ca	6.40 ± 0.07 Aa	124 ± 4.52 Ba
	<i>S.meliloti</i>	708 ± 7.16 Ab	3.12 ± 0.07 Aa	1.63 ± 0.01 Ba	0.77 ± 0.01 BCa	28.4 ± 0.35 Ca	6.62 ± 0.04 Aa	133 ± 5.74 BCa

Note: TN: total nitrogen; AP: available phosphorus; SOC: soil organic carbon; DOC: dissolved organic carbon. The capitalized letters indicate significant differences between different Cu concentrations, whereas the lower-case letters indicate significant differences between non-inoculated and inoculated alfalfa under the same Cu concentration condition ($P < 0.05$). Each value represents the mean ± SE (n = 3).

Figures

Figure 1

The effect of inoculation with *S. meliloti* on length and biomass in alfalfa tissue with different Cu concentration treatments.

Figure 2

The effect of inoculation with *S. meliloti* on MDA and soluble protein in alfalfa tissue with different Cu concentration treatments.

Figure 3

The effect of inoculation with *S. meliloti* on plant antioxidant enzyme activities in alfalfa tissue.

Figure 4

The effect of inoculation with *S. meliloti* on soil enzyme activities under different Cu concentration treatments.

Figure 5

A correlation heat map illustrating pairwise relationships between plant nutrient elements and plant properties in alfalfa shoot (a) and root (b) based on Pearson correlation coefficient analysis.

Figure 6

Partial least squares path modeling (PLS-PM) disentangling major pathways of the effects of *S. meliloti*, soil microbial biomass, enzyme activity, soil nutrient, plant oxidative damage and plant antioxidant enzyme activities on plant growth.

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