Applied Soil Ecology 48 (2011) 117–124

Contents lists available at ScienceDirect

Applied Soil Ecology

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

Alleviation of Cu toxicity in *Oenothera picensis* by copper-adapted arbuscular mycorrhizal fungi and treated agrowaste residue

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ARTICLE INFO

Article history:
Received 6 August 2010
Received in revised form 5 April 2011
Accepted 11 April 2011

Keywords:
Antioxidant enzymes
Arbuscular mycorrhiza
Copper pollution
Oenothera picensis
Sugar beet agrowaste

ABSTRACT

The alleviation of copper (Cu) toxicity in the pseudometallophyte *Oenothera picensis* via arbuscular mycorrhizal fungi (AMF) inoculation and/or sugar beet agrowaste (SB) application was evaluated at increasing soil Cu levels. Plants were grown in Cu-treated soils (0, 100 or 500 mg Cu kg^{-1}), either with or without SB application, and inoculated with: (i) Cu-adapted Glomeromycotan fungi (GA); (ii) *Glomus claroideum* (GC); or (iii) no fungus (uninoculated). Application of SB amendment increased shoot biomass 2–8-fold with respect to the unamended soils, and allowed the survival of non-mycorrhizal- and GC-inoculated plants, even at the highest Cu level. Additionally, SB application increased shoot Cu content at higher Cu levels and shoot P content especially at lower Cu levels. In general, compared to GC-inoculated plants, GA inoculation caused a decrease in both superoxide dismutase and ascorbate peroxidase antioxidant enzyme activities in shoots (up to levels of 100 mg Cu kg^{-1}), as well as glutathione reductase and catalase activities (up to 500 mg Cu kg^{-1}). Finally, in SB treated plants, GA colonization was higher as compared to GC-inoculated plants, especially at the highest Cu level. These results suggest a relevant role of Glomeromycotan fungal populations isolated from Cu-polluted environments in the alleviation of Cu toxicity that could allow their use in remediation programs for Cu-polluted soils.

1. Introduction

Copper-mining activities produce significant detrimental effects on natural ecosystems due to the high amount of Cu-enriched particulate matter deposited in the soil, which reduces plant cover and growth and limits plant establishment (Ginocchio, 2000). For this reason, the long-term success of phytoremediation programs in Cu polluted soils has been limited (Azcón et al., 2009). Among the factors involved, lack of knowledge about the role of microbial communities in soils polluted with metals could explain some failures in the implementation of phytoremediation processes (Arriagada et al., 2009). Metal tolerance has been reported in diverse microorganisms that colonize metal polluted soils (del Val et al., 1999; Ferrol et al., 2009). Furthermore, metal-tolerant plant species (metallophytes, pseudometallophytes) can also grow on metal polluted soils (Ginocchio, 2000), and microorganisms such as metal tolerant arbuscular mycorrhizal fungi (AMF) could be functioning as the dominant population associated with their rhizosphere (Ferrol et al., 2009).

The AMF interact with plants in metal contaminated soils, and some reports conclude that the symbiosis is partly responsible for plant survival in those extreme environments (Carvalho et al., 2006; Hildebrandt et al., 2007). In this sense, the arbuscular mycorrhizal (AM) fungal colonization could enhance metal tolerance by improving plant nutrition and providing a barrier against metals that prevents their uptake by plants (Leyval et al., 1997). Therefore, plants growing under metal stress conditions may require the use and selection of the most effective AMF for surviving. This selection should be supported by the knowledge of metal-tolerant fungal species able to grow and function on polluted soils, and additionally adapted to nutrient-impoverished soils (del Val et al., 1999). To understand the interactions between metals, AMF and plants, it is necessary to: (i) study and compare the AM fungal diversity in both metal-polluted and unpolluted soils, (ii) pay special attention to AMF associated with metal-tolerant plants, and (iii) pinpoint those that are suitable for bioremediation purposes (Vivas et al., 2006).

It is well-known that, when present in excessive amounts, metals cause uncontrolled redox reactions in cells that result in the formation of reactive oxygen species (ROS) (Schutzendubel and Polle, 2002). Results reported by Ouziad et al. (2005) suggest that a primary function of the fungal cells in symbiosis is to cope with this heavy metal-induced oxidative stress. Plant cells contain an array of protective and repair enzyme systems that minimize oxida-

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doi:10.1016/j.apsoil.2011.04.005

tive damage. Smirnoff (1993) has divided these systems into two categories. One category comprises enzymes that interact with active forms of oxygen and keep them at low levels, such as ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) (Smirnoff, 1993). The second category comprises enzymes that generate oxidized antioxidant, such as glutathione reductase (GR). The first group of enzymes are involved in the detoxification of $O_2^{\bullet -}$ radicals and H_2O_2, thereby preventing the formation of $^{\bullet}OH^-$ radicals. Glutathione reductase is an important component of the ascorbate glutathione pathway, which is responsible for the removal of H_2O_2 in different cell compartments (Aebi, 1984).

