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ORIGINAL PAPER

Influence of arbuscular mycorrhizal fungi and copper on growth, accumulation of osmolyte, mineral nutrition and antioxidant enzyme activity of pepper (*Capsicum annuum* L.)

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Abstract The effect of arbuscular mycorrhizal (AM) fungi inoculation on pepper (*Capsicum annuum* L. cv. Zhongjiao 105) plant growth and on some physiological parameters in response to increasing soil Cu concentrations was studied. Treatments consisted of inoculation or not with *Glomus mosseae* and the addition of Cu to soil at the concentrations of 0 (control), 2 (low), 4 (medium), and 8 (high) mM $CuSO_4$. AM fungal inoculation decreased Cu concentrations in plant organs and promoted biomass yields as well as the contents of chlorophyll, soluble sugar, total protein, and the concentrations of P, K, Ca, and Mg. Plants grown in high Cu concentration exhibited a Cu-induced proline accumulation and also an increase in total free amino acid contents; however, both were lower in mycorrhizal pepper. Cu-induced oxidative stress by increasing lipid peroxidation rates and the activity of superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase, and AM symbiosis enhanced these antioxidant enzyme activities and decreased oxidative damage to lipids. In conclusion *G. mosseae* was able to maintain an efficient symbiosis with pepper plants in contaminated Cu soils, improving plant growth under these conditions, which is likely to be due to reduced Cu accumulation in plant tissues, reduced oxidative stress and damage to lipids, or enhanced antioxidant capacity.

Keywords *Arbuscular mycorrhiza* · *Capsicum annuum* · Copper · *Glomus mosseae* · Heavy metals · Oxidative stress · Proline · Soluble sugar

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Introduction

Arbuscular mycorrhizal (AM) fungi, obligate biotrophs of higher plants, constitute one of the most widespread groups of soil microorganisms (Barea 1991). AM fungi colonize the root cortex of most plant species and develop an extraradical mycelium which spreads through the soil surrounding plant roots. By increasing the interface between plants and the soil environment, they contribute to plant uptake of macronutrients (P and N) as well as micronutrients (Cu and Zn; Smith and Read 2008). On the other hand, under conditions of supraoptimal levels of essential metals, or in the presence of toxic ones, AM fungi are able to alleviate metal toxicity in the plant (Leyval et al. 2002). Despite the significant role that AM fungi play in plant interactions with soil metals and the ubiquity of AM fungi in soil environments, only recently progress has been made towards understanding the cellular mechanisms used to control heavy metals and to avoid their toxicity in AM plants (Gohre and Paszkowski 2006; Hildebrandt et al. 2007; González-Guerrero et al. 2009).

Copper (Cu) is an essential element for plant growth and plays a significant role in many physiological processes, including photosynthesis, respiration, carbohydrate distribution, nitrogen fixation, protein metabolism, and antioxidant activity. At the cellular level, copper is a structural and catalytic component of many proteins and enzymes involved in a variety of metabolic pathways (Pilon et al. 2006). Plants usually find an ample supply of copper in soils; but at high concentrations, the metal can be a stress factor triggering physiological responses and copper concentrations in cells need to be maintained at low levels (Yruela 2005).

Nitrogen metabolism has a central role in plant responses to metal stress (Lea and Azevedo 2007). Several nitrogenous metabolites, such as amino acids, can bind metal cations or scavenge reactive oxygen species (ROS) formed in response to excessive metal concentrations in cells (Groppa and Benavides 2008). The accumulation of some amino acids, such as proline has been reported in plants under excess Cu conditions and suggested to act as an ROS scavenger or to be involved in metal chelation (Sharma and Dietz 2006). Production of ROS and free radicals induced by an excess of Cu can in turn damage cell membranes by binding to the sulfhydryl groups of membrane proteins or by increasing rates of lipid peroxidation (Liu et al. 2004). In order to overcome harmful effects of free radicals, plants exposed to high Cu levels may show increased antioxidant responses (Gratão et al. 2008) which result from enzyme activities such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR; Schützendübel and Polle 2002); it has been reported that Cu increases the activities of antioxidative enzymes such as SOD, CAT, and APX (Lombardi and Sebastiani 2005; Ahmed et al. 2010). SOD catalyzes the dismutation of two molecules of superoxide into oxygen and H_2O, CAT is implicated in the removal of H_2O_2, APX reduces H_2O_2 to water, with ascorbate as electron donor (Asada 1992), and GR plays a part in the control of endogenous H_2O_2, through an oxido-reduction cycle involving glutathione and ascorbate (Smith et al. 1989).

