Interactive effect between Cu-adapted arbuscular mycorrhizal fungi and biotreated agrowaste residue to improve the nutritional status of *Oenothera picensis* growing in Cu-polluted soils

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Abstract

The interactive effect of sugar beet (SB) agrowaste and arbuscular mycorrhizal (AM) fungi inoculation in response to increasing Cu levels was evaluated in the metallophyte *Oenothera picensis*. Plants were grown in a Cu-added soil (0, 100, or 500 mg Cu kg^{-1}), in presence or absence of SB, and inoculated with: (1) indigenous Cu adapted mycorrhiza (IM) isolated from Cu-polluted soils; (2) *Claroideoglomus claroideum* (CC); or (3) maintained uninoculated (control). Sugar beet application produced an increase in shoot biomass of 2 to 7 times, improving plant nutritional status and allowing their survival at the highest Cu concentrations. Moreover, AM fungi utilization had a positive effect promoting the plant establishment; nevertheless, Cu plant concentration as well as the mycorrhizal development in terms of AM colonization, AM spore density, and glomalin production were strictly dependent of the AM fungi strains used. Remarkable differences between AM fungi strains were observed at the highest soil Cu level where only plants colonized by IM were able to survive and grow when no SB residue was added. An interactive effect between AM fungi and SB produced a higher plant growth than plants without the amendment application, improving the plant establishment and allowing their survival at highest copper concentrations, suggesting that this combination could be used as a biotechnological tool for the phytoremediation of Cu-polluted soils.

Key words: arbuscular mycorrhiza fungi / copper pollution / *Oenothera picensis* / sugar beet agrowaste

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1 Introduction

Copper (Cu) is an important essential trace element for normal plant growth and development. However, an excessive amount of this element in the soil is highly toxic to the plants, and often results in vegetation degradation producing a decrease in soil quality with the consequent negative effect on the normal ecosystem functions (*Wong*, 2003). In this sense, for the reclamation of contaminated sites, a number of environmental remediation systems such as physical, chemical or biological treatments have been developed in the last decades (*Mulligan* et al., 2001). However, some of these treatments are expensive and may alter the soil physicochemical and biological properties, and therefore are considered environmentally invasive (*Meier* et al., 2012b).

Phytoremediation can be defined as the combined use of plants, soil amendments and environment practices to remove pollutants from the environment, or attenuate their toxicity (*Salt* et al., 1995; *Lu* et al., 2015). Due to the economic costs of growing tolerant plants which are lower than those associated to soil removal and/or replacement, the use of vegetation for landscaping, stabilization and pollution control is probably the most feasible and ecologically sound approach for soil reclamation in metal polluted sites (*Ali* et al., 2013). Nevertheless, one key factor that determines the success of phytoremediation is the initial plant establishment, which is often limited by metal toxicity, low nutrient availability and poor physical structure of the soil (*Ye* et al., 2003).

On the other hand, it is well known that soil biological quality declines in metal contaminated soils as a consequence of the progressive decrease of soil organic matter (SOM) content. Thus, the use of organic amendments such as sugar beet (SB)—an inexpensive lignocellulosic residue—appear to be a feasible alternative in order to improve physical, chemical and biological properties of metal contaminated soils (*Medina* et al., 2005; 2006).

Additionally, some microorganisms can promote the plant establishment in Cu polluted soils. For example, it has been widely reported that arbuscular mycorrhizal (AM) fungi improve plant establishment in metal contaminated soils, and even some investigations concluded that the symbiosis is partly responsible for plant survival in those extreme environments (*Carvalho* et al., 2006; *Hildebrandt* et al., 2007; *Meier*