Because the soil biological quality has been deteriorated in metal-contaminated soils due to the gradual decline in organic matter content, the use of an organic amendment is recommended. Sugar beet waste (SB), an inexpensive lignocellulosic residue, has been used as an effective amendment for improving physical, chemical and biological soil properties (Medina et al., 2006). These SB residues have been transformed by *Aspergillus niger* into simple sugar compounds that can be used by rhizosphere microorganisms to promote metabolic activities and growth (Vassilev et al., 1998). Recently, Azcón et al. (2009) reported a positive interactive effect of such treated SB and AM inocula on the development of plants that were sensitive to toxic metals. These results suggest that the use of SB and AM inocula could attenuate metal stress in metallophytes; thus, these inocula have a potential use in phytoremediation programs. Moreover, if SB and/or AMF (indigenous or non-adapted strains) contribute to the amelioration of metal stress in plant tissues, changes in the plant antioxidant defense system will also be expected.

The aim of this research was to evaluate the role of AM fungal inoculation and/or SB addition on the alleviation of Cu toxicity using a native Chilean pseudometallophyte as a model plant. The effectiveness of AM inoculation, either by populations of Cu-adapted autochthonous Glomeromycotan (GA) fungi or the non-adapted *Glomus claroideum* (GC) fungus (with or without agrowaste residue), was tested by analyzing the plant growth, nutrient acquisition and Cu content. Changes in the activities of SOD, CAT, GR and APX enzymes were also determined to analyze the effects of the different treatments on oxidative stress in *O. picensis*.

2. Materials and methods

2.1. Plant species

The pseudo-metallophyte *Oenothera picensis* (fragrant evening primrose) was used as a model plant for this bioassay. This species (formerly named *O. affinis*) has been previously described as Cu tolerant plant (González et al., 2008). Seeds of *O. picensis* were collected from a Mediterranean ecosystem area strongly affected by the deposit of Cu-enriched particles (up to 830 mg total Cu kg^{-1} soil and 330 mg DTPA extractable Cu kg^{-1} soil; Cornejo et al., 2008) and located approximately 1.5 km southeast from a copper smelter in the Puchuncaví Valley, Central Chile (32°46′30″S; 71°28′17″W).

2.2. Agrowaste

The treated agrowaste used in these experiments was an amendment that had been successfully tested by Vassilev et al. (1998) and Medina et al. (2006). The amendment was prepared with sugar beet waste (SB), which was supplemented with rock phosphate (RP) and mineralized by *Aspergillus niger*. For amendment production, an *A. niger* strain (NB2) was used due to its increased production of organic acids (mainly citric acid) when growing on complex substrates and its ability to mineralize lignocellulosic materials (Vassilev et al., 1998).

2.3. Soil and arbuscular mycorrhizal fungi

The soil used in this assay was collected from a zone in Granada, Spain. The soil had a pH_W of 7.2, and was comprised of 1.6% organic matter, 57.8% sand, 19.0% silt, 23.2% clay, and had the following nutrient concentrations (in mg kg^{-1}): N, 2.1; $NaHCO_3$-extractable-P, 1.7; extractable K, 0.8.

Two inocula of AM fungi were used for this study. A mix of autochthonous Glomeromycotan (GA) fungi was isolated from the rhizosphere soil of *O. picensis* plants growing in Cu polluted areas of the Puchuncaví Valley in central Chile. The fungal reproduction was carried out in an open pot culture using a sepiolite:quartz sand:vermiculite (1:1:1 v:v:v) mix as a substrate, and *O. picensis* and *Plantago lanceolata* were used as host plants. After 6 months of plant growth, the shoots were removed and the soil and root substrate were used as GA inocula. A preliminary morphological analysis revealed that the majority of the spores present in the inocula belonged to the *Glomus* genus, with *Glomus* aff. *intraradices* being the dominant ecotype. In addition, a strain of *Glomus claroideum* (GC) was isolated from agricultural soils of the Araucanía Region in southern Chile and used as reference of presumably non-Cu-adapted AM fungus. The GC inoculum was obtained similarly to the GA inoculum; however, *Sorghum bicolor* and *Trifolium repens* were used as host plants.

2.4. Experimental design and plant growth conditions

There were three AM treatments, two levels of SB amendment, and three Cu levels in a full randomized design with five replicates per combination for a total of 90 experimental units. The AM treatments were: (i) non-AM inoculated plants (NM), (ii) plants inoculated with *Glomus claroideum* (GC), and (iii) plants inoculated with a mixture of Cu-adapted autochthonous Glomeromycotan (GA) fungi. Each one of these treatments was assayed with or without SB amendment, and plants were grown at Cu concentrations of 0, 100 or 500 mg Cu kg^{-1} soil.

Before starting the experiment, the soil was sieved through a 2 mm mesh and diluted with quartz-sand (<1 mm particle size; 2:1 soil:sand, v/v), sterilized by tyndallization for three consecutive days, and air-dried for 24 h. The soil mixture was placed into 200-mL pots. After sterilization, the soil/sand mixture was supplemented with 0, 100 or 500 mg Cu kg^{-1} soil, an adequate amount of $CuCl_2{\cdot}2H_2O$ solution was added, and mixtures were allowed to equilibrate for 2 weeks. The respective treated SB amendment was mixed (5%, w/w) with the soil:sand mixture and left to equilibrate for another 2 weeks at room temperature.

