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Rhizobium inoculation enhances copper tolerance by affecting copper uptake and regulating the ascorbate-glutathione cycle and phytochelatin biosynthesis-related gene expression in *Medicago sativa* seedlings

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ABSTRACT

Despite numerous reports that legume-rhizobium symbiosis alleviates Cu stress in plants, the possible roles of legume-rhizobium symbiosis and the regulatory mechanisms in counteracting Cu toxicity remain unclear. Here, *Sinorhizobium meliloti* CCNWSX0020 was used for analyzing the effects of rhizobium inoculation on plant growth in *Medicago sativa* seedlings under Cu stress. Our results showed that rhizobium inoculation alleviated Cu-induced growth inhibition, and increased nitrogen concentration in *M. sativa* seedlings. Moreover, the total amount of Cu uptake in inoculated plants was significantly increased compared with non-inoculated plants, and the increase in the roots was much higher than that in the shoots, thus decreasing the transfer coefficient and promoting Cu phytostabilization. Cu stress induced lipid peroxidation and reactive oxygen species production, but rhizobium inoculation reduced these components' accumulation through altering antioxidant enzyme activities and regulating ascorbate-glutathione cycles. Furthermore, legume-rhizobium symbiosis regulated the gene expression involved in antioxidant responses, phytochelatin (PC) biosynthesis, and metallothionein biosynthesis in *M. sativa* seedlings under Cu stress. Our results demonstrate that rhizobium inoculation enhanced Cu tolerance by affecting Cu uptake, regulating antioxidant enzyme activities and the ascorbate-glutathione cycle, and influencing PC biosynthesis-related gene expression in *M. sativa*. The results provide an efficient strategy for phytoremediation of Cu-contaminated soils.

1. Introduction

Copper (Cu), is an essential micronutrient, that plays an important role in the normal growth and development of plants, directly participating in various metabolic pathways (Merlos et al., 2016; Yruela, 2009). However, Cu is a toxic agent when it is in excess in plants. Cu toxicity can cause serious damage to the permeability of cell membranes, inhibit protein synthesis and enzyme activities, and finally induce plant senescence and even death (Miotto et al., 2014; Sun et al., 2015; Yruela, 2009; Zhang et al., 2014b). In China, Cu contamination is also very serious, especially for soils near Cu mines and industrial areas (Li, 2006). Moreover, severe Cu contamination inhibits plant growth and results in a nutrient deficiency, threatening the human health (Zhang et al., 2014b).

Legumes are well known for their capacity to form root nodules and increase soil nitrogen through biological nitrogen fixation (Pajuelo et al., 2011). Therefore, legume-rhizobium symbiosis plays an important role in ecologically and agriculturally (Hao et al., 2014; Naveed et al., 2015). Recently, legumes have attracted attention for their roles in remediating toxicity to plants in metal-contaminated soil. Previous studies have shown that rhizobia promote plant growth and enhance the defense of plants against stressful environments (Pajuelo et al., 2011; Wani et al., 2008). For instance, legumes accumulated heavy metals mainly in their roots and showed a low level of metal translocation to shoots (Kong et al., 2015a; Pajuelo et al., 2011). In addition, *Sinorhizobium* inoculation alleviated the toxicity of arsenic by regulating arsenic accumulation in *Medicago sativa* seedlings (Pajuelo et al., 2008, 2011). However, the detailed phytoremediation mechanism of the symbiosis is still largely unknown, and how symbiosis regulates the Cu stress in plants is not clear.

At the cellular level, Cu can interact with membrane proteins, leading to lipid peroxidation and oxidative stress by disturbing the

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balance between oxidation and reduction in plants (Kong et al., 2015a; Sun et al., 2015; Wang et al., 2015; Duan et al., 2018). For example, excess Cu induced the production of superfluous reactive oxygen species (ROS) in plants. Due to the fast metabolism of ROS, their actual level is affected by the balance between generation and removal by antioxidative enzymes, as well as non-enzymatic antioxidants (Mittler, 2002). Antioxidative enzymes include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX), etc. (Mittler, 2002; Mostofa et al., 2014). Non-enzymatic antioxidative defense systems include small molecule antioxidants such as reduced glutathione (GSH), ascorbate (AsA) and others. The AsA-GSH cycle is an important and effective pathway to remove superfluous hydrogen peroxide (H_2O_2) in plants (Foyer and Noctor, 2011; Noctor and Foyer, 1998). GSH alleviated Cu stress through reducing Cu uptake and enhancing antioxidant ability in rice seedlings (Mostofa et al., 2014). The observation suggested that the AsA-GSH cycle might be involved in Cu tolerance in plants. GSH, which has dual roles as an antioxidant or as a precursor of phytochelatins (PCs), plays an important role in heavy metal tolerance (Flores-Caceres et al., 2015; Jozefczak et al., 2012). Furthermore, MTs have low molecular weight, cysteine-rich proteins that bind heavy metals (Leszczyszyn et al., 2013). MT deficiency impacted Cu accumulation and redistribution in leaves and seeds of Arabidopsis (Benatti et al., 2014). Therefore, PCs and MTs play an important role in Cu homeostasis in plants, but it is not clear whether PCs and MTs are involved in regulating the legume-rhizobium symbiosis in response to Cu stress.

However, a detailed mitigation mechanism of legume-rhizobium in regulating Cu stress is remains unknown. Therefore, the Cu-resistant strain *Sinorhizobium meliloti* CCNWSX0020 was selected as the experimental strain (Kong et al., 2015a, 2015b). The aims of this study were as follows: (1) to study the effect of rhizobium inoculation on the growth phenotype and Cu transfer in *Medicago sativa* seedlings; (2) determine how rhizobium inoculation regulates antioxidant enzyme activities and the AsA-GSH cycle under excess Cu stress; and (3) evaluate whether PC biosynthesis and MT gene expression levels are affected by rhizobium inoculation. These results will increase our understanding of the role of legume-rhizobium symbiosis and the regulatory mechanisms in counteracting metal Cu toxicity.

2. Materials and methods

2.1. Plant growth and treatment

Medicago sativa seeds (Beijing Rytway Ecotechnology Co., LTD) were first sterilized in 75% ethanol for 3 min and then in 10% sodium hypochlorite solution for an additional 10 min followed by washing with distilled water and germinating in a vermiculite with soil (1:2) for 14 d. Approximately 14-d-old seedlings were planted in plastic pots (10 cm diameter) filled with approximately 100 g mixture of vermiculite and perlite (2:1). The mixture medium was treated with copper (Cu) in the form of $CuSO_4$ to produce a series of Cu concentration including 0, 50, 100, 200, 300, and 400 mg kg^{-1} (0, 50, 100, 200, 300, and 400 ppm). Thirty seedlings were placed in each pot and each treatment had six pots. The seedlings were watered with the Fåhraeus nitrogen-free mineral nutrient solution when necessary. Seedlings were grown in a controlled growth chamber with a relative humidity of 80%, a light/dark regime of 14/10 h, a photosynthetically active radiation (PAR) of 200 µmol m^{-2} s^{-1}, and temperature of 22/25 °C (night/day).

After plants grew their first leaves, seedlings were inoculated with a cell suspension of wild-type *Sinorhizobium meliloti* CCNWSX0020 (Fan et al., 2011). The growth of *S. meliloti* and the method of treatment was in accordance with the method of Kong et al. (2015a). Plants were harvested at 60 days after inoculation to determine their physiological index and other measurements.

2.2. Determination of copper and nitrogen element contents

Cu concentration was measured using an atomic absorption spectrophotometer (Hitachi Z2000, Hitachi, Japan) according to the method of Chen et al. (2013). The Cu content was calculated using Cu concentrations and sample biomass. To evaluate the transport ability of Cu from roots to shoots under excess Cu conditions in plants, the transfer coefficient was calculated using the following formula,

$$\text{Transfer coefficient} = Cu_{shoot}/Cu_{root}$$

where Cu_{shoot} and Cu_{root} are Cu concentrations in shoots and roots, respectively.

Total N concentration was determined using a Kjeldahl digestion method by a Nitrogen Analyzer System (Kjeltec 8400 Auto System II, Foss Tecator AB, Höganäs, Sweden), according to the method of Kong et al. (2015a).

2.3. Determination of lipid peroxidation, superoxide radical, and hydrogen peroxide contents

Lipid peroxidation was evaluated by measuring the malondialdehyde (MDA) content (Yan et al., 2010). The MDA content was measured using an MDA reagent kit (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China). The production of a superoxide radical ($O_2^{\cdot-}$) was determined using an $O_2^{\cdot-}$ reagent kit (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China). The OD value of the solution was measured with a spectrophotometer (Mapada UV6300PC, Mapada, Shanghai) at 530 nm using $NaNO_2$ as the standard curve. Hydrogen peroxide (H_2O_2) was measured using an H_2O_2 reagent kit (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China). H_2O_2 content was detected by measuring the absorbance at 415 nm with a spectrophotometer.