The objective of this study was to improve understanding of the mechanisms of Cu stress alleviation in AM plants. For this purpose, pepper plants were inoculated with *Glomus mosseae* and compared to non-mycorrhizal plants grown under Cu-stressed conditions. The effect of an established AM association on the growth, chlorophyll, soluble sugar, total protein, proline and amino acid contents of pepper plants was analyzed, as well as the activity of antioxidant enzymes and ion balance in Cu-affected plants.

Material and methods

Experimental design

A greenhouse experiment was conducted using a 2×4 factorial scheme and completely randomized design, with 6 replications. The treatments used were the absence or presence of 2, 4, and 8 mM of $CuSO_4$ in the nutrient solution and the inoculation or not with *G. mosseae*. Treatments were denominated as: control (grown in the absence of Cu and *G. mosseae*), M (grown in the absence of Cu but inoculated with *G. mosseae*), Cu (grown in the presence of 2 (low), 4 (medium), and 8 (high) mM of $CuSO_4$ but not inoculated with *G. mosseae*), and M+Cu (grown in the presence of 2, 4, and 8 mM of $CuSO_4$ and inoculated with *G. mosseae*).

AM fungal inoculum

G. mosseae was obtained from the collection maintained by the Institute of Vegetables and Flowers, CAAS, Beijing, P. R. China. Colonized root fragments, mycelium, and a sand–soil mixture containing spores were used as inoculum. Each pot received approximately 2,500 spores at the time of sowing. The nonmycorrhizal treatments received washings of the soil–inoculum mixture filtered through Whatman no 42 filter paper.

Pot culture experiment

Pepper (*Capsicum annuum* L. cv. Zhongjiao 105) seeds were surface-sterilized with 1:3 (*v*/*v*) of 2.5% sodium hypochlorite solution for 10 min. Six seeds were sown per pot containing 2 kg of an autoclaved mixture of black soil and sand (1:1.5, *v*/*v*). The soil mix was collected from greenhouse of Institute of Vegetables and Flowers and sterilized (160°C, 4 h). Soil properties were pH 7.26, 11.1% organic matter, 150 mg kg^{-1} available phosphorus, 451 mg kg^{-1} available nitrogen, and 518 mg kg^{-1} available potassium. After emergence, seedlings were thinned to two plants per pot. The plants were supplemented with a nutrient solution for once per week in the following composition: KNO_3 5 mM, KH_2PO_4 1 mM, $Ca(NO_3)_2$ 5 mM, $MgSO_4$ 2 mM, H_3BO_3 50 μM, $MnCl_2$ 10 μM, $ZnSO_4$ 1 μM, $CuSO_4$ 0.4 μM, H_2MoO_4 0.1 μM, and Fe-EDTA 20 μM. Distilled water was supplied on alternate days. Plants undergoing Cu treatment received a nutrient solution containing 2, 4, or 8 mM of Cu supplied as $CuSO_4$. The plants were allowed to grow for 8 weeks. Seeds were grown in a green house, with temperature ranging from 22–30°C and relative humidity ranging from 60% to 80%.

Measurements and analyses

Growth criteria

Leaf area was measured using a digital planimeter (Placom KP-90) to the nearest square centimeter. At harvest, root, stem, and leaves were separately washed with tap water to remove any adhering debris. The root and shoot dry weights were determined after over drying at 70°C for 48 h.

