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RESEARCH ARTICLE

Rhizobial symbiosis effect on the growth, metal uptake, and antioxidant responses of *Medicago lupulina* under copper stress

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Abstract The effects of rhizobial symbiosis on the growth, metal uptake, and antioxidant responses of *Medicago lupulina* in the presence of 200 mg kg^{-1} Cu^{2+} throughout different stages of symbiosis development were studied. The symbiosis with *Sinorhizobium meliloti* CCNWSX0020 induced an increase in plant growth and nitrogen content irrespective of the presence of Cu^{2+}. The total amount of Cu uptake of inoculated plants significantly increased by 34.0 and 120.4 % in shoots and roots, respectively, compared with non-inoculated plants. However, although the rhizobial symbiosis promoted Cu accumulation both in shoots and roots, the increase in roots was much higher than in shoots, thus decreasing the translocation factor and helping Cu phytostabilization. The rate of lipid peroxidation was significantly decreased in both shoots and roots of inoculated vs. non-inoculated plants when measured either 8, 13, or 18 days post-inoculation. In comparison with non-inoculated plants, the activities of superoxide dismutase and ascorbate peroxidase of shoots of inoculated plants exposed to excess Cu were significantly elevated at different stages of symbiosis development; similar increases occurred in the activities of superoxide dismutase, catalase, and glutathione reductase of inoculated roots. The symbiosis with *S. meliloti* CCNWSX0020 also upregulated the corresponding genes involved in antioxidant responses in the plants treated with excess Cu. The results indicated that the rhizobial symbiosis with *S. meliloti* CCNWSX0020 not only enhanced plant growth and metal uptake but also improved the responses of plant antioxidant defense to excess Cu stress.

Keywords Antioxidant enzymes · Copper · Legume-*Rhizobium* · Gene expression · Phytoremediation

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Introduction

Copper is an essential micronutrient for plants, as it is directly involved in numerous essential metabolic processes, including respiration, photosynthesis, protein synthesis, cell wall metabolism and lignification, ethylene sensing, and oxidative stress protection (Festa and Thiele 2011). Indeed, these properties make copper ions indispensable for plant growth and development. However, copper can be toxic for plants when it is present at even slightly higher than optimal level (Martins and Mourato 2006; Reichman et al. 2006). Over the centuries, as a result of industrial production, sewage irrigation, and extensive use of chemical fertilizer and pesticides, copper has contaminated numerous soils and waters, especially in industrial areas (Figueira et al. 2002; Lu et al. 2009; Srinivasa Gowd et al. 2010). Copper pollution of both water and soil directly poses a significant toxicity threat to plants, which in turn, impacts negatively on both human and environmental health.

Phytoremediation, representing an environmentally friendly and cost-effective potential alternative to conventional remediation approaches, has been highly touted (Ali et al. 2013; Gerhardt et al. 2009; Rajkumar et al. 2012). However, many of the plants that are most effective at removing metals from the soil are typically characterized by their small sizes and slow growth rates, thus reducing their remediation potential and restricting their practical use in this technology (Baker et al. 1994; Komárek et al. 2007). On the other hand, plant growth-promoting bacteria can act as adjuncts in metal phytoremediation and significantly facilitate the growth of plants in the presence of otherwise inhibitory levels of metals (Gamalero and Glick 2011; Glick 2003; Glick 2010).

Legumes are well known for their ability to form root nodules with compatible rhizobial strains, within which atmospheric nitrogen is reduced to ammonia for the benefit of both bacteria and plants; therefore, the symbiosis is of great environmental and agricultural importance and has been studied extensively (Graham and Vance 2003). As a consequence of the intrinsic potential of rhizobia to facilitate the growth of legumes, in recent years, considerable attention has been paid to understanding how rhizobia benefit their host plants under extreme conditions. Thus, there is evidence of rhizobial bacteria promoting plant growth under heavy metal stress through mechanisms other than improved N nutrient (Brígido and Glick 2015; Reichman 2007). In this regard, rhizobial bacteria also have positive effects on plant growth through other mechanisms, including the solubilization of phosphate (Abril et al. 2007), the secretion of siderophores to sequester iron (Wani et al. 2008b), the production of the phytohormone indole-3-acetic acid (IAA) (Ghosh et al. 2008), and the synthesis of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase to lower stress ethylene levels in plants (Glick et al. 2007).

A number of highly heavy metal-resistant rhizobial strains have been isolated from industrial areas; these strains have significant potential for use in improving plant growth under heavy metal-stressed conditions (Vidal et al. 2009; Wani et al. 2007, 2008a, b, c). In metal phytoremediation studies, different rhizobia may affect plant growth and metal uptake in different ways (Pajuelo et al. 2011), so that attention must be paid to the selection of the appropriate legume-*Rhizobium* symbiosis combination, displaying significant phytoremediation potential in the presence of excess heavy metal. For this purpose, it is important to focus on how a plant's metal tolerance is positively affected by symbiotic rhizobia.

It is generally believed that copper ions can interact with membrane proteins and lead to lipid peroxidation through the photosynthetic electron transport system (Sandmann and Böger 1980). Moreover, high metal concentrations can cause oxidative stress in plants resulting in the overproduction of reactive oxygen species (ROS). To counter ROS, plant cells utilize a wide array of antioxidant defense systems, which can alleviate oxidative damage by scavenging the ROS. One of major defense systems against ROS is involved directly with active forms of oxygen, keeping them at a low level, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX). APX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (MDHAR), as well as glutathione reductase (GR) are involved in the ascorbate-glutathione cycle, which is described as one of important ROS-scavenging pathways in the chloroplasts, cytosol, mitochondria, peroxisomes, and apoplasts (Choudhury et al. 2013; Hasanuzzaman et al. 2012). Copper-induced changes in antioxidative systems have previously been investigated in various plants (Chaoui and El Ferjani 2005; Demirevska-Kepova et al. 2004; Deniz et al. 2007; Drążkiewicz et al. 2004; Jouili and El Ferjani 2003; Posmyk et al. 2009; Tewari et al. 2006; Thounaojam et al. 2012; Zhang et al. 2008). These studies demonstrated that oxidative stress and differential responses of antioxidant enzymes in plants were induced by excess Cu stress. It is generally believed that high activities of antioxidant enzymes improve plant resistance to oxidative damage. In recent years, oxidative stress and antioxidative responses in plants have stimulated increased interest, especially with regard to adverse conditions and plant-microbe interactions. It is well known that reactive oxygen species could be easily elevated in legume root nodules due to the high rates of respiration, the abundance of leghemoglobin, redox proteins, and non-protein Fe during N_2 fixation (Becana et al. 2000). Considerable efforts have been expended into identifying the quantity and diversity of antioxidant defenses in legumes nodules (Becana et al. 2010). However, little work has been done on how antioxidant defense of legumes is affected by the addition of rhizobia.