2.4. Assay of antioxidant enzyme activities

Fresh shoots and roots were homogenized in an ice bath with 1 ml of extraction buffer (50 mM phosphate buffer solution containing 1 mM ascorbic acid and 1 mM EDTA) at 4 °C. The homogenate was centrifuged at 15,000*g* for 15 min at 4 °C, and the supernatant was used for assaying antioxidant enzyme activities (Chen et al., 2013). The activity of total superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), and ascorbate peroxidase (APX, EC 1.11.1.11) were assayed according to the manufacturer's instructions of reagent kits (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China).

2.5. Measurement of non-protein thiols and cysteine contents

The total content of non-protein thiols (NPTs) in *M. sativa* seedlings was measured according to the manufacturer's instructions using the NPTs reagent kit (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China). Fresh shoots and roots were ground in an ice bath with 1 ml extraction buffer, and then 4 ml of methyl alcohol was added and mixed for 10 min at room temperature. The homogenate was centrifuged at 10,000*g* for 10 min at 4 °C and the supernatant was used for assaying NPTs contents. Finally, NPTs content was determined by measuring the absorbance at 412 nm with a spectrophotometer.

The cysteine content was determined using a cysteine reagent kit (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China). Fresh shoots and roots were ground in an ice bath with 1 ml extraction buffer. The homogenate was centrifuged at 8000*g* for 10 min at 4 °C, and then the supernatant was used for measuring cysteine content according to the manufacturer's instructions. The OD value of the solution was measured with a spectrophotometer at 600 nm.

2.6. Determination of glutathione and ascorbate contents

An assay of reduced glutathione (GSH) and disulphide glutathione (GSSG) content was estimated using the kit of GSH and GSSG reagents (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China) according to the manufacturer's instructions. Fresh shoots and roots were each ground and extracted with 1 ml extraction buffer. The homogenate was then centrifuged at 8000*g* for 10 min at 4 °C. The supernatants were used for assaying GSH and GSSG content according to the manufacturer's instructions. The OD value of the solution at 412 nm was used to calculate the GSH and GSSG content of plant tissues. The GSH/GSSG ratio was calculated using the two components' values.

Reduced ascorbate (AsA) and dehydroascorbate (DHA) were determined spectrophotometrically using AsA and DHA reagent kits (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China). Fresh shoot and root samples (0.1 g) were ground in an ice bath and extracted with 1 ml extraction buffer. Subsequently, the homogenate was centrifuged at 8000*g* for 20 min at 4 °C. The supernatants were used for assaying the AsA and DHA content according to the manufacturer's instructions. The AsA and DHA contents were determined by measuring the absorbance at 265 nm with a spectrophotometer. The AsA/DHA ratio was calculated using the two components' values.

2.7. Determination of dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase activities

Enzyme activities were detected using reagent kits (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China). Samples (0.1 g) were ground in liquid nitrogen and extracted with 1 ml of extraction buffer. The homogenates were then centrifuged at 8000*g* for 15 min at 4 °C. The supernatants were used for assaying the activities of dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR), respectively. DHAR activity was measured by assaying the absorbance of OD265 nm. MDHAR activity was determined by analyzing the absorbance of OD340 nm. GR activity was determined by monitoring the change in absorbance at 340 nm. The calculation method of these enzyme activities was performed according to the manufacturer's instructions.

2.8. Total RNA extraction and gene expression analysis

Total RNA was isolated from *M. sativa* shoots and roots (0.3 g) under different treatments with an RNA extraction reagent kit (TaKaRa, Dalian, China) according to the manufacturer's instructions (Chen et al., 2013). Quantitative real-time PCR (qRT-PCR) was used for analyzing the related gene expression using a QuantStudio™ 6 Flex Real-Time PCR System (Life Technologies, Thermos, USA). A 25 μl real-time PCR reaction system was used in our experiment according to Chen et al. (2011) with some minor modifications. Primers (Supporting information Table S1) were designed according to known sequences in the NCBI database. The procedures of dsDNA synthesis used in the qRT-PCR are listed in Supporting information Table S2. Relative quantification values for each target gene were calculated by the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). Gene expression levels were standardized using *Actin* as the internal control. The mRNA quantity from the Control (without Cu and *S. meliloti*) was set as "1" for each gene, and other treated samples were expressed relative to the corresponding control. The average expression abundance of the three biological replicates, including two technical replicates for each biological replicate, was calculated. Then hierarchical clustering of the expression profiles was performed on the log base 2 average expression fold values using R language.

2.9. Statistical analysis

For physiological and biochemical measurements, four replicates were used. Statistical treatment of the data was carried out by one-way analysis of variance (One-way ANOVA) with SPSS 19.0 (SPSS, Chicago, Illinois USA). Duncan's post-test ($P < 0.05$) was used for multiple comparisons. Data are expressed as means ± SE. In all figures, different capital letters indicate significant differences between different treatments, whereas the lowercase letters indicate significant differences between non-inoculated and inoculated *M. sativa* seedlings under the same Cu concentration condition.

3. Results and discussion

3.1. Legume-rhizobium symbiosis alleviates Cu-induced growth phenotype inhibition and Cu accumulation in plants

Severe Cu stress significantly inhibited the growth and development of plants. Moreover, it was noted that seedlings exposed to excess Cu tended to be short and small, with leaf chlorosis (Fig. 1A and Fig. S1, S2). In addition, Cu stress exerted an obvious influence on the legume-rhizobium symbiosis (Fig. S1). For instance, Cu stress notably reduced nodule number and weight in inoculated *M. sativa* seedlings (Fig. S1E and F), which is consistent with previous reports on heavy metal stress in legume-rhizobium symbiosis (Balestrasse et al., 2006; Hao et al., 2014; Kong et al., 2017, 2015b; Kopittke et al., 2007; Pajuelo et al., 2008, 2011; Wani et al., 2008). Interestingly, rhizobium inoculation significantly alleviated Cu-induced inhibition of plant growth in *M. sativa* seedlings. The beneficial effects of rhizobia on legume plant growth in the presence of heavy metals has been noted in previous studies (Kong et al., 2015a, 2015b; Wani et al., 2007), suggesting that legume-rhizobium symbiosis plays an important role in Cu bioremediation of soil.

Cu stress did not significantly decrease shoot N concentration in non-inoculated plants, but in inoculated plants, Cu stress inhibited the shoot N concentration (Fig. 1B). Interestingly, rhizobium inoculation increased shoot N concentration in the control (0 ppm) and in the low Cu concentrations (50 or 100 ppm). However under high Cu concentrations, the shoot N content was not significantly altered by rhizobium inoculation (Fig. 1B), which is consistent with the report of Kong et al. (2015a). Moreover, the effect of rhizobium inoculation on N concentration in the roots was more significantly increased than that in the shoots under different Cu concentrations (Fig. 1C). A possible explanation is that the nodules, the main N fixation organs, function in the roots; the N concentration in the shoots could be considered as the supply of N through N-fixation in the root nodules, since a nitrogen-free nutrient solution was used in this study. This means that the copper-resistant strain, *S. meliloti* CCNSWX0020, could survive under the Cu concentration used in this study, and meanwhile promotes a normal level of plant nitrogen. Similarly, many reports have also indicated that N concentration was increased in other legume plants grown in heavy metal-contaminated soils (Kong et al., 2015b, 2017; Wani et al., 2008). Moreover, in the presence of rhizobium, N concentration was decreased by 10.8% under the high Cu concentration (400 ppm) compared with the control (0 ppm) (Fig. 1C), suggesting that severe Cu stress (400 ppm) inhibited nitrogen fixation in legume plants. Moreover, the N concentration has close relationship with chlorophyll synthesis and photosynthesis in plants. For instance, previous studies have shown that the photosynthetic capacity of leaves is related to their nitrogen content, as the proteins of the Calvin cycle and thylakoids represent the majority of leaf nitrogen. Moreover, there are strong linear relationships between nitrogen and chlorophyll. With increasing nitrogen, chlorophyll content and photosynthesis in the leaves experienced similar increase (Fritschi and Ray, 2007; Shangguan et al., 2000; Evans, 1989). Therefore, we also determined the chlorophyll concentration and photosynthesis in *M. sativa* seedlings. Our results indicate that Cu stress did not significantly affect chlorophyll concentration compared to the control, except for 100 and 400 ppm Cu concentrations (Fig. S2A). At 100 ppm Cu, the chlorophyll concentration was increased by 22.6%