Root colonization by G. mosseae

A fraction of the roots was carefully washed, cut into 1 cm long segments, cleared in 10% KOH at 90°C for 20 min,

acidified in 2% HCl for 5 min, and stained with 0.01% acid fuchsin (Kormanik et al. 1980). Mycorrhizal colonization was evaluated using the gridline intercept method described by Giovannetti and Mosse (1980).

Photosynthetic pigments and metabolite analysis

The concentrations of chlorophyll a, chlorophyll b, and chlorophyll a+b and carotenoids of the youngest fully-expanded leaf were assayed 1 week before harvest according to Zhang and Zhang (2006). Extracts were made from a 200 mg fresh sample in 20 ml ethanol, acetone and water (4.5: 4.5: 1, *v*/*v*/*v*) mixture and measured at 645, 663, and 470 nm with a UV/VIS spectrophotometer.

Soluble sugar content was determined by the anthrone sulfuric acid method described by Badour (1959). The dried tissue of leaves was extracted by distilled water. One milliliter of the carbohydrate extract was mixed with 9 ml of anthrone sulfuric acid reagent in a test tube and heated for 7 min at 100°C. The absorbance was measured spectrophotometrically at 620 nm against a blank containing only distilled water and anthrone reagent.

Total protein of leaves was determined according to the method described by Bradford (1976), in which 5 ml of the protein reagent were added to 0.1 ml of the extract and the contents mixed on a vortex mixer. The absorbance was measured at 595 nm after 1 h. The concentration of protein was calculated from a previously constructed standard curve for bovine serum albumin.

Total free amino acids of leaves were extracted and estimated according to the method of Lee and Takahashi (1966). About 0.1 m1 of the water extract containing free amino acids was mixed with 1.9 ml of ninhydrin–citrate–glycerol mixture in a test tube for 20 min at 100°C. The absorbance was measured at 570 nm against a blank (only distilled water and the same reagent).

Proline content of leaves was estimated according to Bates et al. (1973) for free proline determination. A known weight of dried tissue was homogenized in 10 ml of 3% sulfosalicylic acid and filtered. Two milliliter of the filtrate was reacted with 2 ml glacial acetic acid and 2 ml of acid–ninhydrin reagent in a test tube and heated for 1 h at 100°C. The reaction mixture was extracted with 4 ml toluene. The chromophore was sampled from the aqueous phase and the absorbance was read at 520 nm using toluene as a blank.

Mineral content

Dried samples were ground and digested in concentrated acid (HNO_3: $HClO_4$, 2:3 *v*/*v*) at 140–160°C. After cooling, the extracts were diluted with 1 M HCl and made up to 25 ml (Allen 1989). Reagent blanks were prepared by carrying out the whole extraction procedure but in the absence of sample. Phosphorus concentration in leaves was determined by the ammonium molybdate blue method (Allen 1989). The same digest was also used to determine the concentration of Cu in root and shoot or K, Ca and Mg in leaves by atomic absorption spectrometry.

Lipid peroxidation

Malondialdehyde (MDA) was measured according to the thiobarbituric acid (TBA) reaction as described by Zhang and Qu (2004). Leaf samples were homogenized with 5% trichloroacetic acid and centrifuged at 4,000×*g* for 10 min. Two ml of extract was added to 2 ml 0.6% TBA placed in a boiling water bath for 10 min, and absorbance was read at 532, 600, and 452 nm. The MDA concentration was calculated according to the formula: $6.45\times (A_{532}-A_{600})-0.56\times A_{450}$.

Extraction of antioxidant enzymes

For enzyme extracts and assays, 500 mg fresh leaves were frozen in liquid nitrogen and then ground in 4 ml solution containing 50 mM phosphate buffer (pH 7.0), 1% (*w*/*v*) polyvinylpolypyrrolidone, and 0.2 mM ascorbic acid. The homogenate was centrifuged at 15,000×*g* for 30 min, and the supernatant was collected for enzyme assays.