This study was specifically directed toward understanding how rhizobial symbiosis with *Sinorhizobium meliloti* CCNW SX0020 affected antioxidant responses of Cu-stressed plants. Thus, plant growth, nitrogen content, and Cu content were measured in addition to the activities of SOD, CAT, APX, and GR as well as the rate of lipid peroxidation in both shoots and roots at different symbiosis development stages. With the aim to deepen the knowledge about the effects of symbiosis with *S. meliloti* on the antioxidant defense systems in plants, quantitative real-time PCR (qRT-PCR) was performed to investigate the expression of genes involved in antioxidant defense in both shoots and roots of plants grown in the presence of excess Cu. The results reported here provide some insight into how legume metal (i.e., copper) antioxidantive defense systems were positively affected by the addition of a specific rhizobial strain, thus confirming that rhizobial strains together with their cognate legumes may facilitate the remediation of heavy metal-contaminated soils.

Materials and methods

Bacterial inoculation and plant treatments

The *S. meliloti* strain CCNWSX0020, resistant to 1.4 mM Cu^{2+} in tryptone-yeast extract (TY) medium was isolated from *Medicago lupulina* plants growing in lead-zinc mine tailings in northwest China (Fan et al. 2010). The *S. meliloti* CCNW SX0020 inoculant was grown for 2 days at 28 °C with shaking at 150 rpm in TY liquid medium (5 g tryptone, 3 g yeast extract, and 0.7 g $CaCl_2{\cdot}2H_2O$/l; pH 7.2). *M. lupulina* seeds (provided by Gansu Agricultural University, China) were surface sterilized by treatment in 75 % (*v*/*v*) ethanol for 5 min followed by 10 min in 20 % (*v*/*v*) NaClO (containing 8 % available chlorine). After the seeds were thoroughly rinsed with several changes of sterile distilled water, they were planted in plastic pots (10 cm diameter) filled with 100 g of a sterilized perlite-vermiculite (1:1) mixture. The seedling medium was treated with Cu in the form of $CuSO_4$ to produce a concentration of Cu^{2+} of 200 mg kg^{-1}. After it was thoroughly mixed with the Cu solution, the soil medium was packed into the plastic pots and allowed to equilibrate for 1 week. The concentration of Cu applied was determined through a preliminary experiment to be able to significantly inhibit plant growth but still allow the formation of root nodules (data not shown). The seedlings were then maintained in a plant growth incubator at 25 °C in the light at 200 μmol m^{-2} s^{-1} for 16 h, and 21 °C in the dark for 8 h. Fåhraeus nitrogen-free mineral nutrient solution (Fåhraeus 1957) was used to water the plants when necessary (approximately 150 ml every 5–6 days). After 7 days, seedlings were inoculated with an *S. meliloti* CCNWSX0020 inoculant cell suspension (approximately 10^9 CFU ml^{-1}) corresponding to zero days post-inoculation (0 dpi). Six seedlings were planted in each pot, and three replicates were conducted for each treatment. The plant treatments included: no stress conditions (CN); no stress conditions with *S. meliloti* CCNWSX0020 inoculation (CSM); Cu stress conditions (CuN); and Cu stress conditions plus *S. meliloti* CCNWSX0020 inoculation (CuSM).

Measurement of plant growth, N content and Cu content

Plants were harvested at 18 dpi. The fresh weight, dry weight, plant height, and N content of the shoots and roots were recorded. The total number of nodules and active nodules (pink-red color) were counted, respectively. Pink-red color, because of the presence of leghemoglobin, was considered as an index of potential N-fixation (Ott et al. 2005; Reichman 2007). The shoots and roots were separated, washed in distilled water (ddH_2O) three times to remove any loosely bound Cu^{2+}, and then dried at 65 °C for 48 h before the dry weight was determined. Total N content was measured by Kjeldahl method on a Kjeltec™ 8400 Analyzer Unit (FOSS-Tecator AB, Hoganas, Sweden). Aliquots of precisely 0.2 g powdered plant tissue samples were digested with an acid mixture ($HNO_3/HClO_4$=3:1), and the Cu content was analyzed by atomic absorption spectrophotometry (Z-5000; Hitachi, Tokyo, Japan). To evaluate the transport behavior of Cu from plant roots to shoots under Cu stress conditions, the translocation factor was analyzed (Singh and Agrawal 2007). The translocation factor was calculated using the following formula:

$$\text{Translocation factor}(\text{TF}) = \text{Cu}_\text{s}/\text{Cu}_\text{r}$$

where Cu_s and Cu_r are Cu content in shoots and roots, respectively.

Lipid peroxidation and antioxidant enzyme activity

Taking into account the symbiosis developmental stage, all plant harvested dates were determined based on the timing of root nodule formation in preliminary experiments (Supplemental Fig. S1). Plants were harvested at 3 dpi (no root nodules visible), 8 dpi (tiny white nodules observed), 13 dpi (small pink nodules observed), and 18 dpi (large/full pink nodules observed). At each sampling date, the shoots and roots were separated and weighed. For quantitative real-time PCR analysis, plant tissues were also collected at each sampling date, the fresh weight was measured, and samples were then harvested into liquid nitrogen and stored at −80 °C. Fresh plants samples (200 mg) were homogenized in 5 ml of cold extraction buffer (50 mM potassium phosphate at pH7.8, containing 1 % polyvinyl pyrrolidine). The homogenate was then centrifuged at 12,000×*g* for 15 min at 4 °C using a Sigma 3K15 centrifuge, and the supernatant obtained was used as the crude extract for lipid peroxidation and enzyme determination. All of the spectrophotometric analyses were conducted in a Nicolet 300 UV/Visible spectrophotometer (Thermoelectron, USA). Lipid peroxidation and all enzyme activities were expressed per gram of fresh weight.

Lipid peroxidation was assayed by the method of Heath and Packer (1968) in terms of malondialdehyde (MDA) content following the thiobarbituric acid (TBA) reaction. The activity of superoxide dismutase (SOD; EC.1.15.1.1) was determined by measuring the enzyme's ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) as described by Beauchamp and Fridovich (1971). Catalase (CAT; EC.1.11.1.6) activity was assayed by measurement of the decrease of absorbance at 240 nm according to the method of Aebi (1984). Ascorbate peroxidase (APX; EC.1.11.1.11) activity was assayed spectrophotometrically at 290 nm by the method of Nakano and Asada (1981). The activity of glutathione reductase (GR; EC.1.6.4.2.) was measured by monitoring the rate of oxidation of NADPH at 340 nm (Foyer and Halliwell 1976).

Quantitative real-time PCR analysis

Total RNA was isolated from shoots and roots separately at each sampling date using TRIzol reagent (TAKARA, Dalian, China) following the manufacturer's instructions. PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time, TAKARA, Dalian, China) was used to treat 1 μg total RNA to remove residual DNA, and then reverse transcribe RNA to complementary DNA (cDNA), according to the manufacturer's instructions.

Quantitative real-time PCR was performed using the CFX96 real-time PCR system (Bio-Rad, Hercules CA, USA). SYBR Green real-time PCR assay was performed in a total volume of 20 μl, containing 10 μl of FastStart Essential DNA Green Master (Roche Applied Science, Indianapolis, IN, USA), 0.2 μM of each specific primer, and 2 μl of cDNA solution. For each primer set, a no-template water control was also performed. The PCR conditions consisted of denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. A melting curve was run after each PCR reaction to confirm the accuracy of each amplified product. RT-PCR amplification for each cDNA sample was performed in triplicate wells. The messenger RNA (mRNA) levels of the target genes were normalized against EF*1-α* (Yahyaoui et al. 2004). Specific primer pairs for the genes cytosolic *CuZnSOD*, plastid *CuZnSOD*, plastid *FeSOD*, mitochondrial *MnSOD*, *CAT*, cytosolic *APX*, and cytosolic *GR* were those reported by Naya et al. (2007). Quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method as previously described (Livak and Schmittgen 2001). According to the same criteria that are generally used in cDNA array studies (Yahyaoui et al. 2004), upregulation ratio >2 or downregulation ratio <0.5 in gene expression was considered as significant.