Seeds of *O. picensis* were surface sterilized with 2% Cloramin-T solution for 5 min and rinsed thoroughly. Seeds were germinated and plantlets were grown before transplanting to the greenhouse, where plants were grown under a 16/8 h light/dark photoperiod with 80–90% relative humidity at $25 \pm 3/15 \pm 3$ °C day/night temperatures. At transplanting, the plantlets were either inoculated with AMF or maintained uninoculated. In both cases, a mixture of rhizosphere substrate containing spores (about 250–300 spores per 100 g), hyphae (about 3-4 m per g), and mycorrhizal root fragments was used as an inoculum. Ten grams of each inoculum were added to the respective pots just below the seedlings. Uninoculated plants (NM) received an equivalent amount of autoclaved inoculum. Plants were grown for 3 months under greenhouse conditions before being harvested.

2.5. Measurements

At harvest, the shoots and roots were separated, and shoot subsamples (1 g) of fresh material were stored at −80 °C for antioxidant enzyme activity assays. Plant samples (shoots and roots) were dried

Table 1
F-Values and significance for the main effects and factor interactions for the variable analyzed in an *O. picensis* crop study by means of a multifactorial ANOVA ($n = 90$).

Experimental variable	Cu[a]	SB	AM	Cu × SB	Cu × AM	SB × AM	Cu × SB × AM
Shoot dry weight (g)	26.0***	329.7***	0.2ns	12.1***	1.8ns	2.2ns	0.3ns
Root dry weight (g)	10.5***	0.0ns	5.8**	9.8***	3.8**	8.1**	1.6ns
Shoot Cu content (μg/plant)	9.7***	203.2***	1.1ns	6.9**	1.7ns	0.2ns	2.5ns
Root Cu content (μg/plant)	172.0***	59.6***	52.7***	81.1***	38.1***	21.9***	13.8***
Shoot P content (μg/plant)	92.4***	1209.2***	3.7*	51.3***	5.9***	3.7*	3.7**
Root P content (μg/plant)	24.7***	12.2**	11.5***	17.6***	2.7ns	2.0ns	5.9***
Shoot S content (μg/plant)	73.4***	12.2**	33.0***	40.5***	3.4*	25.3***	1.4ns
Root S content (μg/plant)	37.6***	672.3**	14.7***	55.0***	4.0**	10.1***	1.3ns
Shoot Zn content (μg/plant)	132.9***	849.4***	15.2***	42.5***	3.3*	7.2**	4.4**
Root Zn content (μg/plant)	32.1***	12.4**	36.8***	9.9***	21.5***	19.0***	7.8***
Shoot Mn content (μg/plant)	11.9***	670.2***	7.8**	9.7***	7.7***	16.1***	5.6**
Root Mn content (μg/plant)	12.3***	141.8***	1.8ns	3.9*	9.0***	11.8***	9.8***
GR activity[b] (nmol min^{-1} mg $protein^{-1}$)	31.8***	38.9***	24.4***	1.5ns	14.3***	63.9***	11.8***
SOD activity (units min^{-1} mg $protein^{-1}$)	20.1***	51.4***	0.7ns	24.3***	20.5***	23.8***	4.6**
APX activity (nmol min^{-1} mg $protein^{-1}$)	15.7***	6.9*	3.0ns	15.5***	18.8***	16.2***	6.3**
CAT activity (μmol min^{-1} mg $protein^{-1}$)	10.7***	69.2***	20.2***	6.1**	4.7**	11.7***	0.0ns
Mycorrhization (%)[c]	13.1***	10.8**	96.6***	6.0**	60.5***	5.2*	22.4***

Significance convention: ns = no significant differences.
[a] Cu = different Cu levels; SB = sugar beet treated agrowaste residue application; AM = arbuscular mycorrhizal inoculation.
[b] GR = glutathione reductase; SOD = superoxide dismutase; APX = ascorbate peroxidase; CAT = catalase.
[c] For mycorrhization determination, only were considered the treatments including AM fungi inoculation.
* Significance convention: $P \leq 0.05$.
** Significance convention: $P \leq 0.01$.
*** Significance convention: $P \leq 0.001$.

at 70 °C for 2 days and weighed. Then, the samples were ground, ashed at 550 °C and digested using an $H_2O/HCl/HNO_3$ mixture (8/1/1, v/v/v). The plant extracts were used for the determination of S, Cu, Mn, P and Zn in an ICP plasma analyzer (IRIS Intrepid II XDL, Thermo Electron Corporation). Mineral analyses were carried out by the analytical service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, Murcia, Spain.

Arbuscular mycorrhizal fungal root colonization was quantified using a dissection microscope (20–40×) after clearing a portion of the roots in 10% KOH (w/v) and staining with 0.05% trypan blue in lactic acid (w/v). The gridline intersection method (Giovannetti and Mosse, 1980) was used to determine the proportion of AM root colonization.