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et al., 2011; 2012b; 2012c; *Cornejo* et al., 2013). In this sense, AM fungi have been proven to enhance plant nutrition (*Reinhardt*, 2007). In addition, they are able to improve the soil structure by the actions of external mycelium as well as the production of glomalin, a glycoprotein that can support the sequestering of Cu and other potentially toxic elements (*González-Chávez* et al., 2004; *Cornejo* et al., 2008; *Aguilera* et al., 2011; *Gil-Cardeza* et al., 2014). Therefore, AM fungi colonization constitutes a functional component of the soil–plant system and could be considered as a key factor for attenuation of metal stress in polluted environments (*Azcón* et al., 2009a; 2009b), especially if the use of AM fungal strains with a high capability to produce glomalin is taken into account. However, different AM fungal isolates can differ in their metal tolerance and in their ability to protect plants against metal toxicity (*da Silva* et al., 2003; *Meier* et al., 2012b; 2012c). In addition, some studies have reported that AM fungi from polluted areas are generally more resistant to environmental stresses (*del Val* et al., 1999; *Hildebrandt* et al., 2007; *Meier* et al., 2011; 2012a). For this reason we consider particularly relevant to determine if AM fungi from non-polluted environments possess intrinsic capacities to tolerate elevated Cu levels, and also to predict whether they are able to survive and growth in metal polluted sites.

Moreover, *Azcón* et al. (2009b) reported a positive interactive effect of treated SB and AM inocula on the development of plants non tolerant to toxic metals. This fact suggests that the combined use of SB and AM inocula could attenuate metal stress in plants with potential use in phytoremediation programs. Therefore, if SB and/or AM fungi contribute to ameliorate metal stress in plant tissues, the plant growth promotion and surviving will be also expected. In this context, we hypothesized that the use of AM fungi [either an indigenous Cu-adapted mycorrhizal fungus (IM) or a non-adapted *Claroideoglomus claroideum* (CC) fungus] in combination with SB application could produce a positive interaction attenuating plant Cu stress, promoting plant growth and contributing to facilitate the plant-soil system management in Cu-polluted environments.

Therefore, the aim of this study was to evaluate the interactive effect between the AM fungi inoculation and the SB application on the promotion of the nutritional status and growth of *Oenothera picensis*, a plant that naturally grows in Cu polluted environments from Central Chile. The effectiveness of either AM fungi inoculation or agrowaste residue application was tested by analyzing plant growth, nutrient concentrations in plant tissues, and mycorrhizal parameters such as root colonization, spores density and glomalin accumulation in the soil.

2 Material and methods

2.1 Experimental design

The experiment was performed in a fully randomized design using the following mycorrhizal treatments: non-AM inoculated plants, inoculated either with *Claoideoglomus claroideum* (CC) or with Indigenous mycorrhizal inocula (IM). Each one of these three mycorrhizal treatments was assayed with or without SB amendment, and Cu was added at nominal concentrations of 0, 100, or 500 mg Cu kg^{-1} soil. Five replicates (n = 5) were used for each combination totaling 90 experimental units (n = 90).

2.2 Agrowaste residue

The residue used as amendment was a biotreated sugar beet (SB) agrowaste. The SB characteristics were: cellulose (29%), hemicellulose (23%), and lignin (5%). The solid residue was dried at 60°C and then ground to pass a 2-mm-pore screen. Portions of 15 g of the solid substrate were placed in 250-mL Erlenmeyer flasks. Czapek-DOX mineral salt solution (0.01g L^{-1} $FeSO_4 \cdot 7\ H_2O$; 0.5g L^{-1} $MgSO_4 \cdot 7\ H_2O$; 0.5 g L^{-1} KCl; 3 g L^{-1} $NaNO_3$; 1.0 g L^{-1} $K_2\ HPO_4$, and 30.0 g L^{-1} sucrose) was added (40 mL) to each flask. Rock phosphate (Morocco fluorapatite, 12.8% soluble P, 1 mm mesh) was added at a rate of 0.75 g per flask. These culture media were sterilized by autoclaving at 120°C for 30 min. The *Aspergillus niger* NB2 strain was used to mineralize P in the agrowaste residue (*Armada* et al., 2014; *Medina* et al., 2006). For inoculum preparation, *A. niger* was grown in plates containing spores at 30°C for 7 d, which were scraped in sterile distilled water. A volume of 3 mL of *A. niger* spore suspension (about 1.2×10^6 spores mL^{-1}) was spread carefully over the surface of the respective flask containing SB agrowaste. The fermentation process was carried out at 30°C for 20 d. To prevent further influence of *A. niger* in the study, the SB agrowaste was autoclaved previously to be applied to the experimental units.