Statistical analyses

Data were subjected to statistical evaluation using analysis of variance (ANOVA) followed by Duncan test ($p<0.05$). All of the statistical analyses were performed with SPSS 16.0 statistical software package. All of the data was analyzed using the Origin Pro v8.0 (Origin Lab, Hampton, USA) to create the figures.

Results

Plant growth, nodulation, and N content

The effects of the rhizobial symbiosis with *S. meliloti* CCNW SX0020 on plant growth, nodulation, and N content of *M. lupulina* are presented in Table 1. The presence of Cu

Table 1 The effect of the symbiosis with *Sinorhizobium meliloti* CCNWSX0020 on the growth, nodulation and N content of *Medicago lupulina*

Treatments	Fresh weight (mg plant^{-1})		Dry weight (mg plant^{-1})		Plant height (cm)	Nodulation		N content (% DW plant^{-1})	
	Shoots	Roots	Shoots	Roots		Nodules number (plant^{-1})	Active nodules (%)	Shoots	Roots
CN	49.03±1.16 b	8.07±0.55 b	6.22±0.44 b	1.13±0.15 b	5.76±0.11 b	–	–	4.57±0.24 c	2.52±0.21 c
CSM	57.22±2.40 a	9.90±0.75 a	7.48±0.36 a	1.70±0.58 a	6.72±0.22 a	12.0 a	72.97 a	6.05±0.12 b	3.77±0.07 b
CuN	40.64±1.59 c	5.13±0.30 c	4.00±0.20 d	0.70±0.58 c	4.85±0.12 c	–	–	4.99±0.12 c	2.93±0.20 c
CuSM	46.10±2.97 bc	5.43±0.30 c	5.13±0.31 c	1.17±0.20 b	5.63±0.28 b	7.0 b	66.43 a	6.72±0.06 a	4.56±0.22 a
F values									
Inoculation (*df*=1)	10.083*	4.348	12.257**	15.500**	19.824**	–	–	115.623***	58.973***
Cu treatment (*df*=1)	20.635**	52.318***	45.481***	13.565**	26.004**	–	–	13.256**	10.248*
Inoculation×Cu treatment (*df*=1)	0.405	2.246	0.034	0.145	0.219	–	–	0.715	1.022

The values indicate the mean±SE of three replicates. Different letters (a–d) show significant differences between treatments at $p<0.05$ by Duncan test

CN no stress conditions (non-inoculated), *CSM* no stress conditions (inoculated with *S. meliloti* CCNWSX0020), *CuN* Cu stress conditions (non-inoculated), *CuSM* Cu stress conditions (inoculated with *S. meliloti* CCNWSX0020)

*$p<0.05$; **$p<0.01$; ***$p<0.001$—levels of significance

induced a significant decrease in the total fresh weight, dry weight and plant height of both inoculated and non-inoculated plants. No nodules were observed for the non-inoculated plants during the experiment. For the inoculated plants, the excess Cu also induced a significant reduction in nodules number, but had no effect on active nodules rate. Unexpectedly, the excess Cu induced a slight increase in N content in both shoots and roots of inoculated plants. On the other hand, when *S. meliloti* CCNWSX0020 was inoculated in the presence of Cu, the dry weights of inoculated plants significantly increased by 28.3 and 67.1 % in shoots and roots, respectively, compared with non-inoculated plants under the same conditions. Similar observation was observed in fresh weight between inoculated and non-inoculated plants in the absence of excess Cu. Plant height and N content also showed significant increase in comparison with non-inoculated plants in the absence and presence of Cu. The two-way analysis of variance (ANOVA) showed that the individual effects of inoculation and Cu treatment were significant ($p<0.05$) for all the measure parameters except the individual effect of inoculation on fresh weight of roots. The interactive effect of inoculation and Cu treatment was not significant ($p<0.05$) for all the measure parameters.

Cu content

The Cu content was considerably greater in roots than in shoots for both inoculated and non-inoculated plants exposed to excess Cu (Table 2). The Cu content of inoculated plants significantly increased by 5.52 and 32.23 % in shoots and roots, respectively, compared with non-inoculated plants. Plant dry weight and Cu content were calculated to show the total amount of Cu uptake in each plant .The results showed that inoculation with *S. meliloti* dramatically increased the total Cu uptake in both shoots and roots by 34.0 and 120.4 %, respectively, when plants were grown in the presence of Cu. Concerning the transport behavior of Cu from roots to shoots of plants, the TF of inoculated plants decreased by 16.67 % in the presence of 200 mg kg^{-1} Cu^{2+}, compared with that of non-inoculated plants. The two-way analysis of variance (ANOVA) showed that the individual effects of inoculation and Cu treatment were significant ($p<0.05$) for all the measure parameters except the individual effect of inoculation on TF. The interactive effect of inoculation and Cu treatment was significant ($p<0.05$) for all the measure parameters except on the total amount of Cu uptake in shoots.

Lipid peroxidation

The effects of strain *S. meliloti* CCNWSX0020 on the MDA content of *M. lupulina* plants under Cu stress are presented for both shoots and roots in Fig. 1. In the presence of Cu, inoculation with *S. meliloti* significantly decreased the MDA content in shoots at 8, 13, and 18 dpi, i.e., by15.0, 25.2, and 27.2 %, respectively, compared with non-inoculated plants. A similar situation was observed in the roots of inoculated plants, being significantly decreased by 13.3, 13.2, and 16.0 % at 8, 13, and 18 dpi, respectively.

Antioxidant enzyme activity

The effects of the symbiosis with *S. meliloti* CCNWSX0020 on the activities of SODs, CAT, APX and GR of *M. lupulina* plants under Cu stress are presented for shoots and roots in Fig. 2.

The total SOD activities of roots were much higher than those of shoots upon Cu stress. As shown in Fig. 2a, in the presence of Cu, a significant increase could be observed in the

Table 2 The effect of the symbiosis with *Sinorhizobium meliloti* CCNWSX0020 on Cu uptake of *Medicago lupulina*

Treatments	Cu content (μg g^{-1} dry weight)		Total amount of Cu uptake (ng $plant^{-1}$)		Translocation factor (TF)
	Shoots	Roots	Shoots	Roots	
CN	7.63±0.08 d	19.27±0.19 d	47.46±4.14 d	21.81±2.67 c	0.40±0.007 b
CSM	11.79±0.16 c	28.48±0.18 c	90.12±4.08 c	48.44±1.94 c	0.41±0.008 a
CuN	44.77±0.33 b	361.14±0.15 b	182.67±8.51b	252.80±20.87 b	0.12±0.001 c
CuSM	47.24±0.28 a	477.54±0.66 a	244.68±1.88 a	557.28±97.36 a	0.10±0.001 d
F values					
Inoculation (*df*=1)	204.013***	29,874.461***	23.902**	11.046*	0.426
Cu treatment (*df*=1)	24,482.825***	1,184,532.912***	183.181***	55.152***	2912.752***
Inoculation×Cu treatment (*df*=1)	13.217**	21,757.563***	0.817	7.778*	15.615**