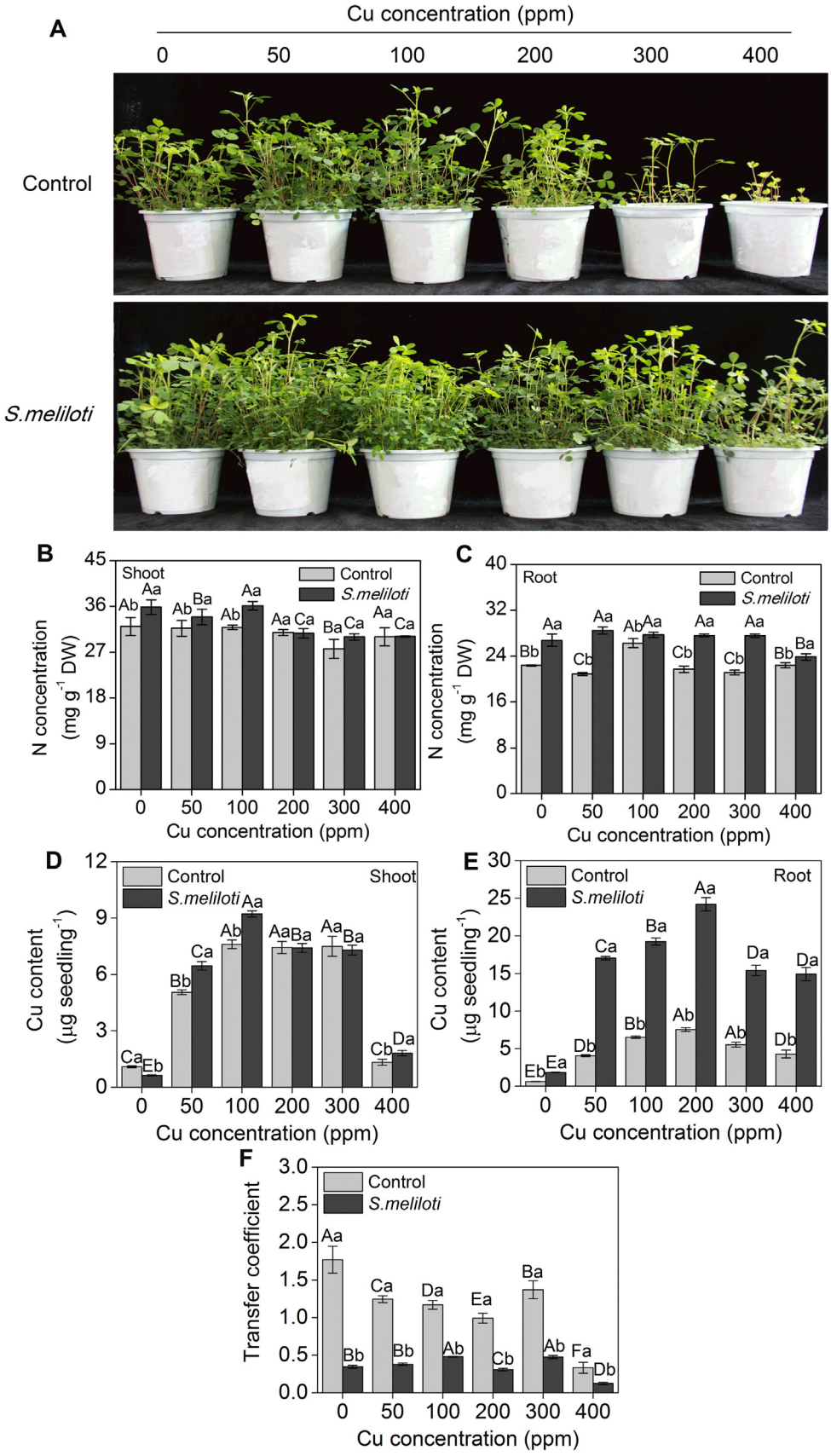

Fig. 1. The effect of rhizobia inoculation on the growth phenotype (A), N concentration in the shoots (B) and roots (C), the uptake amount of Cu in the shoots (D) and roots (E), and transfer coefficient (F) in *Medicago sativa* seedlings treated with different Cu concentration. Each value represents the mean ± SE ($n = 4$). Columns labeled with different letters indicate significant differences with $P < 0.05$. Different capital letters (A, B, C, D, E, and F) indicate significant differences ($P < 0.05$) between different treatments (0, 50, 100, 200, 300, and 400 ppm Cu treatments), whereas the lowercase letters (a and b) indicate significant differences ($P < 0.05$) between non-inoculated and inoculated *M. sativa* seedlings under the same Cu concentration condition.

compared with the control (0 ppm), but a high Cu concentration decreased the chlorophyll content (Fig. S2A). Cu is an essential micronutrient for plants when present at an optimal level. In the present study, 50 or 100 ppm Cu is the optimal concentration for normal plant growth. Rhizobium inoculation increased chlorophyll content compared with non-inoculated plants, especially for 400 ppm Cu (Fig. S2A). These results were consistent with the results on N concentration. Similarly, photosynthesis (Pn) was inhibited by high Cu concentrations (400 ppm) with or without rhizobium-inoculation compared with that of the control. At 400 ppm Cu, Pn was 5.88 μmol m^{-2} s^{-1}, yet after rhizobium inoculation, Pn was increased to 8.22 μmol m^{-2} s^{-1} (Fig. S2B). These results demonstrate that a high Cu concentration inhibits chlorophyll synthesis, photosynthesis, and nitrogen concentration, yet rhizobium inoculation alleviates Cu-induced decreases of these indexes. In other words, legume-rhizobium symbiosis promotes Cu phytoremediation in soils through increasing the N concentration, chlorophyll concentration and photosynthesis of plant tissues.

In metal phytoremediation studies, it has previously been observed that increasing or decreasing the amount of metal taken up by plant tissues is a function of the bacterium, the metal species, the particular plant involved, and the metal concentration (Rajkumar et al., 2009). Moreover, in nitrogen fixation, metal-resistant rhizobia promoted the growth and biomass of plants through regulation of biological nitrogen fixation and further effected metal solubility and bioavailability in soils (Pajuelo et al., 2011). Our results demonstrated that rhizobium inoculation decreased the Cu concentration in shoots under Cu stress (Fig. S3A). In contrast, the root Cu concentration was significantly increased in rhizobium-inoculated plants compared with non-inoculated plants (Fig. S3B). In addition, we also analyzed the total amount of Cu uptake in *M. sativa* seedlings. Cu uptake slightly increased on several levels in the shoots of inoculated plants compared with non-inoculated plants (Fig. 1D). The effect of rhizobium inoculation on the total amount of Cu uptake was more pronounced in the roots than in the shoots under Cu stress (Fig. 1E). In addition, the Cu transfer coefficient in rhizobium-inoculated plants was significantly reduced under Cu stress compared with non-inoculated plants (Fig. 1F). This suggested that Cu accumulated mainly in the roots, while a very low level of Cu was translocated to the shoots, which was beneficial to the phytostabilization of heavy metals. Similar results have been noted in the studies of Kong et al. (2015b), Delgadillo et al. (2015) and Morina et al. (2016). In brief, the above results demonstrate that *Medicago*-rhizobium symbiosis is potentially used for Cu phytostabilization, which plays an important role in avoiding leaching and toxic metal transfer into the food chain and ultimately affecting human health.

3.2. Legume-rhizobium symbiosis reduces Cu-induced oxidative stress through enhancing antioxidant enzyme activities in plants

Reactive oxygen species (ROS) are unavoidable by-products of aerobic metabolism in plants. Their accumulation is not only a negative stress-induced factor, but also regarded as messengers involved in signal transduction pathways for enzyme activities, gene expression, programmed cell death, and other processes (Clemens et al., 2002; Mittler, 2002). In this study, excess Cu accumulation resulted in superfluous production of ROS in both shoots and roots of *M. sativa* seedlings, which in turn induced oxidative stress (Table 1). Compared with non-inoculated plants, ROS accumulation induced by Cu was significantly lower in the shoots and roots of inoculated plants (Table 1), suggesting that rhizobium inoculation alleviated the oxidative stress of excess Cu by controlling $O_2^{\cdot-}$ and H_2O_2 levels in plants. Previous studies indicated that the application of exogenous nitrogen relieved heavy metal-induced oxidative stress in plants (Hu et al., 2015; Zhang et al., 2014a). Moreover, Cu stress increased the MDA contents in both shoots and roots of *M. sativa* seedlings (Table 1). The MDA contents in both shoots and roots significantly decreased in rhizobium-inoculated plants compared with non-inoculated plants in the presence of Cu, suggesting that the shoots and roots of rhizobium-inoculated plants suffered from lower damage levels and showed higher antioxidant activities. These results revealed that rhizobium-inoculated plants are more efficient than non-inoculated plants in mitigating the damage caused by ROS in *M. sativa* seedlings.