In the shoots, total SOD activity (EC 1.15.1.1; Beyer and Fridovich, 1987) was measured on the basis of the SOD-dependent reduction of nitroblue tetrazolium (NBT) by photochemically generated superoxide radicals. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25 °C (Beyer and Fridovich, 1987). The CAT activity (EC 1.11.1.6) was measured by H_2O_2 consumption (extinction coefficient of 39.6 mM^{-1} cm^{-1}) at 240 nm for 1 min (Aebi, 1984). The reaction mixture consisted of 50 mM phosphate buffer (pH 7.0) containing 10 mM H_2O_2 and 100 μL of enzyme extract in a 2 mL volume. The APX activity (EC 1.11.1.11) was measured in a 1-mL reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2 and 0.5 mM ascorbate. The H_2O_2 was added to start the reaction, and the absorbance decrease at 290 nm was recorded for 1 min to determine the oxidation rate for ascorbate (Amako et al., 1994). The GR activity (EC 1.20.4.2.) was estimated by measuring the decrease in absorbance at 340 nm at 25 °C due to the oxidation of NADPH (Carlberg and Mannervik, 1985). The reaction mixture (1 mL) contained 0.1 M HEPES-NaOH (pH 7.8), 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM oxidized glutathione, 150 μL enzyme extract, and 0.2 mM NADPH was added with thorough mixing to begin the reaction. The results were expressed in μmol NADPH per oxidized g of fresh plant material per minute, and the activity was calcu-

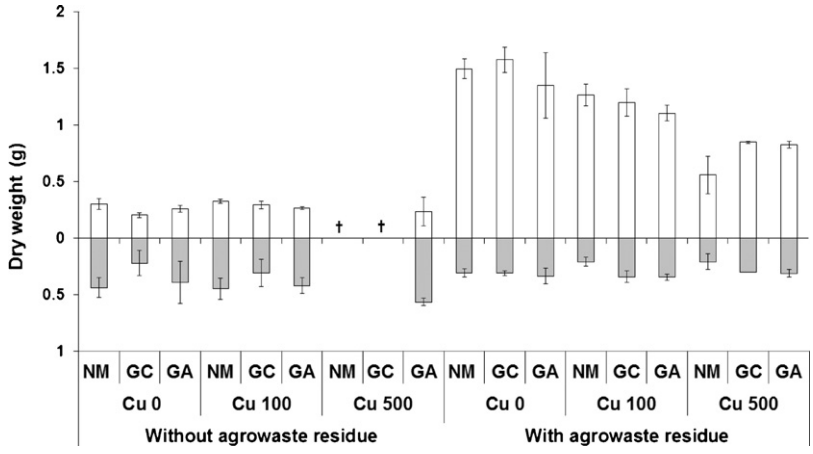

Fig. 1. Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on shoot and root dry weight in *O. picensis*. NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; $n = 5$.

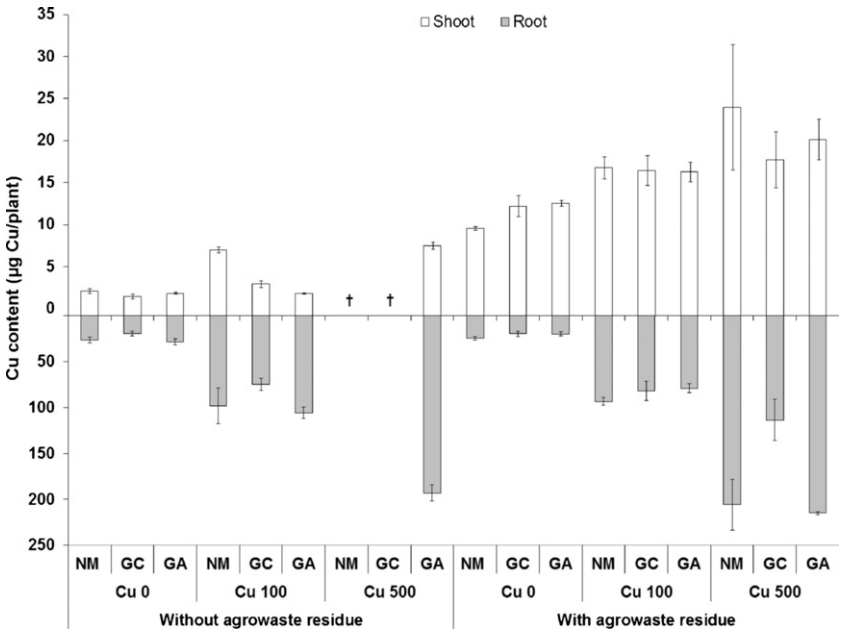

Fig. 2. Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on shoot and root Cu content (µg/plant) in *O. picensis*. NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; $n = 5$.

lated from the initial speed of reaction and the molar extinction coefficient of NADPH ($\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$).

2.6. Statistical analyses

Data regarding Cu levels, AM inoculation, agrowaste residue application and its interactions were tested by means of a multifactorial ANOVA. Means were compared using the orthogonal contrast test (Petersen, 1977). Data sets not meeting assumptions for ANOVA were transformed as required, but the results are presented in the original scale of measurement. Statistical significance was determined at $P \leq 0.05$.