The values indicate the mean±SE of three replicates. Different letters (a–d) show significant differences between treatments at $p<0.05$ by Duncan test

CN no stress conditions (non-inoculated), *CSM* no stress conditions (inoculated with *S. meliloti* CCNWSX0020), *CuN* Cu stress conditions (non-inoculated); *CuSM* Cu stress conditions (inoculated with *S. meliloti* CCNWSX0020)

*$p<0.05$; **$p<0.01$; ***$p<0.001$—levels of significance

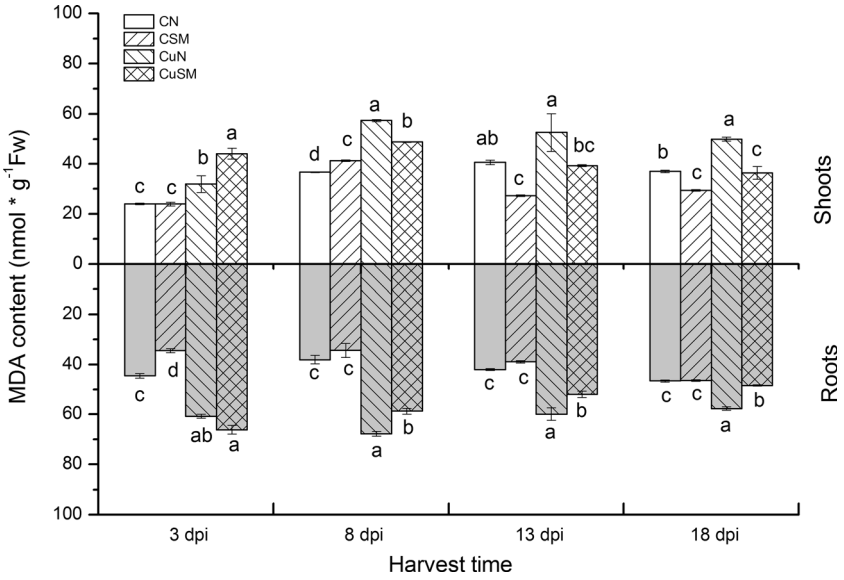

Fig. 1 The effect of the symbiosis with *Sinorhizobium meliloti* CCNWSX0020 on malondialdehyde (*MDA*) content in shoots (*white bar*) and roots (*gray bar*) of *Medicago lupulina* plants under Cu stress. *CN* no stress conditions (non-inoculated), *CSM* no stress conditions (inoculated with *S. meliloti*), *CuN* Cu stress conditions (non-inoculated), *CuSM* Cu stress conditions (inoculated with *S. meliloti*). The values indicate the mean±SE of three replicates. *Bars carrying different letters* are significantly different at $p<0.05$ by Duncan test

shoots of inoculated plants in comparison with non-inoculated plants at each harvested day except 8 dpi. Similarly, the total SOD activity of the roots of inoculated plants significantly increased, i.e., by 21.8, 43.1, and 12.4 % at 8, 13, and 18 dpi, respectively, compared with non-inoculated plants. However, the total SOD activity in roots in the absence of Cu stress was not detectable.

As shown in Fig. 2b, there were no regular changes in CAT activity in either shoots or roots as a consequence of Cu stress throughout the symbiosis development stage. On the other hand, CAT activity in the roots of inoculated plants under Cu stress was significantly elevated, i.e., by 44.8, 28.5, and 31.2 % at 3, 13, and 18 dpi, respectively, compared with non-inoculated plants. A significant increase in the shoots of inoculated plants was observed at 3 dpi.

In terms of APX activity, no significant changes were observed in either shoots or roots between inoculated and non-inoculated plants without Cu stress at each sampling time (Fig. 2c). However, the APX activity of inoculated shoots upon Cu stress was significantly increased by 93.4 and 23.8 %, respectively at 8 and 18 dpi compared with the plants without inoculation. There were no significant differences observed between the roots of inoculated plants and non-inoculated plants exposed to excess Cu.

As shown in Fig. 2d, no regular changes were observed in the GR activity as a result of excess Cu in either inoculated or non-inoculated plants. On the other hand, in the presence of Cu, the GR activity of the roots of inoculated plants increased significantly by 49.8, 54.2, and 14.3 %, respectively, at 8, 13, and 18 dpi compared with the plants without inoculation. A significant increase was also observed in shoots between non-inoculated and inoculated plants upon Cu stress at 18 dpi.

Expression profile analysis by real-time PCR

The expression profiles of mRNAs encoding various antioxidant genes (*CuZnSODc*, *CuZnSODp*, *FeSODp*, *MnSOD*, *CAT*, *APXc*, and *GRc*) involved in plant's defense system in both shoots and roots were analyzed using quantitative real-time PCR. The differentially expressed transcripts in the shoots and roots of inoculated plants compared with non-inoculated plants with or without Cu stress are presented in Tables 3 and 4, respectively. In the absence of Cu, the rhizobial symbiosis significantly upregulated the expression level of *CuZnSODc* and *CuZnSODp* in shoots at dpi 8, while only *CuZnSODc* were significantly upregulated in roots at dpi 13. On the other hand, in the presence of Cu, inoculation with *S. meliloti* CCNWSX0020 induced a different expression profile. Although the expression profile varied quite greatly at different symbiosis developmental stages, the symbiosis with *S. meliloti* CCNWSX0020 induced a significant increase in the expression of *CuZnSODc*, *CuZnSODp*, *CAT,* and *APXc* in shoots, and apparent increase in the expression level of *CuZnSODc*, *CuZnSODp*, *CAT*, *APXc*, and *GRc* in roots could also be observed. The rhizobial symbiosis significantly downregulated the expression level of *MnSOD* in both shoots and roots but has little effect on the expression of *FeSODp*.

Discussion

Considerably greater Cu accumulation in roots than in aerial organs was observed for both inoculated and non-inoculated plants exposed to 200 mg kg^{-1} Cu. The decline in plant biomass was presumably due to the adverse effects of excess Cu on root formation and growth, resulting in a reduced water and