Plants possessed high activities of antioxidant enzymes to protect their cells from the damage of superfluous ROS induced by environmental stress (Hojati et al., 2017; Hu, 2016; Morina et al., 2016). Under a low Cu concentration, rhizobium inoculation suddenly promoted SOD activities in the shoots, but the trend in SOD changes was opposite under high Cu concentrations (Fig. 2A). These results demonstrate that high SOD activity is better at reducing excess Cu-induced superfluous $O_2^{\cdot-}$ in the shoots of *M. sativa* seedlings. However, application of rhizobium decreased the Cu-induced SOD activities in the roots of *M. sativa* seedlings (Fig. 2B). It suggests that rhizobium-inoculation in the roots inhibits the damage caused by superfluous $O_2^{\cdot-}$ and hence there is no need to induce more SOD activities to remove the production of $O_2^{\cdot-}$ associated with Cu concentration in the shoots and roots of *M. sativa* seedlings. Likely, similar results also had been found that the change trends of SOD activities in shoots were different from that in roots in arbuscular mycorrhizal fungus-inoculated maize seedling under Cu stress (Merlos et al., 2016). Moreover, previous studies also reported a positive relationship between Cu concentration and SOD activity in plants (Cao et al., 2017; Demirevska-Kepova et al., 2004; Weckx and

Table 1
The effect of rhizobia inoculation on the MDA content, the production of $O_2^{\cdot-}$, and the H_2O_2 content in the shoots and roots in *Medicago sativa* seedlings treated with different Cu concentrations. Each value represents the mean ± SE ($n = 4$). Different letters indicate significant differences with $P < 0.05$. Different capital letters (A, B, C, D, E, and F) indicate significant differences ($P < 0.05$) between different treatments (0, 50, 100, 200, 300, and 400 ppm Cu treatments), whereas the lowercase letters (a and b) indicate significant differences ($P < 0.05$) between non-inoculated and inoculated *M. sativa* seedlings under the same Cu concentration condition. NS: Control, S: *S. meliloti*.

Treatments	*S. meliloti*	MDA content (nmol g^{-1} FW)		$O_2^{\cdot-}$ (μmol g^{-1} FW)		H_2O_2 (μmol g^{-1} FW)	
Cu (ppm)		Shoot	Root	Shoot	Root	Shoot	Root
0	NS	83.51 ± 1.73 Fa	87.44 ± 1.22 Fa	1.923 ± 0.008 Da	1.793 ± 0.017 Da	84.92 ± 1.08 Fa	48.23 ± 1.04 Ea
	S	41.06 ± 0.81 Fb	91.27 ± 2.40 Fa	1.851 ± 0.007 Db	1.759 ± 0.026 Da	76.29 ± 1.21 Eb	40.48 ± 2.08 Fb
50	NS	98.21 ± 0.88 Ea	98.37 ± 0.91 Ea	1.921 ± 0.007 Da	1.785 ± 0.007 Da	87.06 ± 0.65 Ea	50.73 ± 0.62 Da
	S	56.96 ± 1.70 Eb	98.05 ± 0.78 Ea	1.826 ± 0.004 Eb	1.776 ± 0.017 Da	81.37 ± 1.54 Db	46.13 ± 0.53 Eb
100	NS	105.4 ± 2.46 Da	131.3 ± 1.11 Da	1.915 ± 0.008 Da	1.776 ± 0.009 Da	91.62 ± 1.32 Da	51.77 ± 0.42 Da
	S	81.93 ± 2.05 Db	106.5 ± 0.92 Db	1.838 ± 0.005 Eb	1.759 ± 0.017 Da	84.92 ± 1.08 Cb	47.90 ± 0.89 Db
200	NS	134.5 ± 2.31 Ca	141.4 ± 1.83 Ca	1.979 ± 0.014 Ca	1.849 ± 0.013 Ca	98.55 ± 1.06 Ca	56.60 ± 1.13 Ca
	S	88.59 ± 2.44 Cb	117.9 ± 2.77 Cb	1.915 ± 0.009 Cb	1.794 ± 0.009 Cb	85.92 ± 0.33 Cb	51.45 ± 0.50 Cb
300	NS	147.2 ± 2.68 Ba	167.1 ± 1.69 Ba	2.046 ± 0.028 Ba	1.924 ± 0.008 Ba	105.2 ± 0.42 Ba	62.01 ± 0.70 Ba
	S	100.6 ± 1.32 Bb	137.0 ± 2.91 Bb	1.950 ± 0.036 Bb	1.889 ± 0.010 Bb	89.56 ± 0.33 Bb	54.35 ± 0.77 Bb
400	NS	217.3 ± 1.98 Aa	218.1 ± 1.26 Aa	2.104 ± 0.009 Aa	2.014 ± 0.029 Aa	111.9 ± 1.11 Aa	70.56 ± 2.14 Aa
	S	125.0 ± 1.51 Ab	171.3 ± 3.85 Ab	2.011 ± 0.014 Ab	1.924 ± 0.017 Ab	96.17 ± 0.86 Ab	58.22 ± 0.59 Ab