2.3 Biological material

The pseudo-metallophyte *Oenothera picensis* (Onagraceae, formerly named *O. affinis*), which has been described as the Chilean plant species with the highest copper accumulation (up to 614 mg Cu kg^{-1}; *González* et al., 2008), was used for this bioassay. Seeds of *O. picensis* and rhizosphere soil were collected from a Cu-polluted area in a Mediterranean ecosystem strongly affected by atmospheric deposition 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). It was located approx. 1.5 km SE from the Ventanas copper smelter (CODELCO) in the Puchuncaví Valley, Central Chile (32°46′30″ S, 71°28′17″ W).

Indigenous mycorrhizal inocula (IM) were isolated from the rhizosphere of a Cu-polluted soil following the methodology proposed by *Vivas* et al. (2003a; 2003b). The fungal reproduction was made in an open pot culture using sterile sepiolite:quartz sand:vermiculite (1:1:1 v:v:v) mix as substrate, and *O. picensis* together with *Plantago lanceolata* were used as host plants.

Claroideoglomus claroideum (CC) was used as reference of a non-Cu adapted AM fungus. CC strain was isolated from soils in the Araucanía Region (S Chile) and maintained as pure culture in the collection of the Laboratorio de Micorrizas, Universidad de La Frontera, Temuco, Chile. The CC inoculum was obtained similarly to that described for IM, but using *Sorghum bicolor* together with *Trifolium repens* as host plants. In both cases, after 6 months of plant growth, shoots were elimi-

nated and a mixture of rhizosphere substrate was used as inoculum, containing spores (about 250 to 300 spores per 100 g), hyphae (about 3 to 4 m per g) and mycorrhized root fragments.

2.4 Plant growth conditions

Seeds of *O. picensis*, collected from Cu-polluted areas, were surface sterilized using 2% Cloramin-T solution for 5 min and rinsed thoroughly. After that, they were germinated in sterile sepiolite:sand:vermiculite (1:1:1 v:v:v) mix and grown under greenhouse conditions (25 ± 3/15 ± 3°C day/night temperatures; 16/8 h light/dark photoperiod; 80–90% relative humidity during 3 weeks before transplanting.

The soil used in this assay was collected in a semi-arid ecosystem in Granada (SE Spain). Soil physicochemical characteristics were reported by *Marulanda-Aguirre* et al. (2008) and some of these were pH_W 7.2, 1.6% OM, 57.8% sand, 19.0% silt, 23.2% clay, and the following nutrient concentrations (in mg kg^{-1}): N, 2.1; $NaHCO_3$-extractable-P, 1.7; and K, 0.8. The soil was sieved through a 2 mm mesh and diluted with quartz-sand (2:1 soil:sand, v/v), steam-sterilized for three times, and after 24 h air-dried. Soil/sand mixture was placed in 300 mL pots. After sterilization, the soil/sand mixture was supplemented with 0, 100, or 500 mg Cu kg^{-1} by adding the adequate amounts of $CuCl_2 \cdot 2\ H_2O$ and then left to stabilize for 2 weeks before plant transplanting. In the pots treated with SB amendment it was mixed (5%, w/w; about 15 g pot^{-1}) with the soil:sand mixture and left to stabilize for another two weeks at room temperature before transplanting. Lately, at transplanting time, 10 g of each inoculum (either IM or GC) were applied 2 cm under the surface (near to the roots) to the respective pots, and uninoculated plants (NM) received an equivalent amount of autoclaved inoculum mix.

After transplantation, *O. picensis* plants were grown for 3 months in a greenhouse with temperatures ranging from 25 ± 3/15 ± 3°C day/night; 16/8 h light/dark photoperiod; and 80–90% relative humidity. Plants were watered daily with distilled water, after that the shoots and roots were harvested, weighted and processed for chemical analyses.

2.5 Measurements

At harvest, shoots and roots were separated and dried at 70°C for 2 d and after that weighed. Then, the samples were ground, converted into ashes at 550°C and digested by using a $H_2O/HCl/HNO_3$ mixture (8/1/1; v/v/v). The plant extracts were used for the determination of K, Mg, Cu, B, Mn, P, and Fe in an ICP plasma analyzer (IRIS Intrepid II XDL, Thermo Electron Corporation). The Analytical Service of the Centro de Edafología y Biología Aplicada del Segura, CSIC, MURCIA, Spain, carried out mineral analyses. The Cu translocation factor was defined as the ratio between Cu foliar concentration and Cu root concentration (*Mattina* et al., 2003).