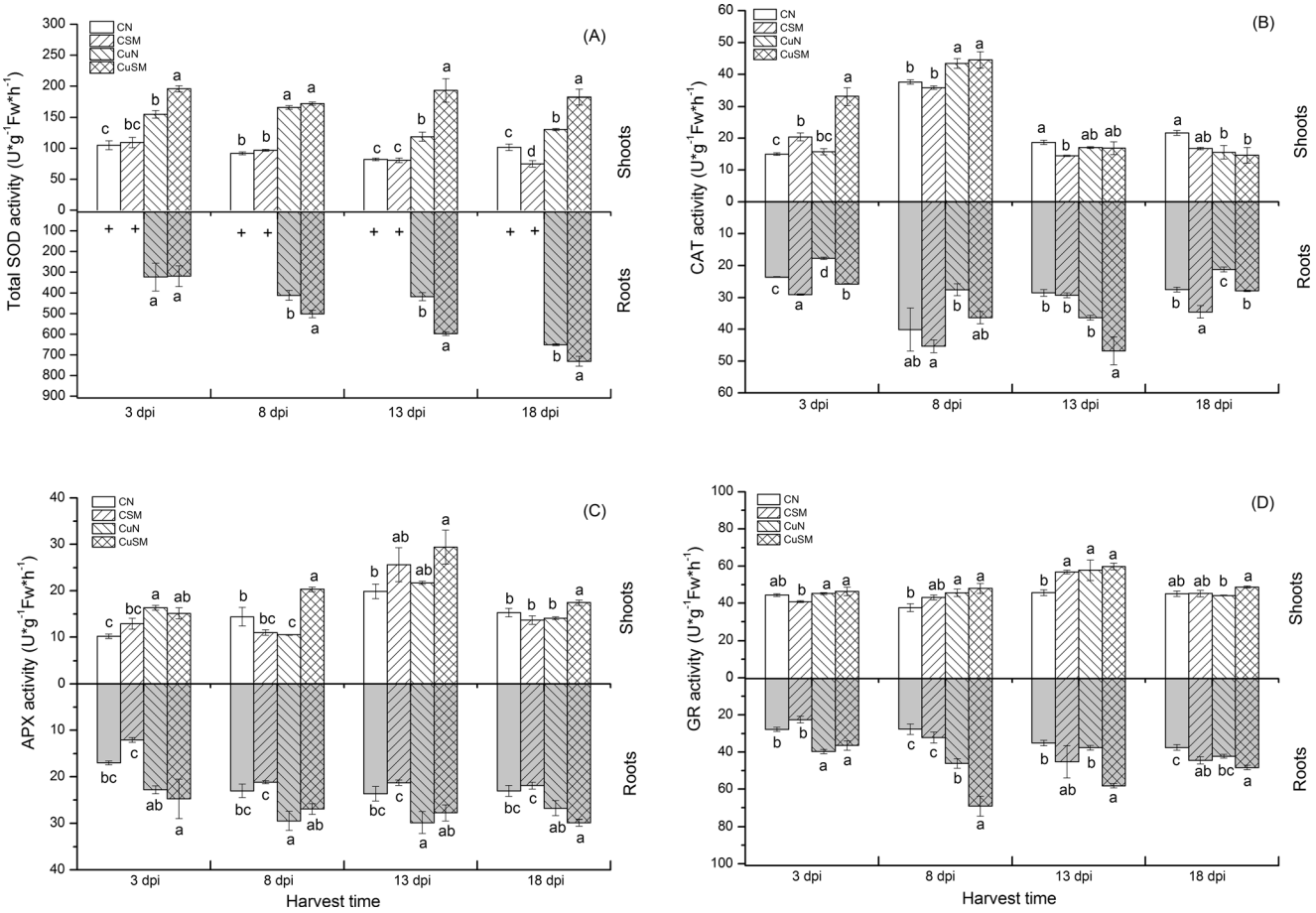

Fig. 2 The effect of the symbiosis with *Sinorhizobium meliloti* CCNW SX0020 on the antioxidant activities of SODs (**a**), CAT (**b**), APX (**c**), and GR (**d**) in shoots (*white bar*) and roots (*grey bar*) of *Medicago lupulina* plants under Cu stress. *CN* no stress conditions (non-inoculated), *CSM* no stress conditions (inoculated with *S. meliloti*), *CuN* Cu stress conditions (non-inoculated), *CuSM* Cu stress conditions (inoculated with *S. meliloti*), "+" not detected. The values indicate the mean±SE of three replicates. *Bars carrying different letters* are significantly different at $p<0.05$ by Duncan test

nutrient uptake by damaged roots (Kopittke 2006; Sheldon 2009). It was noted that the roots exposed to excess Cu tended to be short, thick, and dark brown and produce few roots hairs (data not shown). On the other hand, plants grown in the presence of symbiotic inoculants considerably increased the plant biomass in the presence of excess Cu. The beneficial effects of rhizobia on legume plant growth in the presence of arsenic (Reichman 2007), nickel, zinc (Wani et al. 2007; Wani et al. 2008c), and chromium (Wani et al. 2008a) have been reported in previous studies. Although the exact mechanisms of plant growth enhancement by plant growth-promoting rhizobacteria largely remain elusive, it is known that they differ between bacterial strains and depend on the various plant growth-promoting substances synthesized by the different bacterial strains, such as phytohormone, siderophores, IAA, and ACC deaminase (Dimkpa et al. 2009). Therefore, the positive siderophore activity, a high level of IAA production and a moderate level of ACC deaminase activity of *S. meliloti* CCNWSX0020 might enhance the overall growth of plants (Kong et al. 2015). The inoculated *M. lupulina* plants grown in the presence of excess Cu also showed a slight increase in N content in both shoots and roots. An increase in N content in other legume plants grown in heavy metal-contaminated soils has also been reported (Lasat 2001; Wani et al. 2008c). The N content in shoots could be considered as the supply of N through N-fixation in root nodules, since nitrogen-free nutrient solution was used in this work. This means that the copper-resistant strain *S. meliloti* CCNWSX0020 is able to survive under the Cu concentration used in this study, and thus promotes a normal level of plant nitrogen. This interpretation is supported by the observation that the presence of excess Cu had no effect on active nodules rate. Although some rhizobia have been reported to be sensitive to heavy metals, metal-resistant rhizobia are known as fully effective in nodulation and N_2-fixation of legumes (Pajuelo et al. 2011).

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

Table 3 The effect of the symbiosis with *Sinorhizobium meliloti* CCNWSX0020 on the relative expression (fold change) of antioxidant genes determined by qRT-PCR in shoots of *Medicago lupulina* plants in the presence of copper stress (CK) or absence of copper stress (Cu)

Genes	3 dpi		8 dpi		13 dpi		18 dpi	
	CK	Cu	CK	Cu	CK	Cu	CK	Cu
CuZnSODc	1.11±0.03	1.77±0.11	2.02±0.48*	2.57±0.37*	1.16±0.04	2.30±0.27*	1.09±0.09	2.91±0.07*
CuZnSODp	1.18±0.30	2.15±0.04*	4.81±0.60*	3.75±0.54*	1.65±0.06	1.05±0.06	1.46±0.11	1.63±0.08
FeSODp	0.71±0.37	1.34±0.11	1.94±0.27	1.21±0.51	0.77±0.07	1.54±0.19	0.74±0.18	1.45±0.16
MnSOD	1.01±0.25	0.14±0.08*	1.29±0.18	0.62±0.15	0.75±0.04	0.66±0.11	1.16±0.39	0.47±0.15*
CAT	1.87±0.61	1.45±0.15	1.20±0.13	15.75±2.52*	1.27±0.09	2.03±0.33*	0.81±0.12	1.53±0.28
APXc	1.73±0.09	1.77±0.11	1.09±0.39	6.73±1.29*	0.82±0.25	2.70±0.12*	1.77±0.41	2.73±0.33*
GRc	1.43±0.46	1.23±0.24	1.37±0.25	1.53±0.20	0.59±0.13	1.55±0.27	1.79±0.49	0.82±0.09

The values are indicated with an asterisk when >2 (upregulation) or <0.5 (downregulation)

CuZnSODc cytosolic *CuZnSOD*, *CuZnSODp* plastid *CuZnSOD*, *FeSODp* plastid *FeSOD*, *MnSOD* mitochondrial *MnSOD*, *APXc* cytosolic *APX*, *GRc* cytosolic *GR*

concentration (Rajkumar et al. 2009). In addition to nitrogen fixation, metal-resistant rhizobia demonstrate the production of plant growth-regulating substances or effects on metal solubility and bioavailability both of which affect on plant metal uptake (Pajuelo et al. 2011). In this study, inoculation with *S. meliloti* CCNWSX0020 significantly increased the total amount of Cu uptake in both shoots and roots. However, although the bacterium promotes metal accumulation both in shoots and roots, the increase in roots is much higher than in shoots, thus decreasing the translocation factor and helping phytostabilization, as previously showed for *Medicago*-rhizobia interactions in the presence of Cu (Delgadillo et al. 2015). These findings demonstrate the potential use of *Medicago*-rhizobia symbiosis for Cu phytostabilization, which could avoid leaching, soil erosion, and toxic metals transfer into the food chain (Ghosh and Singh 2005).