Assays for antioxidant enzymes activities

The activity of SOD (EC 1.15.1.1) was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT), according to Stewart and Bewley (1980). The reaction mixture (3 ml) contained 13 mM methionine, 75 mM NBT, 100 mM EDTA, 50 μl of enzyme extract within 50 mM phosphate buffer (pH 7.8). The reaction was started with 2 mM riboflavin by exposing the cuvette to a 15 W fluorescent tube for 10 min. The absorbance of each reaction mixture was measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme which causes 50% inhibition of the photochemical reduction of NBT.

The activity of CAT (EC 1.11.1.6) was determined as a decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2 (Chance and Meahly 1955). The reaction mixture contained 50 mM phosphate buffer (pH 7.0) and 15 mM H_2O_2, and 0.15 ml enzyme extract.

The activity of APX (EC 1.11.1.11) was measured as a decrease in absorbance at 290 nm for 1 min (Nakano and Asada 1981). The assay mixture consisted of 0.5 mM ASA, 0.1 mM H_2O_2, 0.1 mM EDTA, 50 mM sodium phosphate buffer (pH 7.0), and 0.15 ml enzyme extract.

The activity of GR (EC 1.6.4.2) was determined by following the decrease in absorbance at 340 nm for 1 min

due to the glutathione-dependence of NADPH (Cakmak et al. 1993). The reaction mixture contained 1 mM EDTA, 0.5 mM GSSG, 0.15 mM NADPH, 100 mM sodium phosphate buffer (pH 7.8), and 0.15 ml enzyme extract.

Statistical analysis

The experimental data were subjected to analysis and two-way analysis of variance using ANOVA. Means were compared by Duncan's test at the 5% level using SPSS program.

Results

Pepper shoot and root dry weight, shoot/root ratio and leaf area were generally higher for mycorrhizal (M) than nonmycorrhizal (NM) plants (Table 1), but the differences were not significant for shoot dry weight at the medium Cu level or for root dry weight and leaf area up to 4 mM $CuSO_4$. Moreover, *G. mosseae* colonization had no significant effect on shoot/root ratio at low and high Cu levels (Table 1). The positive effects of mycorrhization on shoot and root dry weight and leaf area were recorded at high levels of Cu treatment compared to NM plants (Table 1). The enhancement in shoot and root dry weight due to *G. mosseae* inoculation was more than twofold under a high level of Cu compared to NM plants (Table 1). Roots of NM plants did not exhibit AM fungal structures. The mean proportion of root length with *G. mosseae* colonization ranged from 15% to 57%. Cu addition negatively influenced mycorrhizal root colonization which decreased with increasing metal concentration in soil (Table 1).

Copper treatments drastically lowered leaf chlorophyll content of NM plants (Table 2). Colonization of roots by *G. mosseae* improved chlorophyll content at low and high levels of Cu as well as under control conditions. At the high Cu level, M plants exhibited 56%, 27%, 43%, and 65% increase in chl a, chl b, chl a+b, and carotenoids, respectively, when compared to NM plants (Table. 2). On the other hand, *G. mosseae* inoculation had no significant effect on chlorophyll content at the medium level of Cu versus NM plants (Table 2). The soluble sugar and total protein contents of leaves decreased with increasing Cu concentration in the nutrient solution. However, M plants compared to NM plants, contained greater amounts of sugar and protein especially at the high level of Cu. The enhancement in soluble sugar and total protein due to *G. mosseae* inoculation was 91% and 64%, respectively, under the high Cu level compared to NM plants (Table 3). Total free amino acid and proline contents in leaves were drastically increased in both NM and M plants grown under treatments with copper additions, but the extent of modifications due to Cu addition to soil was higher in NM than M plants (Table 3).