Arbuscular mycorrhizal fungal colonization was measured using an aliquot of about 1 g of fresh roots cut to a length of 1 cm. Then, the root fragments were cleared using 10% KOH (w/v) and stained using a trypan blue solution 0.05% w/v in lactic acid. Finally, they were quantified using a dissection microscope (20–40X) (*Phillips* and *Hayman*, 1970) using the gridline intersection method (*Giovannetti* and *Mosse*, 1980). Arbuscular mycorrhizal spores were isolated from 50 g of soil through the wet sieving and decanting method, followed by sucrose centrifugation at 2,500 rpm for 10 min. After centrifugation, the supernatant was poured through 50-μm-pore-size mesh and quickly rinsed with tap water. Spores were counted in a Doncaster dish under the dissecting microscope (*Sieverding*, 1991).

Glomalin-related soil protein (GRSP), operationally measured as Bradford-reactive soil protein (*Rillig*, 2004), was recovered from soil according to the method described by *Wright* and *Upadhyaya* (1998) with minor modifications. For the easily extractable fraction of GRSP (EE-GRSP), samples of 1 g soil were subjected to extraction with 8 mL of 20 mM citrate (pH 7.0) and autoclaving for 30 min at 121°C. The total GRSP (T-GRSP) was extracted from 1 g of soil with 8 ml of 50 mM citrate (pH 8.0) and autoclaving for 1 h at 121°C. For T-GRSP, the procedure described above was repeated several times on the same sample until the reddish-brown color typical of GRSP disappeared from the supernatant, combining all extracts from a soil sample. In both cases, the supernatant was separated by centrifugation at 8,000 *g* for 20 min and filtrated using filter paper Whatman No 1. The protein content in the crude extract was determined by Bradford assay (Bio Rad Protein Assay, Bio Rad Laboratories) with bovine serum albumin as the standard. Considering that the Bradford procedure is nonspecific for glomalin determination, the amount quantified in non-inoculated treatment was used as background for the other treatments.

2.6 Statistical analyses

The data of main effects of Cu levels, AM inoculation, SB application and its interactions were tested by means of a multifactorial ANOVA. Means were compared by 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 $p \leq 0.05$. In all cases, statistical analyses were performed using the SPSS software v. 10.0 (SPSS Inc., Chicago, IL, USA).

3 Results

All the analyzed variables were highly affected by the applied treatments and/or the interactions between the factors (Table 1). In particular, the multiple interactions produced significant changes in biomass production and nutritional variables analyzed.

3.1 Growth and elements content

A positive effect on shoot biomass production was found as a consequence of SB application (Table 2). In fact, SB application increased shoot biomass production by 2- to 7-fold, allowing also plant survival at the highest Cu concentrations. However, this positive effect was not observed at the root level (Tables 1 and 2). In addition, no differences on shoot and