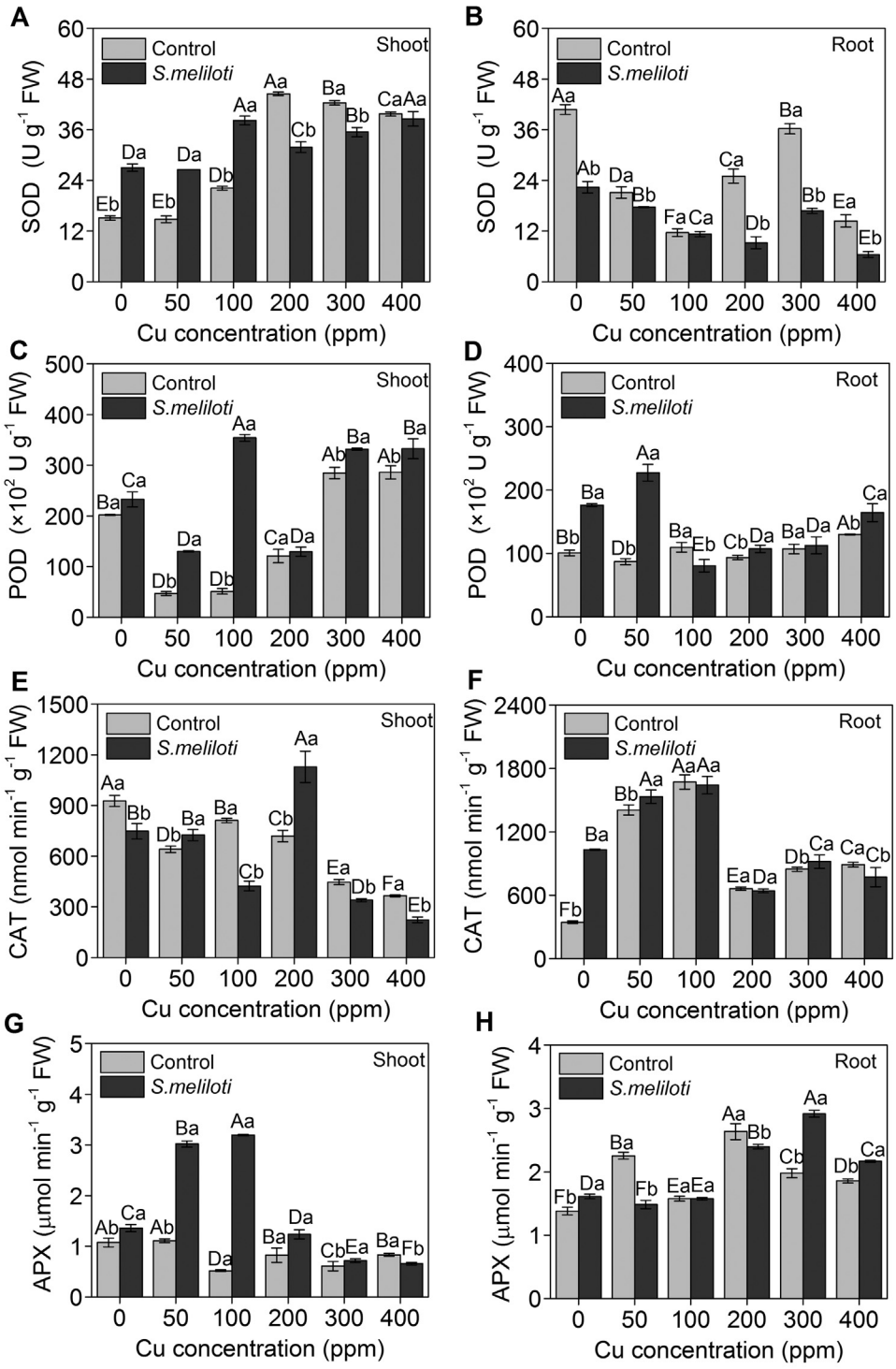

Fig. 2. The effect of rhizobia inoculation on the SOD activity in the shoots (A) and roots (B), the POD activity in the shoots (C) and roots (D), the CAT activity in the shoots (E) and roots (F), and the APX activity in the shoots (G) and roots (H) in *Medicago sativa* seedlings treated with different Cu concentrations. Each value represents the mean ± SE ($n = 4$). Columns labeled with different letters indicate significant differences with $P < 0.05$. Different capital letters (A, B, C, D, E, and F) indicate significant differences ($P < 0.05$) between different treatments (0, 50, 100, 200, 300, and 400 ppm Cu treatments), whereas the lowercase letters (a and b) indicate significant differences ($P < 0.05$) between non-inoculated and inoculated *M. sativa* seedlings under the same Cu concentration condition.

Clijsters, 1996). POD, CAT, and APX play vital roles in plant cells in regulating H_2O_2 levels for signaling during metabolic changes, but POD and APX are proposed to be predominantly responsible for modulating H_2O_2 levels, especially under stressful environments (Mittler, 2002; Weckx and Clijsters, 1996). Our data demonstrates that rhizobium inoculation promoted POD activities in the shoots (Fig. 2C). However, rhizobium inoculation increased POD activities under 0 and 50 ppm Cu, yet at other Cu concentrations, POD activities were not significantly affected in the roots (Fig. 2D), which showed that rhizobium inoculation can maintain the balance of H_2O_2 through affecting POD activity in the shoots and roots. Additionally, high Cu concentrations (300 and 400 ppm) inhibited CAT activities in the shoots of non-inoculated *M. sativa* seedlings compared with the control (0 ppm), and application of rhizobium reduced CAT activities in the shoots (Fig. 2E). APX activities

slightly decreased with increasing Cu concentrations in the shoots of non-inoculated *M. sativa* seedlings (Fig. 2G). APX activities in the roots increased under high Cu concentrations compared to the controls with or without rhizobium inoculation (Fig. 2H). Meanwhile, rhizobium inoculation significantly promoted APX activities at 300 and 400 ppm Cu (Fig. 2H). These results demonstrate that rhizobium inoculation removed excess Cu-induced H_2O_2 in the shoots and roots through affecting the POD, CAT, and APX activities. Additionally, the gene expression levels of *MsCu/Zn SOD*, *MsFe SOD*, *MsMn SOD*, and *MsAPX* were significantly induced by rhizobium inoculation under Cu stress conditions in the shoots of *M. sativa* seedlings, but in the absence of rhizobium, these gene expression levels were not altered by Cu stress (Fig. 5A). In addition, in the roots, gene expression levels of *MsCu/Zn SOD*, *MsFe SOD*, and *MsMn SOD* significantly increased with increasing Cu concentrations, and rhizobium inoculation inhibited Cu-induced changes in the roots of *M. sativa* seedlings under 50–400 ppm Cu conditions (Fig. 5B). It suggests that Cu stress induced high oxidative damage in the roots, but rhizobium inoculation alleviated this damage through regulating SOD-related gene expression abundances. Moreover, the change trends of *MsCu/Zn SOD*, *MsFe SOD*, and *MsMn SOD* gene expression were consistent with the SOD activities in the shoots and roots (Fig. 2A, B and Fig. 5A, B). The change trend between the roots and shoots were opposing, a possible explanation being that rhizobium inoculation causes different responses in the roots and shoots under Cu stress. A similar response mechanism was also reported by previous studies regarding the SOD enzyme activities in shoots and roots of *Medicago lupulina* under Cu stress (Kong et al., 2015b). Besides, previous studies had showed that exogenous nitrogen alleviated oxidative stress associated with heavy metal stress (Zhang et al., 2014a, 2014b). Moreover, nitrogen deficiency caused a decrease in enzyme activities involved in energy metabolism, such as photosynthesis (de Groot et al., 2003). Similarly, since no external N supply was added, our observations in this study are in agreement with these previous findings, along with the observation that the N concentration in inoculated plants under excess Cu remarkably increased as compared with non-inoculated plants. Thus, the above results indicate that nitrogen fixation promoting plant normal growth, increased the activities and related gene expression abundances of antioxidants enzymes, thereby alleviating Cu toxicity in plants through legume-rhizobium symbiosis.

3.3. Legume-rhizobium symbiosis decreases Cu-induced oxidative stress through regulating the AsA-GSH cycle in plants

Ascorbate (AsA) and glutathione (GSH) have been shown to play a vital role in preventing the induction of oxidative damage in heavy metal-stressed plants (Liu et al., 2016a, 2016b; Wang et al., 2017). The application of exogenous AsA stimulated root elongation of pea seedlings (Citterio et al., 1994). Previous studies have shown that transgenic plants overexpressing the key enzyme of the AsA-GSH cycle, improved stress tolerance by increasing AsA and GSH levels (Eltayeb et al., 2007). Moreover, Liu et al. (2016a, 2016b) reported that an enhanced GSH content was related to heavy metal Cd tolerance in Arabidopsis. In the present study, rhizobium significantly decreased the GSH content under 0 and 50 ppm Cu concentration conditions in the shoots, suggesting that the 50 ppm Cu concentration was optimal for plant growth, and does not cause the damage of Cu toxicity (Fig. 3A), but under high Cu concentrations, the GSH content significantly increased in inoculated plants. Similarly, rhizobium inoculation significantly enhanced the GSSG content in the shoots under different Cu concentrations (Fig. 3B). In addition, the GSH/GSSG ratio is often used to indicate the redox state in organisms. A lower GSH/GSSG ratio implies more severe oxidative stress (Smeets et al., 2005). In the present study, the GSH/GSSG ratio in the shoots was affected by Cu stress, while rhizobium inoculation significantly enhanced the GSH/GSSG ratio compared with non-inoculated plants, when exposed to 300 ppm Cu (Fig. 3C). It indicates that rhizobium enhanced the oxidative stress through enhancing the redox state in the shoots of *M. sativa* seedlings. Moreover, rhizobium inoculation greatly enhanced the GSH and GSSG content in the roots compared with non-inoculated plants under Cu stress (Fig. 3D and E). Interestingly, the GSH/GSSG ratio in the roots of rhizobium-inoculated seedlings had an obvious increase compared with non-inoculated plant under Cu stress (Fig. 3C and F). The above results demonstrate that rhizobium inoculation enhanced Cu tolerance through increasing GSH, GSSG contents and the GSH/GSSG ratio in the shoots and roots of *M. sativa* seedlings. Additionally, Rhizobium inoculation reduced the AsA content in the shoots under low Cu concentrations (50 and 100 ppm). In contrast, under high Cu concentrations, the AsA content was slightly increased (Fig. 3G). Similarly, the DHA content was not significantly altered under low Cu concentrations in the shoots of non-inoculated *M. sativa* seedlings (Fig. 3H). Rhizobium inoculation also increased the AsA content under Cu-treated conditions in the roots (Fig. 3J), indicating that rhizobium inoculation promoted the AsA levels in the roots to confer excess Cu stress in the legume-rhizobium symbiosis. The DHA content was significantly reduced by Cu stress, except for 400 ppm Cu, in the roots of non-inoculated *M. sativa* seedlings (Fig. 3K). The AsA/DHA ratio was altered by Cu stress with or without rhizobium inoculation in the roots of *M. sativa* seedlings (Fig. 3L). These results indicate that the increased contents of GSH and AsA in the shoots and roots were regulated by rhizobium inoculation under high Cu concentration stress (Fig. 3A, D, G, and J), thus reducing ROS accumulation and lipid peroxidation (Table 1). These results suggest that legume-rhizobium symbiosis was of important in increasing the components of the AsA-GSH cycle, subsequently protecting against oxidative stress caused by Cu in *M. sativa* seedlings, thus improving plant growth and development under Cu stress.