Cu concentration in root and shoot of both NM and M plants increased as metal additions to soil increased (Table 4). It was lower in M than NM plants at medium and high Cu levels, but differences were not significant at the low Cu level and in the control treatment. The phosphorous and potassium status of shoots showed that P and K uptake was stimulated by *G. mosseae* inoculation. Shoot P and K concentrations were higher in M than NM plants for all experimental treatments (Table 4). Cu addition to soil reduced the P and K concentrations in shoot of both NM and M plants, but no significant differences were noted for shoot P and K concentrations up to 2 mM $CuSO_4$ regardless of whether plants were mycorrhizal or not (Table 4). Shoot concentrations of Ca and Mg were apparently higher for M than NM plants regardless of Cu treatments, but the differences for Ca and Mg concentrations were not significant for the control treatment (Table 4). However, shoot concentrations of Ca and Mg in both NM and M plants decreased with increasing Cu in the soil, but no significant differences were noted for shoot Ca and Mg concentrations at 4 mM $CuSO_4$ regardless of the mycorrhizal status of plants (Table 4).

Table 1 Shoot and root dry weight (g plant^{-1}), shoot/root ratio, leaf area (cm^2), and AMF colonization (%) of nonmycorrhizal (NM) and mycorrhizal (M) pepper (*Capsicum annuum* L.) plants grown in soil with increasing Cu concentrations

Treatments		Shoot dry weight	Root dry weight	Shoot/root	Leaf area	AMF colonization
$CuSO_4$ (mM)	AMF					
0	NM	1.72 c	0.82 ab	2.10 bc	380 a	57 a
	M	2.62 a	0.95 a	2.76 a	400 a	
2	NM	1.92 bc	0.88 ab	2.18 b	388 a	48 b
	M	2.19 b	0.99 a	2.21 b	405 a	
4	NM	0.95 de	0.65 c	1.46 f	275 bc	40 c
	M	1.22 d	0.73 c	1.67 e	294 bc	
8	NM	0.40 f	0.21 g	1.90 d	140 f	15 e
	M	0.81 h	0.45 e	1.80 d	204 d	

Mean pairs followed by different letters are significantly different ($p<0.05$) by Duncan's test; $n=6$

Table 2 Chlorophyll a, Chl b, Chl a+b and carotenoids contents (mg g^{-1}) in leaves of nonmycorrhizal (NM) and mycorrhizal (M) pepper (*Capsicum annuum* L.) plants grown in soil with increasing Cu concentrations

Treatments $CuSO_4$ (mM)	AMF	Chl a	Chl b	Chl a+b	Carotenoids
0	NM	1.65 b	1.21 b	2.86 b	0.59 b
	M	1.99 a	1.64 a	3.63 a	0.75 a
2	NM	1.54 b	1.01 bc	2.55 bc	0.53 bc
	M	1.95 a	1.58 a	3.53 a	0.64 ab
4	NM	0.86 d	0.74 cd	1.60 e	0.36 e
	M	0.95 d	0.89 c	1.84 de	0.43 de
8	NM	0.50 f	0.43 ef	0.93 g	0.20 f
	M	0.78 de	0.55 e	1.33 f	0.33 e

Mean pairs followed by different letters are significantly different ($p<0.05$) by Duncan's test; $n=6$

MDA, an indicator of lipid peroxidation, was measured to determine whether application of excessive Cu caused oxidative stress in leaves of pepper. $CuSO_4$ treatments resulted in a significant increase in MDA content in both NM and M plants (Table 5), but it remained lower in M than NM plants for all treatments. At the high level of Cu, the increase in MDA content was 2.4-fold and 60% in NM and M plants, respectively, compared to control plants. $CuSO_4$ addition to soil resulted in a marked increase in the activity of antioxidant enzymes in both NM and M plants (Table 5) which was higher in M than NM plants for all treatments. The enhancement in the activity of SOD, CAT, APX, and GR due to AMF inoculation was 20%, 50%, 16%, and 29%, respectively, under the high Cu level compared to NM plants (Table 5).