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

Experimental variable	Cu[a]	SB[a]	AM[a]	Cu x SB	Cu x AM	SB x AM	Cu x SB x AM
Shoot dry weight / g	507.4***	3288.8***	196.7***	1050.0***	30.0***	731.9***	464.0***
Root dry weight / g	65.6***	2.2ns	38.6***	19.4***	19.1***	37.0***	36.3***
Shoot Cu concentration / $\mu g\ g^{-1}$	15.7***	26.6***	4.4*	20.5***	3.2*	1.9ns	9.4***
Root Cu concentration / $\mu g\ g^{-1}$	161.3***	143.1***	31.1***	91.4***	23.5***	33.2***	16.1***
Cu translocation factor	64.5***	5.1*	0.5ns	6.2**	3.4*	9.0***	5.9***
Shoot P concentration / $\mu g\ g^{-1}$	55.5***	442.5***	4.6*	36.3***	2.5*	10.6***	5.5**
Root P concentration / $\mu g\ g^{-1}$	13.8***	31.5***	6.3**	10.8***	3.3*	6.0**	5.4**
Shoot K concentration / $\mu g\ g^{-1}$	205.6***	728.2***	26.5***	3.2*	20.8***	3.6*	15.8***
Root K concentration / $\mu g\ g^{-1}$	96.1***	43.7***	10.7***	43.8***	6.2***	16.8***	7.7***
Shoot Mg concentration / $\mu g\ g^{-1}$	6.3**	119.6***	59.2***	257.9***	85.6***	58.9***	101.0***
Root Mg concentration / $\mu g\ g^{-1}$	28.5***	25.0***	11.7***	47.0***	7.1***	13.0***	2.2ns
Shoot Fe concentration / $\mu g\ g^{-1}$	1.6ns	3.8ns	2.0ns	13.6***	6.3***	1.5ns	6.0***
Root Fe concentration / $\mu g\ g^{-1}$	2.6*	0.8ns	18.7***	0.0ns	54.7***	8.0**	6.7***
Shoot Mn concentration / $\mu g\ g^{-1}$	5.5**	234.4***	2.0ns	34.7***	15.7***	28.9***	19.5***
Root Mn concentration / $\mu g\ g^{-1}$	4.8*	148.6***	4.5*	0.5ns	9.4***	12.8***	8.5***
Shoot B concentration / $\mu g\ g^{-1}$	69.8***	27.9***	5.8**	189.8***	11.0***	9.1***	8.4***
Root B concentration / $\mu g\ g^{-1}$	21.6***	246.5***	3.9*	10.7***	12.1***	6.7**	6.9***
Mycorrhizal colonization / %	126.0***	149.9***	4767.6***	85.4***	532.4***	80.3***	180.3***
AM spore density (spores in 50 g of soil)	13.3***	12.0**	1673.4***	32.0***	186.5***	5.3**	26.7***
EE–GRSP / $mg\ g^{-1}$	10.6***	159.5***	0.0ns	3.8*	10.2***	8.0**	2.6*
T–GRSP / $mg\ g^{-1}$	63.0***	334.7***	25.3***	41.3***	16.9***	13.8***	15.4***

Significance conventions: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns = no significant differences.
[a]Cu = different Cu levels; SB = sugar beet biotransformed residue application; AM = arbuscular mycorrhizal inoculation.

root biomass production were found in AM inoculated plants (either inoculated with CC or IM) in comparison with the control treatments (NM) at whatever Cu supply levels (Table 2). However, a relevant result was observed at the highest Cu concentration and without SB application, where only the plants colonized by IM were able to survive (Table 2).

Plant Cu concentration was increased due to Cu addition and it was accumulated principally at root level (about 95% of the total Cu in plants). Thus, a low translocation factor was observed independent of the Cu supply level applied (Table 3). A significant decrease of shoot Cu concentration was also observed in SB treated plants inoculated with AM fungi at the highest Cu supply level. The above effect was more evident at the root level with SB addition, in which the highest Cu supply level of the NM plants accumulated 2.3 and 1.3 times higher Cu amounts than CC and IM inoculated plants, respectively (Table 3).

Shoot macronutrient concentrations were more affected by the amendment application than AM colonization. Thus, SB produced an increase between 1.7- to 2.3-fold in P and K concentration, respectively (Fig. 1). Nevertheless, such effect was not generally detected at the root level. In the case of micronutrients, SB significantly increased B and Mn concentrations, especially at the root level (Fig. 2).

3.2 Arbuscular mycorrhizal parameters

As expected, no root colonization was found in all control treatments (*i.e.*, without AM fungi inoculation). The other two factors (Cu supply levels and SB application) significantly influenced fungal colonization, spore number and GRSP production (Tables 1 and 4; Figs. 3 and 4). In particular, plants inoculated with Cu adapted IM inoculum increased their root colonization and spore density at high Cu supply levels, irrespectively of the SB addition (Fig. 3), whereas an opposite effect was observed in CC inoculated plants.

For IM inoculated plants, the root colonization and spore production were increased by about 1.7 and 2 times, respectively, when the highest Cu dose was applied compared to the control treatment (Table 4). In contrast, in SB treated soil, CC spore density decreased significantly at increasing Cu supply

Table 2: Effect of agrowaste residue application, Cu levels (0, 100, or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Claroideoglomus claroideum* (CC) or Cu adapted fungal populations (IM) on shoot and root dry weight (g) in *Oenothera picensis.* Values are mean ± standard error. † = dead plants. Different letters on each row indicate significant differences ($P < 0.05$) using orthogonal contrasts test ($n = 5$).