The increased level of MDA content and SOD activity in the plants treated with 200 mg kg^{-1} Cu^{2+} indicated that excessive Cu accumulation caused production of ROS, which in turn induced oxidative stress. Furthermore, the SOD activity was found to be increased in both shoots and roots of plants with and without inoculation at each sampling date, while the activities of CAT, APX, and GR varied quite greatly during the period studied. This indicated that the effect of excess Cu seems to be more specific on SOD activity at different developmental stages in *M. lupulina* plants. A close positive correlation between Cu concentration in growth medium and SOD activity in *Arabidopsis thaliana* leaves was also found by Drążkiewicz et al. (2004), which suggested that excess Cu uptake affects on this enzyme activity directly. Due to large amounts of reactive oxygen species (ROS) and possibly also reactive nitrogen species (RNS) generated in legume root nodules during the lifetime of symbiosis development, an important asset of antioxidant enzymes is present in both symbiotic partners (Matamoros et al. 2003). Becana et al. (2000) suggested that the promotion of plant antioxidant defenses by rhizobial bacteria may improve symbiotic performance, especially under non-optimal conditions. The results of this current

Table 4 The effect of the symbiosis with *Sinorhizobium meliloti* CCNWSX0020 on the relative expression (fold change) of antioxidant genes determined by qRT-PCR in roots of *Medicago lupulina* plants in the presence of copper stress (CK) or absence of copper stress (Cu)

Genes	3 dpi		8 dpi		13 dpi		18 dpi	
	CK	Cu	CK	Cu	CK	Cu	CK	Cu
CuZnSODc	0.59±0.13	2.10±0.20*	1.00±0.47	2.49±0.28*	5.18±0.47*	2.94±0.64*	0.62±0.14	9.32±3.33*
CuZnSODp	1.12±0.19	1.26±0.34	0.73±0.10	0.96±0.19	1.80±0.53	1.08±0.09	0.83±0.05	2.04±0.18*
FeSODp	0.63±0.41	1.02±0.54	0.61±0.06	0.62±0.10	0.70±0.05	0.57±0.13	0.65±0.05	0.90±0.11
MnSOD	0.77±0.10	0.45±0.06*	1.61±0.13	0.39±0.08*	1.56±0.18	1.89±0.29	0.62±0.09	0.90±0.23
CAT	0.55±0.10	1.78±0.23	1.25±0.07	2.01±0.23*	1.50±0.15	2.25±0.40*	0.61±0.08	1.91±0.13
APXc	0.73±0.19	9.17±2.09*	0.67±0.01	1.66±0.16	0.91±0.04	1.80±0.15	0.85±0.18	1.55±0.05
GRc	0.56±0.16	3.03±0.10*	1.58±0.04	2.26±0.59*	1.34±0.05	2.27±0.06*	0.60±0.08	1.74±0.11

The values are indicated with an asterisk when >2 (upregulation) or <0.5 (downregulation)

CuZnSODc cytosolic *CuZnSOD*, *CuZnSODp* plastid *CuZnSOD*, *FeSODp* plastid *FeSOD*, *MnSOD* mitochondrial *MnSOD*, *APXc* cytosolic *APX*, *GRc* cytosolic *GR*

study showed that the MDA content promoted by excess Cu was significantly lower in both shoots and roots of inoculated *M. lupulina* plants compared with non-inoculated plants harvested at the same times (Fig. 1). In addition, in comparison with non-inoculated plants, the activities of SOD and APX of shoots of inoculated plants exposed to excess Cu were elevated at different stages of symbiosis development; similar increases occurred in the activities of SOD, CAT, and GR of inoculated roots. According to the expression profile analyses of antioxidant genes, it indicated that symbiosis with *S. meliloti* generally increased the expression level of antioxidant genes in the presence of excess Cu, i.e., *CuZnSODc*, *CuZnSODp*, *CAT*, and *APXc* in shoots and *CuZnSODc*, *CuZnSODp*, *CAT*, *APXc*, and *GRc* in roots (Tables 3 and 4). These findings suggested that the increase in enzyme activities measured in the inoculated plants is not a consequence of the bacterial origin of similar enzymes. The changes of gene expression varied a great deal during symbiosis development in both inoculated and non-inoculated plants with or without copper. This indicated that the overall balance among various antioxidants is strictly controlled all times. Earlier study has reported that exogenous nitrogen could alleviate the oxidative stress associated with heavy metal exposure (Hassan et al. 2005; Zhang et al. 2014). On the other hand, nitrogen deficiency is associated with decreased enzyme activities that are involved in energy metabolism (e.g., photosynthesis and respiration) (de Groot et al. 2003). The observations in this study are in agreement with these previous findings, since no external N supply was added, along with the observation that the nitrogen content in the inoculated plants under excess Cu remarkably increased as compared with non-inoculated plants. In a recent study, nitrogen fixation via *Bradyrhizobium*-legume symbiosis also plays an important role in mitigating Cu toxicity, especially in white lupin plants (Sánchez-Pardo and Zornoza 2014). Thus, it is speculated that nitrogen fixation leading to increased plant health promotes the activities of antioxidant enzymes, thereby mitigating the copper toxicity in the plants through symbiosis with rhizobia.

On the basis of the experimental results in this study, it is possible to conclude that the genes coding for antioxidant enzymes were upregulated in the plants inoculated with *S. meliloti* CCNWSX0020 to produce a higher level of antioxidant enzymes and subsequently alleviate the adverse effects induced copper toxicity. However, the data presented are not sufficient to ascertain whether symbiotic rhizobia act directly on the oxidative processes in plants or through induction of some signaling pathways. Hence, further work on the specific mechanism of *S. meliloti* CCNWSX0020 promote plant tolerance to oxidative stress is required for the practical use of legume-*Rhizobium* symbiosis in phytoremediation in metal-contaminated soils.