Discussion

It is well known that the association of plants with soil microorganisms, including mycorrhizal fungi, modifies plant responses to metal-induced stresses, increasing tolerance in metal-contaminated soils (Göhre and Paszkowski 2006). In the present study, both *G. mosseae* and pepper plants exhibited relative tolerance to Cu at soil concentrations considered within the risk assessment range (Mirlean et al. 2007). However, decreases in dry weight production and leaf area were observed as Cu additions to soil increased, suggesting that metabolic processes might be impaired by high Cu contents in the soil. Mycorrhizal colonization promoted plant dry weight accumulation and leaf area when compared to nonmycorrhizal plants at most levels of Cu additions, indicating an efficient symbiosis. Despite the fungitoxic properties attributed to Cu, the colonization of pepper roots by *G. mosseae* was not affected by the addition of high Cu concentrations to soil, suggesting that roots provided an adequate environment protecting fungal growth from the toxic effects of Cu. Mycorrhizal colonization of pepper roots was significantly decreased at the highest Cu concentration added to the soil. Reduction or even inhibition of mycorrhizal colonization by heavy metals has been reported by several authors

Table 3 Soluble sugar, total protein, total free amino acids and proline contents (mg g^{-1}) in leaves of nonmycorrhizal (NM) and mycorrhizal (M) pepper (*Capsicum annuum* L.) plants grown in soil with increasing Cu concentrations

Treatments $CuSO_4$ (mM)	AMF	Soluble sugar	Total protein	Total Free amino acids	Proline
0	NM	7.65 b	44.35 b	11.34 gh	0.63 fg
	M	9.12 a	57.06 a	9.87 h	0.29 hi
2	NM	6.34 c	40.21 b	13.24 g	0.71 fg
	M	8.93 a	55.45 a	10.35 h	0.34 hi
4	NM	5.03 d	28.75 c	20.45 cd	0.95 de
	M	6.19 c	36.12 bc	15.65 ef	0.65 fg
8	NM	2.33 fg	16.55 d	34.64 a	2.45 a
	M	4.45 de	27.12 c	23.56 c	1.09 d

Mean pairs followed by different letters are significantly different ($p<0.05$) by Duncan's test; $n=6$

Table 4 Cu concentration in root and shoot (mg kg^{-1}) and concentrations of P, K, Ca, and Mg (mg g^{-1}) in shoot of nonmycorrhizal (NM) and mycorrhizal (M) pepper (*Capsicum annuum* L.) plants grown in soil with increasing Cu concentrations

Treatments $CuSO_4$ (mM)	AMF	Cu root	Cu shoot	P	K	Ca	Mg
0	NM	43.98 h	7.85 i	3.43 d	34.03 c	24.15 ab	8.85 a
	M	35.54 hi	6.98 i	6.65 a	49.12 a	28.67 a	9.76 a
2	NM	63.87 g	12.98 g	2.98 de	31.63 c	20.45 bc	6.90 b
	M	52.89 gh	10.86 gh	6.34 a	44.12 a	31.65 a	8.87 a
4	NM	92.98 e	35.87 e	1.45 fg	24.03 d	15.35 cd	4.97 c
	M	77.98 f	23.87 f	3.19 d	30.45 c	25.04 ab	7.96 ab
8	NM	486.08 a	75.76 a	1.06 g	16.15 e	10.60 e	2.03 e
	M	332.87 c	55.98 c	1.99 f	23.34 d	17.87 c	4.08 cd

Mean pairs followed by different letters are significantly different ($p<0.05$) by Duncan's test; $n=6$

(Marques et al. 2007; Andrade et al. 2008). However, other authors did not find significant reductions in mycorrhizal colonization in plants growing in metal-contaminated soils and in some cases higher mycorrhizal colonization was found (Hildebrandt et al. 1999). These variations probably depend on the AM fungus and plant involved, as well as soil properties.