Furthermore, in the AsA-GSH cycle, H_2O_2 is reduced to H_2O, catalyzed by APX using AsA as a reductant (Mittler, 2002; Peto et al., 2013). Our studies demonstrate that rhizobium-inoculated plants increased APX activities by different degrees in the shoots and roots of *M. sativa* seedlings, suggesting that ROS levels were efficiently controlled under excess Cu stress, thereby inhibiting Cu-induced oxidative damage. Additionally, DHA and MDHA were produced in the disposal process of H_2O_2 by APX. Moreover, AsA regeneration from DHA and MDHA is catalyzed by MDHAR and DHAR, respectively (Hu et al., 2015; Mittler, 2002). Our data demonstrates that Cu stress inhibited DHAR activities in the shoots. Rhizobium inoculation changed DHAR activities by different degrees in the shoots of *M. sativa* seedlings (Fig. 4A). In contrast, DHAR activities in the roots were induced by excess Cu, and rhizobium inoculation significantly declined DHAR activities compared with non-inoculated plants under Cu stress (Fig. 4B). It suggests that the change in DHAR activities significantly differed between the shoots and roots, which was associated with the degree of oxidative damage in different plant tissues. Additionally, in the absence of rhizobium, the change in trend of MDHAR activities was similar to the GR activities under Cu-treated conditions in the shoots of *M. sativa* seedlings and rhizobium inoculation also promoted MDHAR activities at several levels in the shoots (Fig. 4C and E). Similarly, MDHAR activities in 100 ppm Cu were the highest compared with other Cu concentrations with or without rhizobium inoculation. In the presence of rhizobium, MDHAR activities were notably elevated compared to non-inoculated plants, except for 100 ppm Cu (Fig. 4D), indicating that rhizobium inoculation enhanced MDHAR activities in the Cu-treated shoots and roots of *M. sativa* seedlings. Similarly, Wang et al. (2017) reported that the high MDHAR activities also increased the Al tolerance in the roots of soybean

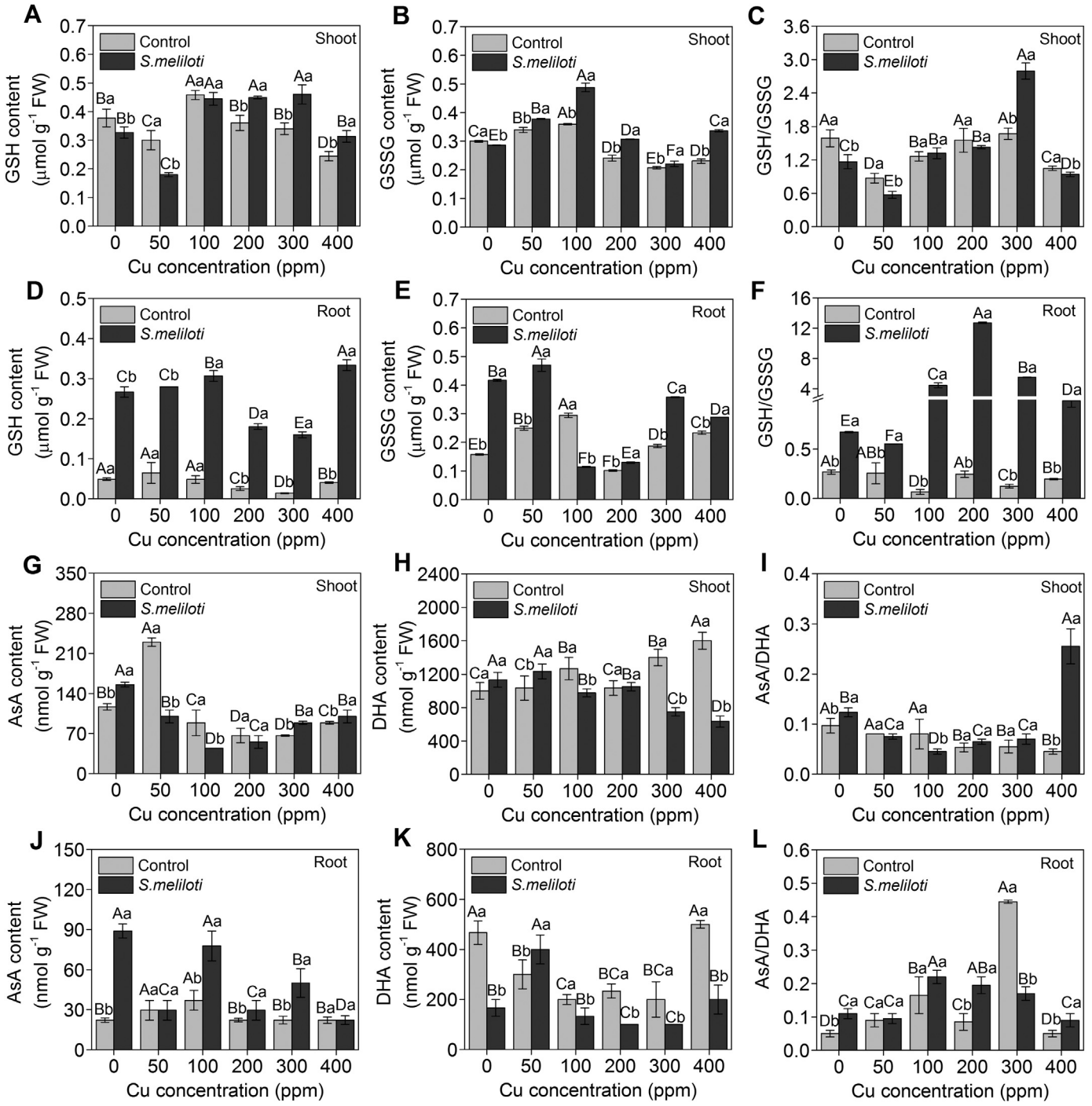

Fig. 3. The effect of rhizobia inoculation on the GSH content in the shoots (A) and roots (D), the GSSG content in the shoots (B) and roots (E), the GSH/GSSG ratio in the shoots (C) and roots (F), the AsA content in the shoots (G) and roots (J), the DHA content in the shoots (H) and roots (K), and the AsA/DHA ratio in the shoots (I) and roots (L) in *Medicago sativa* seedlings treated with different Cu concentrations. Each value represents the mean ± SE ($n = 4$). Columns labeled with different letters indicate significant differences with $P < 0.05$. Different capital letters (A, B, C, D, E, and F) indicate significant differences ($P < 0.05$) between different treatments (0, 50, 100, 200, 300, and 400 ppm Cu treatments), whereas the lowercase letters (a and b) indicate significant differences ($P < 0.05$) between non-inoculated and inoculated *M. sativa* seedlings under the same Cu concentration condition.

seedlings. However, Cu stress did not significantly alter gene expression levels of *MsPXDA*, *MsPXDC*, and *MsMDHAR* in the shoots of non-inoculated *M. sativa* seedlings. However, application of rhizobium sharply promoted expression of these two genes in the shoots (Fig. 5A). In addition, gene expression levels of *MsAPX*, *MsPXDA, MsPXDC*, and *MsMDHAR* were up-regulated by rhizobium inoculation in the roots of *M. sativa* seedlings (Fig. 5B). These results suggest that MDHAR plays a dominant role in antioxidant defenses compared with DHAR in *M. sativa* seedlings. Therefore, we conclude that the increased activities of MDHAR may be responsible for AsA regeneration to resist oxidative stress under excess Cu stress. In addition, GSH, as an electron donor, is involved in the conversion of DHA to AsA in plants (Jozefczak et al., 2012; Semane et al., 2007). Our data demonstrates that rhizobium inoculation promoted GSH levels in the shoots and roots of *M. sativa* seedlings under Cu stress (Fig. 3A and D), suggesting that rhizobium was of great important in maintaining GSH pools in the shoots and roots