3. Results

Most of the measured variables responded significantly to the different treatments applied, and the interaction of the different factors was analyzed (Table 1). Whereas the application of increasing Cu and the addition of SB agrowaste produced differences in almost all variables, non-significant changes were observed in shoot Cu content, root Mn content, SOD and APX activities as a consequence of inoculation with different AM strains. The triple interaction Cu × SB × AM generated significant changes in the amounts of Cu, P, S, Zn and Mn taken up by plants, and also led to changes in the antioxidant enzyme activities, with the exception of CAT (Table 1).

3.1. Dry matter yield and nutrient content

The application of SB increased shoot dry weights by 2–8-fold compared to plants not exposed to SB, even when toxic amounts of Cu were applied (500 mg Cu kg^{-1}; Fig. 1). However, the above positive effect was not observed in root biomass production, which was lower in plants exposed to SB. Neither NM- nor GC-colonized plants were able to survive at the highest Cu level in the absence of SB. In addition, 100 or 500 mg Cu kg^{-1} in SB treatments significantly increased root dry weight in GA colonized plants by about 60% with respect to NM treatment. Differences in dry matter yield among the treatments generated changes in the nutrient content of plant tissues. Nevertheless, no apparent dilution effect of the nutrient concentration was observed due to the increase in dry matter production, and both accumulation and concentration of macro- and micronutrients followed a similar trend (data not shown).

Plant Cu contents were increased by addition of Cu to the soil (Fig. 2). At 100 mg Cu kg^{-1}, in the absence of SB, Cu content in the shoots of NM plants was 3.9-fold higher than GA-colonized plants. In contrast, non-significant differences were observed in root Cu contents between NM and GA-colonized plants (Fig. 2). Thus, the amount of Cu translocated from root to shoot was greatly reduced (from 9.8% to 2.7%) by mycorrhizal GA inoculation. For SB treatments, non-mycorrhizal plants contained 80% more Cu in roots than GC-colonized plants, and had similar Cu levels as compared to GA plants at the highest Cu level (Fig. 2).

Application of SB significantly increased shoot P content, particularly at the lowest soil Cu level (Fig. 3). The effect of GC and GA on plant P uptake was similar irrespective of Cu treatments. Sugar beet residue greatly increased the S uptake of GA- and GC-colonized plants compared with non-mycorrhizal plants, with larger differences observed in the shoots compared to the roots (data not shown). SB amendment greatly increased Mn uptake by plants (data not shown); however, at the highest Cu level, the lowest Mn acquisition was observed, particularly in the roots of mycorrhizal plants. Similarly, SB residue enhanced the Zn uptake by shoots, and reduced uptake in the roots (data not shown).

3.2. Antioxidant enzyme activities

Figs. 4 and 5 illustrate the activities of antioxidant enzymes in shoots under different combinations of SB addition, Cu supply and AM inoculation treatments. Changes in antioxidant enzyme activities were more closely related to the AM and Cu treatments rather than the addition of SB. In general, GR activity increased as Cu concentration increased (Fig. 4A). For SB treated plants, at 0 or 100 mg Cu kg^{-1}, GC inoculation enhanced GR activity by about 3.6- and 5.1-fold, respectively, and non-significant differences were found between NM- and GA-colonized plants. Changes in GR activity at the highest Cu level were also observed; the enzyme activity increased by 2.5-fold in NM plants subjected to SB amendment,

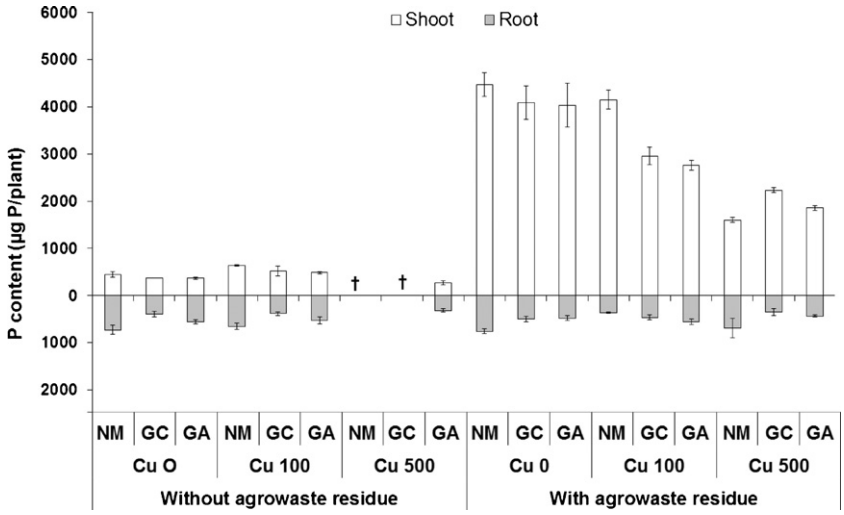

Fig. 3. Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on shoot and root P content (µg/plant) in *O. picensis*. NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; $n = 5$.

whereas in GA-colonized plants, GR activity increased by only 80%.