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References

Abril A, Zurdo-Piñeiro JL, Peix A, Rivas R, Velázquez E (2007) Solubilization of phosphate by a strain of *Rhizobium leguminosarum* bv. *trifolii* isolated from *Phaseolus vulgaris* in El Chaco Arido soil (Argentina). In: Velázquez E, Rodríguez-Barrueco C (eds) First international meeting on microbial phosphate solubilization, vol 102. Developments in plant and soil sciences, vol 102. Springer, Netherlands, pp 135–138. doi:10.1007/978-1-4020-5765-6_19

Aebi H (1984) Catalase in vitro. Meth Enzymol 105:121–126

Ali H, Khan E, Sajad MA (2013) Phytoremediation of heavy metals-concepts and applications. Chemosphere 91:869–881. doi:10.1016/j.chemosphere.2013.01.075

Baker AJM, McGrath SP, Sidoli CMD, Reeves RD (1994) The possibility of in situ heavy metal decontamination of polluted soils using crops of metal-accumulating plants. Resour Conserv Recy 11:41–49. doi:10.1016/0921-3449(94)90077-9

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287. doi:10.1016/0003-2697(71)90370-8

Becana M, Dalton DA, Moran JF, Iturbe-Ormaetxe I, Matamoros MA, Rubio CM (2000) Reactive oxygen species and antioxidants in legume nodules. Physiol Plant 109:372–381. doi:10.1034/j.1399-3054.2000.100402.x

Becana M, Matamoros MA, Udvardi M, Dalton DA (2010) Recent insights into antioxidant defenses of legume root nodules. New Phytol 188:960–976. doi:10.1111/j.1469-8137.2010.03512.x

Brígido C, Glick B (2015) Phytoremediation using rhizobia. In: Ansari AA, Gill SS, Gill R, Lanza GR, Newman L (eds) Phytoremediation. Springer International Publishing, pp 95-114. doi:10.1007/978-3-319-10969-5_9

Chaoui A, El Ferjani E (2005) Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L.) seedlings. CR Biol 328:23–31. doi:10.1016/j.crvi.2004.10.001

Choudhury S, Panda P, Sahoo L, Panda SK (2013) Reactive oxygen species signaling in plants under abiotic stress. Plant Signal Behav 8, e23681. doi:10.4161/psb.23681

de Groot C, Marcelis LM, van den Boogaard R, Kaiser W, Lambers H (2003) Interaction of nitrogen and phosphorus nutrition in determining growth. Plant Soil 248:257–268. doi:10.1023/a:1022323215010

Delgadillo J, Lafuente A, Doukkali B, Redondo-Gómez S, Mateos-Naranjo E, Caviedes MA, Pajuelo E, Rodríguez-Llorente ID (2015) Improving legume nodulation and Cu rhizostabilization using a genetically modified rhizobia. Environ Technol 36:1237–1245. doi:10.1080/09593330.2014.983990

Demirevska-Kepova K, Simova-Stoilovaa L, Stoyanovaa Z, Hölzerb R, Fellerb U (2004) Biochemical changes in barley plants after excessive supply of copper and manganese. Environ Exp Bot 52:253–266. doi:10.1016/j.envexpbot.2004.02.004

Deniz T, Yasemin E, Şeniz Ü (2007) Changes in photochemical and antioxidant enzyme activities in maize (*Zea mays* L.) leaves exposed to excess copper. Chemosphere 67:89–98. doi:10.1016/j.chemosphere.2006.09.052

Dimkpa C, Weinand T, Asch F (2009) Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ 32:1682–1694. doi:10.1111/j.1365-3040.2009.02028.x

Drążkiewicz M, Sk'orzy'nska-Polit E, Krupa Z (2004) Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana*. Biometals 17:379–387. doi:10.1023/b:biom.0000029417.18154.22

Fåhraeus G (1957) The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol 16:374–381. doi:10.1099/00221287-16-2-374

Fan LM, Ma ZQ, Liang JQ, Li HF, Wang ET, Wei GH (2010) Characterization of a copper-resistant symbiotic bacterium isolated from *Medicago lupulina* growing in mine tailings. Bioresour Technol 102:703–709. doi:10.1016/j.biortech.2010.08.046

Festa RA, Thiele DJ (2011) Copper: an essential metal in biology. Curr Biol 21:R877–R883. doi:10.1016/j.cub.2011.09.040

Figueira R, Sérgio C, Sousa AJ (2002) Distribution of trace metals in moss biomonitors and assessment of contamination sources in Portugal. Environ Pollut 118:153–163. doi:10.1016/S0269-7491(01)00203-2

Foyer CH, Halliwell B (1976) The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133:21–25. doi:10.1007/BF00386001

Gamalero E, Glick BR (2011) Mechanisms used by plant growth-promoting bacteria. In: Maheshwari DK (ed) Bacteria in Agrobiology: Plant nutrient management. Springer Berlin, Heidelberg, pp 17–-46. doi:10.1007/978-3-642-21061-7_2c

Gerhardt KE, Huang XD, Glick BR, Greenberg BM (2009) Phytoremediation and rhizoremediation of organic soil contaminants: potential and challenges. Plant Sci 176:20–30. doi:10.1016/j.plantsci.2008.09.014

Ghosh M, Singh SP (2005) A review on phytoremediation of heavy metals and utilization of its by products. Appl Ecol Env Res 3:1–18

Ghosh S, Sengupta C, Maiti TK, Basu PS (2008) Production of 3-indolylacetic acid in root nodules and culture by a *Rhizobium* species isolated from root nodules of the leguminous pulse *Phaseolus mungo*. Folia Microbiol 53:351–355. doi:10.1007/s12223-008-0054-6

Glick BR (2003) Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol Adv 21:383–393. doi:10.1016/S0734-9750(03)00055-7

Glick BR (2010) Using soil bacteria to facilitate phytoremediation. Biotechnol Adv 28:367–374. doi:10.1016/j.biotechadv.2010.02.001

Glick BR, Cheng Z, Czarny J, Duan J (2007) Promotion of plant growth by ACC deaminase-producing soil bacteria. Eur J Plant Pathol 119:329–339. doi:10.1007/978-1-4020-6776-1_8

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. Plant Physiol 131:872–877. doi:10.1104/pp.017004

Hasanuzzaman M, Hossain M, da Silva JT, Fujita M (2012) Plant response and tolerance to abiotic oxidative stress: antioxidant defense is a key factor. In: Venkateswarlu B, Shanker AK, Shanker C, Maheswari M (eds) Crop stress and its management: perspectives and strategies. Springer, Netherlands, pp 261–315. doi:10.1007/978-94-007-2220-0_8

Hassan M, Wang F, Ali S, Zhang G (2005) Toxic effect of cadmium on rice as affected by nitrogen fertilizer form. Plant Soil 277:359–365. doi:10.1007/s11104-005-8160-6

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198. doi:10.1016/0003-9861(68)90654-1

Jouili H, El Ferjani E (2003) Changes in antioxidant and lignifying enzyme activities in sunflower roots (*Helianthus annuus* L.) stressed with copper excess. CR Biol 326:639–644. doi:10.1016/S1631-0691(03)00157-4

Komárek M, Tlustoš P, Száková J, Chrastný V, Ettler V (2007) The use of maize and poplar in chelant-enhanced phytoextraction of lead from contaminated agricultural soils. Chemosphere 67:640–651. doi:10.1016/j.chemosphere.2006.11.010

Kong ZY, Glick BR, Duan J, Ding SL, Tian J, McConkey BJ, Wei GH (2015) Effects of 1-aminocyclopropane-1-carboxylate (ACC) deaminase-overproducing *Sinorhizobium meliloti* on plant growth and copper tolerance of *Medicago lupulina*. Plant Soil. doi:10.1007/s11104-015-2434-4