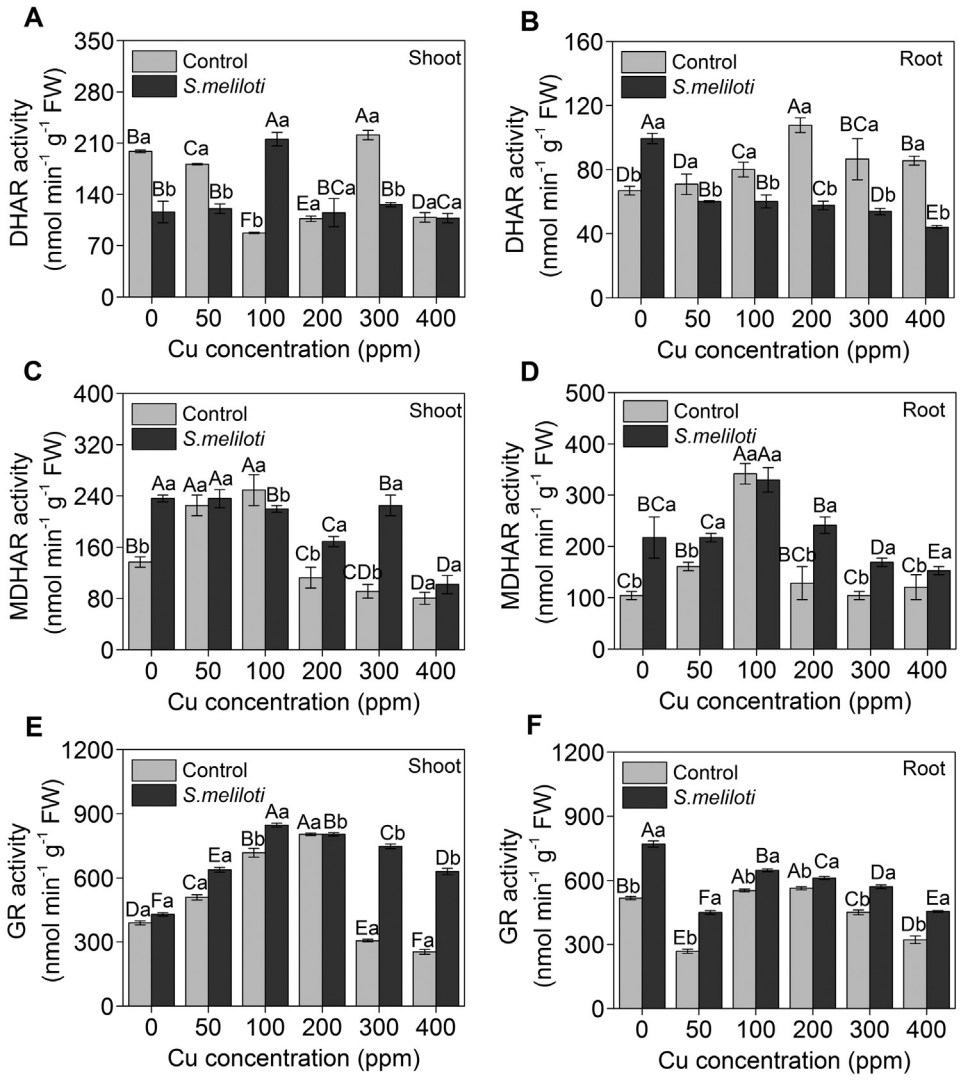

Fig. 4. The effect of rhizobia inoculation on the DHAR activity in the shoots (A) and roots (B), the MDHAR activity in the shoots (C) and roots (D), and the GR activity in the shoots (E) and roots (F) in *Medicago sativa* seedlings treated with different Cu concentrations. Each value represents the mean ± SE ($n = 4$). Columns labeled with different letters indicate significant differences with $P < 0.05$. Different capital letters (A, B, C, D, E, and F) indicate significant differences ($P < 0.05$) between different treatments (0, 50, 100, 200, 300, and 400 ppm Cu treatments), whereas the lowercase letters (a and b) indicate significant differences ($P < 0.05$) between non-inoculated and inoculated *M. sativa* seedlings under the same Cu concentration condition.

under Cu stress. The regeneration of GSH from oxidized glutathione (GSSG) is catalyzed by GR. Previous studies have shown that over-expression of GR in tobacco and poplar resulted in higher leaf AsA contents and improved tolerance to oxidative stress (Sharma et al., 2012). Our data demonstrates that application of rhizobium significantly promoted GR activities in the shoots of *M. sativa* seedlings under different Cu concentrations (Fig. 4E). Similarly, GR activities were also affected by Cu stress in the roots with or without rhizobium inoculation. Moreover, the effect of rhizobium on GR activities in the roots was similar to the changes in the shoots under Cu stress (Fig. 4F). Moreover, the elevated activities of MDHAR and GR in the shoots and roots of *M. sativa* seedlings explained the observation of the increased ratio of AsA/DHA and GSH/GSSG (Fig. 3C, F, I and L). We conclude that legume-rhizobium symbiosis enhanced enzyme activities through controlling the levels of AsA and GSH to protect *M. sativa* seedlings from excess Cu damage.

3.4. Legume-rhizobium symbiosis ameliorates Cu stress through affecting the cysteine level and regulating PC and MT biosynthesis-related gene expression in plants

Thiol compounds are crucial to heavy metal detoxification and one of the most important compounds for binding metal ions is non-protein thiols (NPTs). Previous studies have shown that heavy metal enhanced NPTs formation in plants (Chen et al., 2013; Liu et al., 2016a, 2016b). Similarly, Rhizobium increased NPTs content in the shoots under Cu stress, especially in 50 and 200 ppm Cu concentrations (Table 2). However, the NPTs content was decreased by rhizobium inoculation compared with non-inoculated plants under high Cu stress in the roots of *M. sativa* seedlings (Table 2), indicating that shoots and roots had different response to Cu stress. Cysteine, as a predominant NPTs and the precursor molecule for GSH biosynthesis, plays an important role in the response to plant stress (Liu et al., 2016b). Moreover, cysteine is a potent chelator of heavy metals ions and heavy metal exposure can increase cysteine content (Grill et al., 1987). In addition, the accumulation of free cysteine is not always beneficial to plants. Cysteine-metal ion complexes can trigger the Fenton reaction and produce the highly toxic $^{\cdot}OH$ radical (Zagorchev et al., 2013). Our data demonstrates that rhizobium inoculation significantly increased the cysteine content under 50–300 ppm Cu concentration conditions (Table 2). In addition, the cysteine content in the roots was induced with increasing Cu concentrations and rhizobium inoculation affected the cysteine content in the roots of *M. sativa* seedlings by different degrees (Table 2). Therefore, over-accumulation of cysteine in the roots could be another important reason why Cu-treated seedlings suffered from severe oxidative stress in the non-inoculated *M. sativa* seedlings.