SOD activity increased as Cu levels increased in SB-treated and non-treated plants (Fig. 4B). Under SB treatment, similarities in SOD activity were observed between NM- and AM- (GC or GA) colonized plants at the highest Cu level. However, GA-colonized plants growing at 0 or 100 mg Cu kg^{-1} exhibited SOD activity that was at least 3.6-fold lower than the activity observed in NM- or GC-colonized plants.

Ascorbate peroxidase activity also increased due to the addition of Cu to the soil (Fig. 5A). When SB and 100 mg Cu kg^{-1} were supplied, APX activity was reduced by about 5.5-fold in

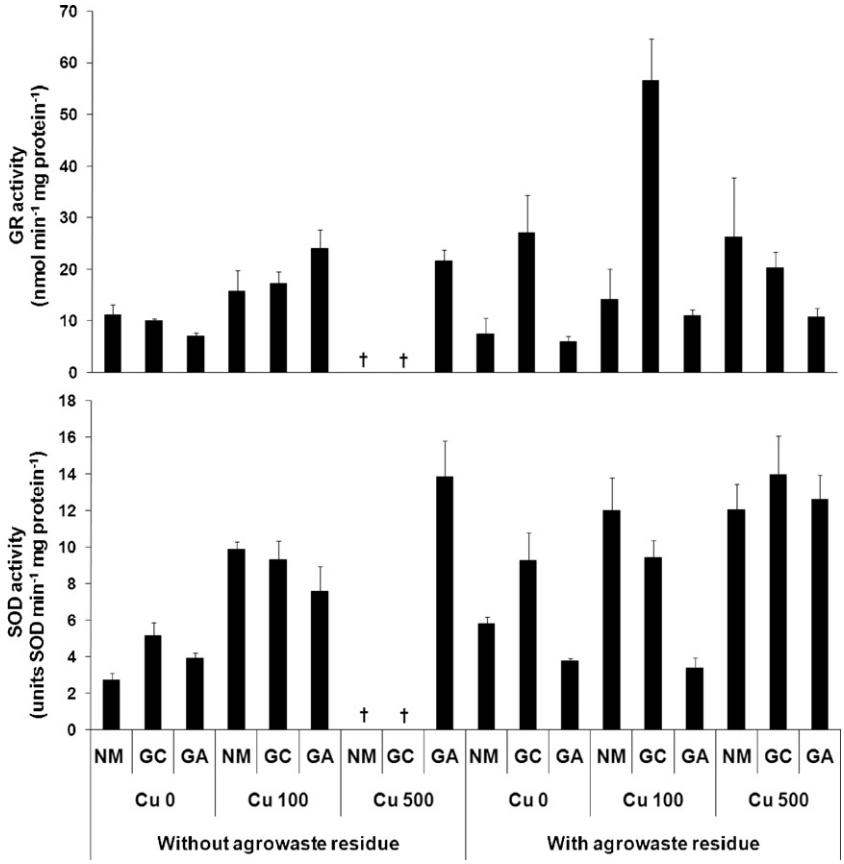

Fig. 4. Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on antioxidant activities in *O. picensis*. Glutathione reductase (GR) activity, Superoxide dismutase (SOD) activity. NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; $n = 5$. N.D = None detected.

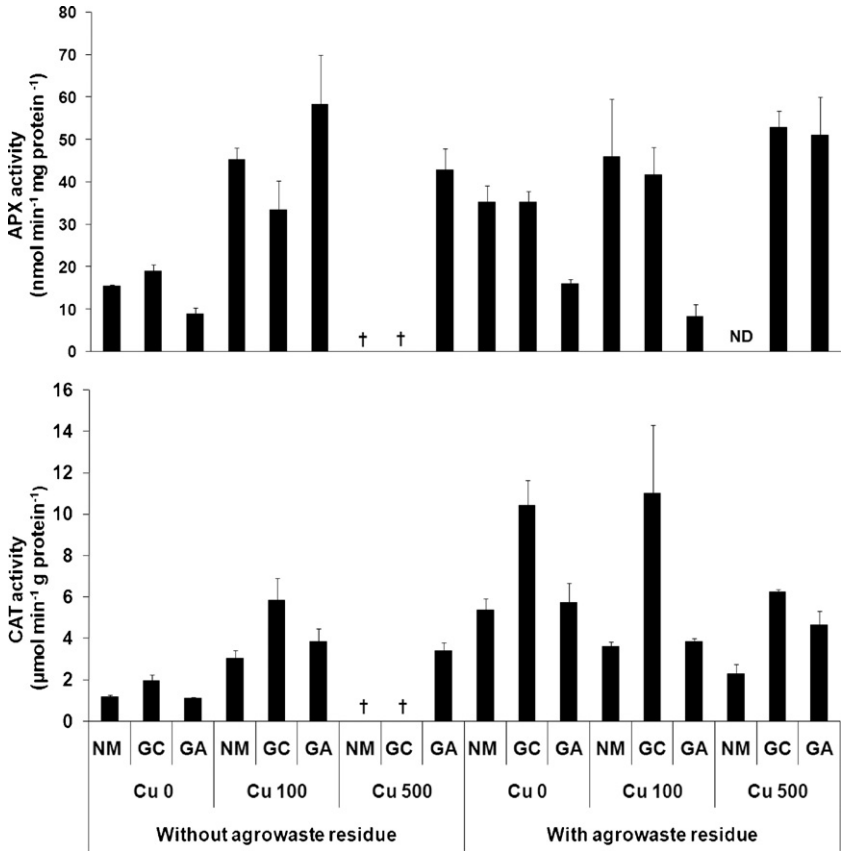

Fig. 5. Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on antioxidant activities in *O. picensis*. Ascorbate peroxidase (APX) activity and Catalase (CAT) activity. NM = non-mycorrhizal plants, † = death plants. Bars denote means ± S.E.; $n = 5$. N.D = None detected.