Kopittke P (2006) Effect of Cu toxicity on growth of cowpea (*Vigna unguiculata*). Plant Soil 279:287–296. doi:10.1007/s11104-005-1578-z

Lasat MM (2001) Phytoextraction of metals from contaminated soil: a review of plant/soil/metal interaction and assessment of pertinent agronomic issues. J Hazard Sub Res 2:1–21

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408. doi:10.1006/meth.2001.1262

Lu X, Wang L, Lei K, Huang J, Zhai Y (2009) Contamination assessment of copper, lead, zinc, manganese and nickel in street dust of Baoji, NW China. J Hazard Mater 161:1058–1062. doi:10.1016/j.jhazmat.2008.04.052

Martins LL, Mourato MP (2006) Effect of excess copper on tomato plants: growth parameters, enzyme activities, chlorophyll, and mineral content. J Plant Nutr 29:2179–2198. doi:10.1080/01904160600972845

Matamoros MA, Dalton DA, Ramos J, Clemente MR, Rubio MC, Becana M (2003) Biochemistry and molecular biology of antioxidants in the rhizobia-legume symbiosis. Plant Physiol 133:499–509. doi:10.1104/pp.103.025619

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867–880

Naya L, Ladrera R, Ramos J, Gonz a, lez EM, Arrese-Igor C, Minchin FR, Becana M (2007) The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. Plant Physiol 144:1104–1114

Ott T, van Dongen JT, Gu¨nther C, Krusell L, Desbrosses G, Vigeolas H, Bock V, Czechowski T, Geigenberger P, Udvardi MK (2005) Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. Curr Biol 15:531–535. doi:10.1016/j.cub.2005.01.042

Pajuelo E, Rodríguez-Llorente ID, Lafuente A, Caviedes MÁ (2011) Legume-*Rhizobium* symbioses as a tool for bioremediation of heavy metal polluted soils. In: Khan MS, Zaidi A, Goel R, Musarrat J (eds) Biomanagement of metal-contaminated soils, vol 20. Springer, Netherlands, pp 95–123. doi:10.1007/978-94-007-1914-9_4c

Posmyk MM, Kontek R, Janas KM (2009) Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. Ecotoxicol Environ Saf 72:596–602. doi:10.1016/j.ecoenv.2008.04.024

Rajkumar M, Ae N, Freitas H (2009) Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77:153–160. doi:10.1016/j.chemosphere.2009.06.047

Rajkumar M, Sandhya S, Prasad MNV, Freitas H (2012) Perspectives of plant-associated microbes in heavy metal phytoremediation. Biotechnol Adv 30:1562–1574. doi:10.1016/j.biotechadv.2012.04.011

Reichman SM, Menzies NW, Asher CJ, Mulligan DR (2006) Responses of four Australian tree species to toxic concentrations of copper in solution culture. J Plant Nutr 29:1127–1141. doi:10.1080/01904160600689274

Reichman SM (2007) The potential use of the legume-*Rhizobium* symbiosis for the remediation of arsenic contaminated sites. Soil Biol Biochem 39:2587–2593. doi:10.1016/j.soilbio.2007.04.030

Sánchez-Pardo B, Zornoza P (2014) Mitigation of Cu stress by legume-*Rhizobium* symbiosis in white lupin and soybean plants. Ecotoxicol Environ Saf 102:1–5. doi:10.1016/j.ecoenv.2014.01.016

Sandmann G, Böger P (1980) Copper-mediated lipid peroxidation processes in photosynthetic membranes. Plant Physiol 66:797–800. doi:10.1104/pp.66.5.797

Sheldon A (2009) The effect of copper toxicity on the growth and root morphology of Rhodes grass (*Chloris gayana Knuth*.) in resin buffered solution culture. Plant Soil 278:341–349. doi:10.1007/s11104-005-8815-3

Singh RP, Agrawal M (2007) Effects of sewage sludge amendment on heavy metal accumulation and consequent responses of *Beta vulgaris* plants. Chemosphere 67:2229–2240. doi:10.1016/j.chemosphere.2006.12.019

Srinivasa Gowd S, Ramakrishna Reddy M, Govil PK (2010) Assessment of heavy metal contamination in soils at Jajmau (Kanpur) and Unnao industrial areas of the Ganga Plain, Uttar Pradesh, India. J Hazard Mater 174:113–121. doi:10.1016/j.jhazmat.2009.09.024

Tewari RK, Kumar P, Sharma PN (2006) Antioxidant responses to enhanced generation of superoxide anion radical and hydrogen peroxide in the copper-stressed mulberry plants. Planta 223:1145–1153. doi:10.1007/s00425-005-0160-5

Thounaojam TC, Panda P, Mazumdar P, Kumar D, Sharma GD, Sahoo L, Sanjib P (2012) Excess copper induced oxidative stress and response of antioxidants in rice. Plant Physiol Biochem 53:33–39. doi:10.1016/j.plaphy.2012.01.006

Vidal C, Chantreuil C, Berge O, Mauré L, Escarré J, Béna G, Brunel B, Cleyet-Marel J-C (2009) *Mesorhizobium metallidurans* sp. nov., a metal-resistant symbiont of *Anthyllis vulneraria* growing on metallicolous soil in Languedoc, France. Int J Syst Evol Microbiol 59:850–855. doi:10.1099/ijs.0.003327-0

Wani PA, Khan MS, Zaidi A (2007) Effect of metal tolerant plant growth promoting *Bradyrhizobium* sp. (vigna) on growth, symbiosis, seed yield and metal uptake by greengram plants. Chemosphere 70:36–45. doi:10.1016/j.chemosphere.2007.07.028

Wani PA, Khan MS, Zaidi A (2008a) Chromium-reducing and plant growth-promoting *Mesorhizobium* improves chickpea growth in chromium-amended soil. Biotechnol Lett 30:159–163. doi:10.1007/s10529-007-9515-2

Wani PA, Khan MS, Zaidi A (2008b) Effect of metal-tolerant plant growth-promoting *Rhizobium* on the performance of pea grown in metal-amended soil. Arch Environ Contam Toxicol 55:33–42. doi:10.1007/s00244-007-9097-y

Wani PA, Khan MS, Zaidi A (2008c) Effects of heavy metal toxicity on growth, symbiosis, seed yield and metal uptake in pea grown in metal amended soil. Bull Environ Contam Toxicol 81:152–158. doi:10.1007/s00128-008-9383-z

Yahyaoui FE, Küster H, Amor BB, Hohnjec N, Pühler A, Becker A, Gouzy J, Vernié T, Gough C, Niebel A, Godiard L, Gamas P (2004) Expression profiling in *Medicago truncatula* identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. Plant Physiol 136:3159–3176. doi:10.1104/pp.104.043612

Zhang F, Wan X, Zheng Y, Sun L, Chen Q, Zhu X, Guo Y, Liu M (2014) Effects of nitrogen on the activity of antioxidant enzymes and gene expression in leaves of *Populus* plants subjected to cadmium stress. J Plant Interact 9:599–609. doi:10.1080/17429145.2013.879676

Zhang H, Xia Y, Wang G, Shen Z (2008) Excess copper induces accumulation of hydrogen peroxide and increases lipid peroxidation and total activity of copper-zinc superoxide dismutase in roots of *Elsholtzia haichowensis*. Planta 227:465–475. doi:10.1007/s00425-007-0632-x