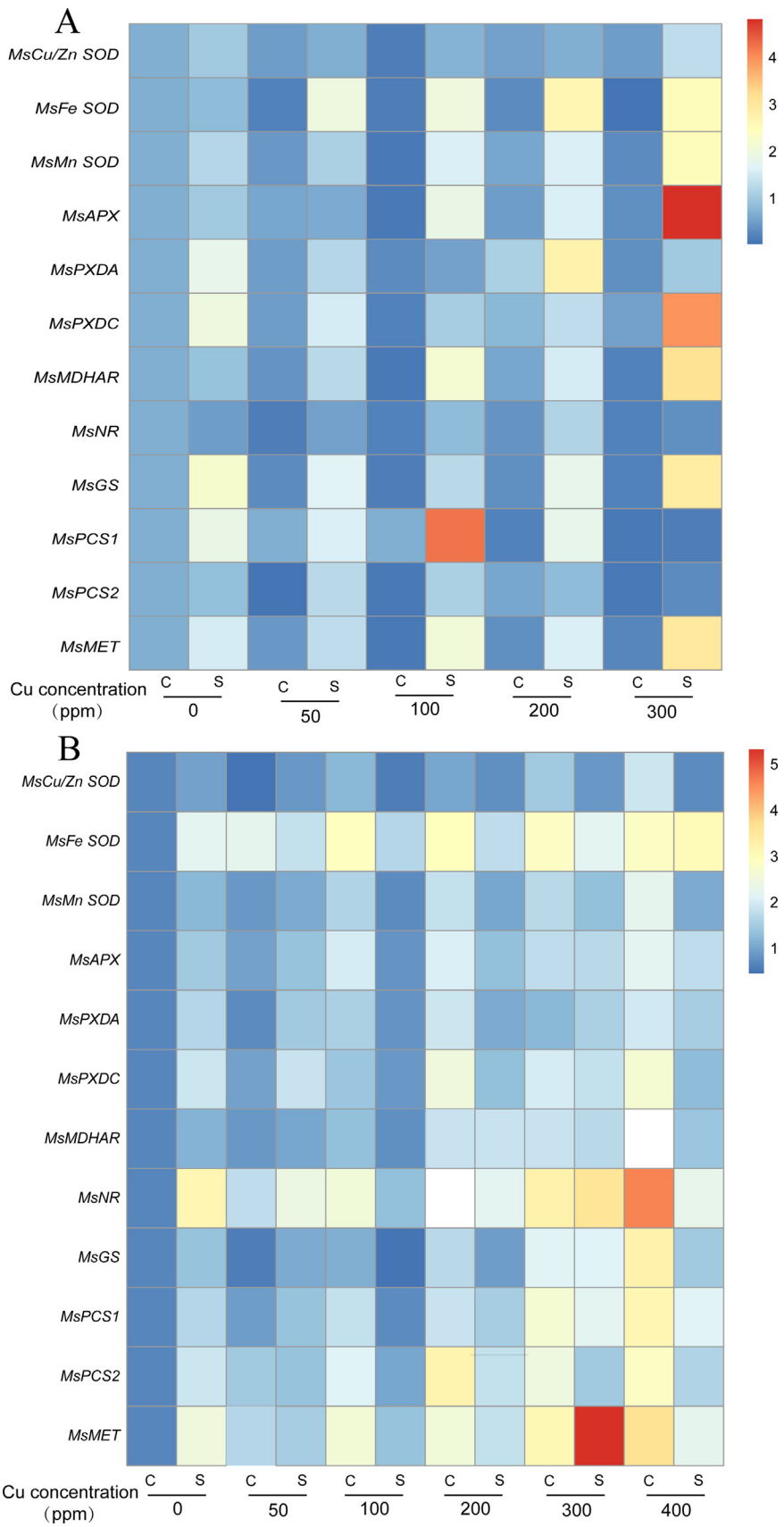

Fig. 5. The effect of rhizobia inoculation on the antioxidant enzyme-related gene expression PC biosynthesis-related gene expression and MT gene expression in the shoots (A) and roots (B) in *Medicago sativa* seedlings treated with different Cu concentrations. Red and blue indicate up- and down-regulation in the treated plants compared with the control, respectively. The intensity of the colors is proportional to the absolute value of the fold difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The effect of rhizobia inoculation on the NPTs content and the cysteine content in the shoots and roots in *Medicago sativa* seedlings treated with different Cu concentrations. Each value represents the mean ± SE ($n = 4$). Different letters indicate significant differences with $P < 0.05$. Different capital letters (A, B, C, D, E, and F) indicate significant differences ($P < 0.05$) between different treatments (0, 50, 100, 200, 300, and 400 ppm Cu treatments), whereas the lowercase letters (a and b) indicate significant differences ($P < 0.05$) between non-inoculated and inoculated *M. sativa* seedlings under the same Cu concentration condition. NS: Control, S: *S. meliloti*.

Treatments	*S. meliloti*	NPTs (μmol g^{-1} FW)		Cysteine (μmol g^{-1} FW)	
Cu (ppm)		Shoot	Root	Shoot	Root
0	NS	14.69 ± 1.35 Ab	12.69 ± 1.18 Ba	50.00 ± 2.88 Aa	29.16 ± 2.20 Cb
	S	20.69 ± 2.01 Ca	13.05 ± 1.45 Aa	37.50 ± 1.44 Db	40.83 ± 1.67 Ba
50	NS	8.847 ± 0.72 Db	13.56 ± 0.82 Ba	35.01 ± 1.42 Cb	30.83 ± 3.00 Cb
	S	25.67 ± 0.72 Ba	12.61 ± 0.65 Aa	45.83 ± 1.66 Ca	40.01 ± 0.017 Ba
100	NS	13.34 ± 0.29 Ba	10.83 ± 1.42 Cb	32.51 ± 2.51 Cb	40.83 ± 2.20 Ba
	S	12.47 ± 0.87 Ea	12.95 ± 1.07 Aa	46.67 ± 0.83 Ca	23.33 ± 2.20 Cb
200	NS	10.08 ± 0.56 Cb	14.50 ± 0.44 Ba	45.01 ± 2.88 Bb	47.51 ± 0.013 Aa
	S	30.02 ± 0.72 Aa	10.34 ± 1.23 Bb	49.16 ± 0.83 Ba	47.50 ± 1.443 Aa
300	NS	6.768 ± 0.48 Eb	15.95 ± 0.91 Aa	35.02 ± 1.44 Cb	39.16 ± 2.20 Bb
	S	9.137 ± 0.14 Fa	12.76 ± 0.66 Ab	27.50 ± 2.51 Aa	46.25 ± 1.25 Aa
400	NS	17.11 ± 3.16 Aa	13.70 ± 1.22 Ba	46.25 ± 1.25 Ba	48.33 ± 3.00 Aa
	S	15.95 ± 0.76 Da	10.44 ± 0.58 Bb	33.33 ± 0.83 Eb	40.01 ± 2.28 Bb

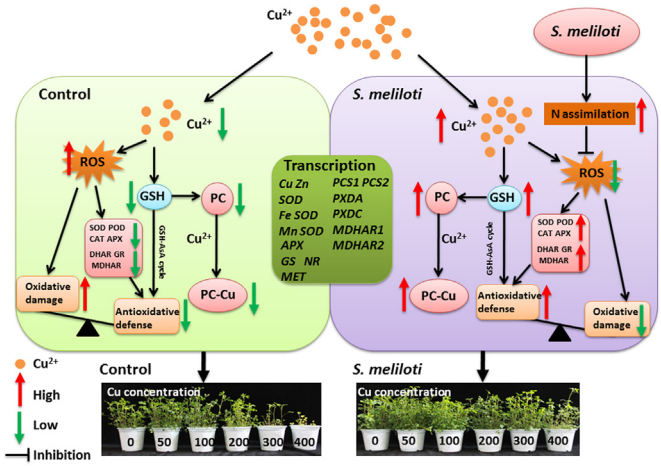

Fig. 6. The proposed model for the role of rhizobium inoculation in alleviating Cu-induced oxidative damage in *Medicago sativa* seedlings. Increased components are marked by an upward arrow and red color, and decreased components are marked by a downward arrow and green color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Chelation by PCs is considered as another efficient mechanism against heavy metal toxicity. PCs bind metal ions via the thiol group of cysteine (Benatti et al., 2014). In our study, we detected that gene expression of *MsPCS1* and *MsPCS2* was up-regulated after Cu exposure in the roots of non-inoculated *M. sativa* seedlings (Fig. 5B), suggesting that Cu stress induced gene expression of *MsPCS1* and *MsPCS2*, which was consistent with previous studies in Arabidopsis (Liu et al., 2016b). Interestingly, rhizobium inoculation significantly increased gene expression abundances of *MsPCS1* and *MsPCS2* in shoots, but reduced their expression levels in the roots (Fig. 5A and B), which was associated with Cu concentrations in the shoots and roots (Fig. S3A and B). In addition, plant MTs have been proposed to participate in a variety of processes including metal ion homeostasis and tolerance, oxidative stress protection, root development and seed germination, etc. (Cobbett and Goldsbrough, 2002; Zimeri et al., 2005; Benatti et al., 2014). A number of studies suggest that plant MTs may participate in metal ion homeostasis, especially for Cu, during the process of vegetative growth (Benatti et al., 2014). In Arabidopsis, rice and the metal hyperaccumulator *Noccaea caerulescens*, MT RNA expression was strongly induced by Cu treatment (Zhou and Goldsbrough, 1994; Hsieh et al., 1995). Our results suggest that gene expression of *MsMET* was down-regulated in the shoots, but up-regulated in the roots after Cu exposure, suggesting that gene expression of *MsMET* is a tissue-specific expression in *M. sativa* seedlings. Moreover, rhizobium inoculation significantly promoted gene expression of *MsMET* in shoots of *M. sativa* seedlings (Fig. 5), indicating that rhizobium inoculation enhanced Cu tolerance through affecting the gene expression of *MsMET*. Taken together, the above results demonstrate that legume-rhizobium symbiosis could promote Cu accumulation in plant tissues through regulating NPTs and cysteine content, PC biosynthesis-related gene expression, and MT-related gene expression.

4. Conclusions

Our results demonstrate that excess Cu stimulated ROS production and destroyed the H_2O_2 scavenging system in *M. sativa* by inhibiting the activities and gene expression of antioxidant enzymes and affecting the AsA-GSH cycle. Cu affected GSH content and PC biosynthesis-related gene expression, and inhibited the formation of PC-Cu. Rhizobium-inoculation reduced ROS accumulation, increased GSH level, and affected the AsA-GSH cycle and the ability of antioxidant defenses. Rhizobium inoculation regulated PC biosynthesis and MT-related gene expression to protect *M. sativa* from excess Cu stress (Fig. 6). These results add fundamental knowledge to our understanding of the mitigation mechanism of legume-rhizobium symbiosis in counteracting Cu toxicity.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2018.07.001.

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