GA-colonized plants, with respect to NM or GC treatments. Nevertheless, for GA-colonized plants, APX was activated by the addition of 500 mg Cu kg^{-1}, and no differences were detected in enzyme activity among GC- and GA-treated plants.

A differential CAT activity response to Cu addition occurred among SB-treated and non-treated plants, which was dependent on the AM inoculation treatments (Fig. 5B). Without SB addition, CAT activity was enhanced by the addition of 100 mg Cu kg^{-1} in AM-colonized and non-colonized plants. Under SB treatment, CAT activity decreased as Cu levels increased in NM plants. When SB and 100 mg Cu kg^{-1} were added, CAT activity was not altered in GC-colonized plants, but was significantly reduced at the highest Cu level. Comparatively, the application of SB slightly inhibited CAT in GA-colonized plants at 100 mg Cu kg^{-1}, and non-significant differences in the enzyme activity were observed at either 0 or 500 mg Cu kg^{-1}.

3.3. Mycorrhizal root colonization

Mycorrhization remained relatively constant as Cu levels increased in GC-colonized plants (Fig. 6), and the addition of SB slightly decreased the percentage of GC root colonization at all Cu levels. In contrast, the above-mentioned negative effect of Cu on symbiotic development was not observed in GA-colonized plants, which exhibited a Cu-tolerant increase in AM colonization by GA inoculum. This effect was observed at 100 and 500 mg Cu kg^{-1} and was irrespective of the presence of the SB amendment. The most evident differences in mycorrhizal colonization were observed at the highest Cu level (500 mg Cu kg^{-1}) with SB agrowaste residue application. Under such conditions, the colonizing ability of autochthonous fungi (GA) was much higher than GC.

4. Discussion

The presence and toxicity of copper in plant tissues depends on complex interactions between soil and plants, as well as microbial rhizospheric activities. AM fungi appear to play a central modulating role in protecting plants from metal toxicity (Schutzendubel and Polle, 2002). According to our results, the most important effect of the Cu-adapted AM inoculum was observed at the highest Cu levels assayed (500 mg Cu kg^{-1}). Under these conditions, only plants colonized by Cu-adapted mycorrhizal strains (GA) were able to survive and grow when no SB residue was added (Fig. 1). This mycorrhizal effect at 500 mg Cu kg^{-1} was not observed in SB-treated plants colonized by GC (a strain assumed to be Cu sensitive). The effect of tolerance of indigenous AM fungi versus non-adapted fungus in promoting plant establishment and survival on contaminated soil has been previously reported for soils polluted with different metals like Zn (Hildebrandt et al., 2007), Pb (Sudová and Vosátka, 2007), and Cu (Leung et al., 2006).

Different mechanisms seem to be functioning in the mycorrhizal stimulating effect of GA-colonized plants at the highest Cu concentration. Additionally, the SB amendment was required for non-mycorrhizal (NM) or GC-colonized plant survival and growth, especially at the highest Cu concentration. At 500 mg Cu kg^{-1} plus SB, root Cu accumulation of GA-colonized plants was greater than that of GC-colonized plants, but their Cu content in the shoots was similar (Fig. 2). In accordance with these results, in the SB treated soil, NM plants had 8-fold increased shoot Cu acquisi-

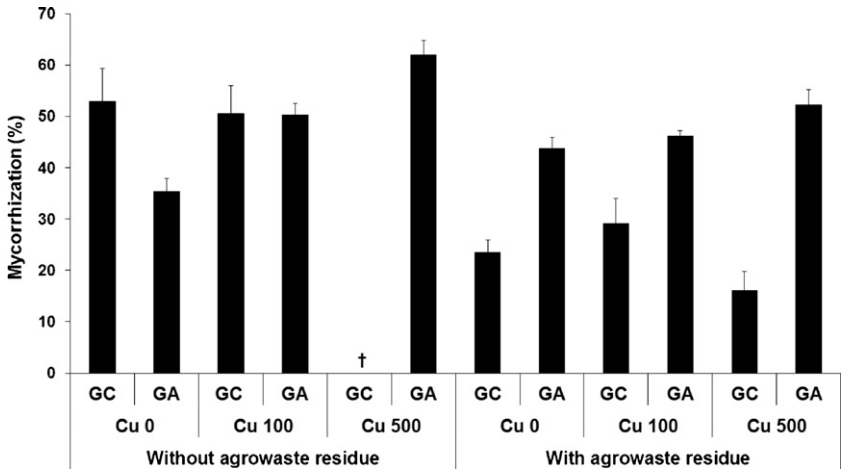

Fig. 6. Effect of agrowaste residue application, Cu levels (0, 100 or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Glomus claroideum* (GC) or Cu adapted autochthonous Glomeromycotan fungal populations (GA) on mycorrhizal root colonization in *O. picensis*. NM = non-mycorrhizal plants, † = plants death. Bars denote means ± S.E.; $n = 5$.