The improved P supply and the higher growth in AMF-associated plants is one of the most beneficial effects observed in plants exposed to metal stress; however, not all the benefits may be attributed only to the improved nutrient status. At low, or no, Cu additions, plants exhibited similar Cu concentrations in root and shoot but with high Cu additions, mycorrhiza formation reduced Cu acquisition by the plants, diminishing the concentrations in the root and shoot by around 30% when compared with NM plants. This altered Cu uptake suggests that, under elevated Cu concentrations, *G. mosseae* might be conferring the plants with some degree of tolerance to Cu. Decreased susceptibility to metal toxicity is one of the mycorrhizal benefits observed in plants growing in metal-contaminated soils in spite of the fungal effect on metal uptake by the plants (Andrade et al. 2008). Cu immobilization in fungal structures like extra-radical hyphae, or metal binding to extracellular glomalin, may be contributing to the prevention of Cu transfer to plant tissues, so that AM fungi are acting as a biological barrier. This is supported by studies that report a high Cu-binding capacity of AM fungal hyphae (Joner et al. 2000; Toler et al. 2005). Previous studies have shown that AM fungi also improve plant tolerance to heavy metal stress in polluted soils by decreasing metal accumulation and translocation from roots to shoots, thus protecting plants against heavy metal toxicity and enabling normal plant growth (Joner and Leyval 1997).

The reduction in soluble sugar levels of NM plants may be due to the decrease in chlorophyll contents, which leads to low photosynthesis efficiency. It has been suggested that mycorrhizal plants alleviate the severe effect of metals by changing the repartition patterns and by sequestration in the AM fungal hypha, so the toxic effects on photosynthesis and carbohydrates metabolism might decrease (Ryan and

Table 5 Malondialdehyde (MDA) content (nmol g^{-1}), superoxide dismutase (SOD) activity (U min^{-1} mg^{-1} protein), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities (μmol min^{-1} mg^{-1} protein) in leaves of nonmycorrhizal (NM) and mycorrhizal (M) pepper (*Capsicum annuum* L.) plants grown in soil with increasing Cu concentrations

Treatments $CuSO_4$ (mM)	AMF	MDA	SOD	CAT	APX	GR
0	NM	8.65 fg	20.34 e	10.30 g	0.34 e	0.83 e
	M	6.87 h	25.65 c	14.65 e	0.63 c	0.94 d
2	NM	11.65 e	23.65 c	12.12 f	0.45 d	0.98 d
	M	8.74 fg	28.78 b	18.98 d	0.89 b	1.43 c
4	NM	15.34 bc	28.87 b	15.17 e	0.62 c	1.36 c
	M	11.96 e	34.65 a	22.22 c	0.96 a	1.78 b
8	NM	20.98 a	30.76 b	19.12 d	0.87 b	1.62 bc
	M	13.87 d	36.87 a	28.56 a	1.01 a	2.09 a

Mean pairs followed by different letters are significantly different ($p<0.05$) by Duncan's test; $n=6$

Angus 2003; Vogel-Mikus et al. 2005). The total protein content in leaves of M plants was higher than in NM ones, confirming the results of other authors (e.g., Gianinazzi-Pearson and Gianinazzi 1995; Andrade et al. 2008). Different physiological and protein expression patterns have already been reported in mycorrhizal plants in response to metal exposure (Ouziad et al. 2005; Hildebrandt et al. 2007) or under other abiotic stresses (Sannazzaro et al. 2007). Stimulation of de novo protein synthesis by mycorrhization (Barker et al. 1998) and marked alterations in gene expression known to occur in other M plants (Wyss et al. 1990) may explain the higher protein concentrations found in mycorrhizal pepper leaves. The distinct induction of genes encoding for proteins potentially involved in heavy metal tolerance has been observed in mycorrhizal plants, and these may contribute to enhanced metal tolerance in AM plants (Hildebrandt et al. 2007). Accordingly, high proline accumulation in response to Cu in soil suggests a detoxification role for this amino acid especially in mycorrhizal pepper leaves where there was a strong Cu-induced proline accumulation. Proline has been previously shown to accumulate in plants under heavy metal stress conditions, indicating a protective or a regulatory role (Sharma and Dietz 2006; Fariduddin et al. 2009). One of the proposed roles of proline is to reduce free radical levels generated as a result of toxicity in a manner similar to other molecules like glutathione, ascorbic acid, or α-tocopherol. In the present study, proline and total free amino acid contents in leaves of M and NM pepper plants increased in response to Cu addition to the soil suggesting that both may have a similar stress response to excess Cu in the soil, and that amino acids may be an adequate indicator of Cu toxicity.

Mycorrhization also conferred increased concentrations of K, Ca, and Mg to pepper plants. These results are also in agreement with previous studies carried out with other plant species and metals (Toler et al. 2005; Andrade et al. 2008), revealing the important contribution of AMF to plant development under metal stress conditions.

The increase in MDA observed in pepper leaves signifies oxidative stress; excessive Cu has been reported to enhance the activity of lipoxygenase (Ahmed et al. 2010) which catalyzes lipid peroxidation, indicated by an increase in MDA levels. Reduced growth in plants following heavy metal toxicity has been related to ultrastructural damage and physiological changes due to oxidative stress. Such cellular stress conditions could lead to an enhancement of ROS production, resulting in irreversible damage to macromolecules (Lombardi and Sebastiani 2005). It has also been proposed that ROS production due to Cu toxicity may lead to damages to the thylakoidal membranes and alterations in the electron transport chain (Lombardi and Sebastiani 2005). This may corroborate the drastically lowered leaf chlorophyll content of NM pepper plants and its improvement in M plants which would contribute to their better tolerance of Cu stress.

ROS scavenging can be achieved by antioxidant enzymes like SOD, CAT, APX, and GR. In the present study, Cu treatment of pepper plants resulted in an increase in the activities of these enzymes, which can be a manifestation of the initiation of antioxidant defense (Marquez-Garcia and Cordoba 2010). Furthermore, SOD, CAT, APX, and GR exhibited higher activity patterns in M than NM plants in the presence of added Cu. SOD is an antioxidant enzyme that plays a key role in cellular defense against ROS (Bowler et al. 1992) and CAT is considered to be the most important enzyme which eliminates H_2O_2 from cells. Cu is particularly toxic to membranes (Ahmed et al. 2010) and the stimulation of SOD activity along with CAT in Cu-treated M plants indicates a protective role against membrane damage. APX, which plays a role converting H_2O_2 to water, can be found in different cells compartments, such as the cytosol and plastids, possibly participating in the fine modulation of ROS for signaling. Thus, the increase in the activities of APX in Cu-treated M plants suggests increased production of H_2O_2, whilst the increase in GR activity may be related to the maintenance of the intracellular levels of reduced glutathione which is required for phytochelatin biosynthesis (Gomes-Junior et al. 2006), or to H_2O_2 removal by the activation of ascorbate-glutathione cycle (Asada 1999). It is important to bear in mind that other antioxidant systems may also be involved and should be considered in future studies.

In conclusion, differences between mycorrhizal and non-mycorrhizal pepper plants were found in response to increasing Cu additions to soil. Cu-induced antioxidant enzymes mainly in plants associated with *G. mosseae* and the accumulation of proline and MDA levels was the most evident response to excess Cu in nonmycorrhizal plants. It was also clear in the present study that at high Cu additions, mycorrhizal pepper plants exhibited lower Cu concentrations in root and shoot and those inoculated with *G. mosseae* exhibited better growth. In addition, *G. mosseae* was able to maintain an efficient mycorrhizal symbiosis with pepper plants in moderate Cu stress conditions, promoting not only growth but also chlorophyll content, osmolyte accumulation, and mineral nutrition, which indicates that beneficial mechanisms are operating and suggests that the promotion of this symbiotic association could aid pepper plants to cope with high Cu conditions in soils.

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