tion, whereas those colonized by GA had only 3-fold increased Cu at 500 mg Cu kg^{-1} soil. The role of the agrowaste amendment in improving plant growth at toxic Cu levels could, in part, be ascribed to the formation of metal-citrate complexes (Bolan and Duraisamy, 2003). In addition, the rich organic matter composition of the amendment, particularly dissolved organic carbon (DOC), plays a vital role in the immobilization of metals (including Cu) by forming metal-DOC complexes, thereby decreasing metal phytotoxicity (Bolan et al., 2003, 2011). Nevertheless, our results did not support a Cu-chelating activity for SB. In this case, the agrowaste residue could be playing mainly a nutritional role and increasing the Cu tolerance by means of plant growth stimulation (Caravaca et al., 2004), as was observed for P (Fig. 3) and S.

In this study we were also interested in the different antioxidant responses of plants colonized by Cu adapted/tolerant or Cu sensitive AM fungal strains. This comparative study under non-toxic and increasing Cu levels will allow a better understanding of the Cu stress-tolerance mechanisms of mycorrhizal colonized plants. The role of the AM fungi (GC or GA) and/or Cu level was very important for the activity of the antioxidant enzymes evaluated here. It is well-known that the ability of the plant antioxidative system to counteract toxic levels of ROS determines the extent of oxidative stress. Superoxide dismutase is the first enzyme of defense against ROS; subsequently, CAT and APX act to detoxify the H_2O_2 produced by SOD (Bowler et al., 1992), and GR regenerates glutathione disulfide (GSSG) to GSH in the Asada–Halliwell pathway. In general, the Cu applied to the soil at increasing concentrations steadily activated GR, SOD and APX in the shoots, irrespective of the AM treatment (Figs. 4 and 5). However, at 100 mg Cu kg^{-1}, GA-colonized plants exhibited lower enzyme activities than NM- or GC-colonized plants. In addition, the root growth was stimulated approximately 60% by GA inoculation as compared to NM plants at this Cu level (Fig. 1). These results give reason to infer that, at a Cu concentration of 100 mg Cu kg^{-1} soil, symbiosis with native AM fungi decreased the need of such enzymes to detoxify ROS in plant tissues.

At the highest Cu level, a noticeable reduction of shoot growth occurred (Fig. 1), which was accompanied by a significant increase in shoot Cu concentration (data not shown). Furthermore, the addition of 500 mg Cu kg^{-1} significantly activated the antioxidant responses in GA-colonized plants, and in most of cases non-significant differences in the enzyme activities were observed when plants were inoculated with AM. These facts indicate that Cu toxicity occurred at the highest Cu dose, and this toxicity was likely due to: (i) increased ROS production by the Fenton reaction (Schutzendubel and Polle, 2002) and/or (ii) the impairment of photosynthetic function (Küpper et al., 2002). Furthermore, these results suggest that the availability of Cu to the plant, Cu toxicity, and plant antioxidant responses depend on exchange processes between soil, plants and root-colonizing microorganisms (Azcón et al., 2009).

On the other hand, Cu levels did not affect GC root colonization, regardless of whether SB was applied or not (Fig. 6). The opposite trend was observed in the roots of plants colonized by GA. Thus, GA colonization increased by about 15% at the highest soil Cu concentration. These results provide evidence that native AM fungus adapted to Cu polluted soils may have developed a differential mechanism that improves their tolerance to a wide range of metal concentrations in soil (Hildebrandt et al., 2007). In addition, reports of González-Chávez et al. (2002) suggest that extraradical AM mycelia are able to sorb and/or accumulate Cu, and different tolerant isolates from the same polluted soil have different metal tolerance abilities. This provides further evidence for functional diversity within AM fungal populations in Cu-polluted soils (del Val et al., 1999).

5. Conclusion

Marked differences between mycorrhizal and non-mycorrhizal *Oenothera picensis* plants were observed at increasing soil Cu concentrations, with the use (or omission) of a treated SB amendment together with the inoculation of both a Cu-adapted and a non-adapted AMF strain. Data obtained suggest that Cu-adapted mycorrhizal fungi (GA) provide physiological traits that allow for plant survival at phytotoxic Cu levels, possibly through exclusion mechanisms favoring a decrease of the oxidative stress produced by this element. The mechanism of resistance triggered by Cu-adapted AMF strains requires further study before *O. picensis* can be used in remediation of soils polluted with Cu.

Acknowledgements

This research was supported by the Plan National CICYT AGL2003-05619-CO2-02 (Spain) and by the National Commission for Scientific and Technological Research through Project FONDECYT 11080131 (Chile). Sebastian Meier acknowledges the support of MAE-AECID (Spain).

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