Cu levels	AM Inoculation	Shoot dry weight / g		Root dry weight / g	
		–SB	+SB	–SB	+SB
Control	NM	$0.33 \pm 0.02^{(f)}$	$1.43 \pm 0.06^{(b)}$	$0.54 \pm 0.06^{(a)}$	$0.38 \pm 0.02^{(de)}$
	CC	$0.20 \pm 0.01^{(g)}$	$1.52 \pm 0.08^{(ab)}$	$0.18 \pm 0.01^{(k)}$	$0.33 \pm 0.02^{(efg)}$
	IM	$0.26 \pm 0.02^{(f)}$	$1.63 \pm 0.01^{(a)}$	$0.43 \pm 0.02^{(bc)}$	$0.32 \pm 0.03^{(fgh)}$
Cu 100	NM	$0.30 \pm 0.01^{(fg)}$	$1.10 \pm 0.08^{(c)}$	$0.49 \pm 0.09^{(ab)}$	$0.22 \pm 0.03^{(jk)}$
	CC	$0.29 \pm 0.02^{(fg)}$	$1.10 \pm 0.06^{(c)}$	$0.29 \pm 0.02^{(fghi)}$	$0.34 \pm 0.02^{(ef)}$
	IM	$0.23 \pm 0.02^{(fg)}$	$1.01 \pm 0.07^{(c)}$	$0.43 \pm 0.04^{(cd)}$	$0.27 \pm 0.01^{(hij)}$
Cu 500	NM	†	$0.67 \pm 0.11^{(de)}$	†	$0.28 \pm 0.06^{(ghi)}$
	CC	†	$0.77 \pm 0.01^{(de)}$	†	$0.29 \pm 0.03^{(fghi)}$
	IM	$0.66 \pm 0.05^{(e)}$	$0.79 \pm 0.02^{(d)}$	$0.44 \pm 0.02^{(bcd)}$	$0.23 \pm 0.01^{(ijk)}$

Table 3: Effect of agrowaste residue application (+SB with application; –SB without application of the amendment), Cu levels (0, 100, or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Claroideoglomus claroideum* (CC) or Cu adapted autochthonous fungal populations (IM) on shoot and root Cu concentration (μg g^{-1}) and metal translocation factor in *Oenothera picensis.* Values are mean ± standard error. † = dead plants. Different letters on each row indicate significant differences ($P < 0.05$) using orthogonal contrasts test ($n = 5$).

Cu levels	AM Inoculation	Shoot Cu concentration / μg g^{-1}		Root Cu concentration / μg g^{-1}		Translocation factor	
		–SB	+SB	–SB	+SB	–SB	+SB
Control	NM	$7.75 \pm 0.2^{(f)}$	$6.41 \pm 0.2^{(f)}$	$60.7 \pm 2.1^{(g)}$	$72.7 \pm 2.8^{(g)}$	$0.12^{(abc)}$	$0.09^{(bcdef)}$
	CC	$7.12 \pm 0.09^{(f)}$	$7.74 \pm 0.6^{(f)}$	$89.1 \pm 3.2^{(g)}$	$59.5 \pm 3.1^{(g)}$	$0.08^{(bcdef)}$	$0.14^{(ab)}$
	IM	$7.11 \pm 0.2^{(f)}$	$9.28 \pm 0.3^{(f)}$	$75.1 \pm 3.1^{(g)}$	$62.7 \pm 2.2^{(g)}$	$0.10^{(abcde)}$	$0.17^{(a)}$
Cu 100	NM	$21.4 \pm 0.2^{(bc)}$	$13.5 \pm 0.7^{(de)}$	$219 \pm 13^{(f)}$	$416 \pm 13^{(d)}$	$0.12^{(abcd)}$	$0.03^{(g)}$
	CC	$9.95 \pm 0.5^{(ef)}$	$14.0 \pm 0.9^{(d)}$	$244 \pm 7.9^{(f)}$	$240 \pm 8.8^{(f)}$	$0.04^{(efg)}$	$0.06^{(cdef)}$
	IM	$6.78 \pm 0.2^{(b)}$	$14.7 \pm 1.2^{(d)}$	$252 \pm 6.0^{(f)}$	$325 \pm 8.9^{(e)}$	$0.03^{(efg)}$	$0.05^{(efg)}$
Cu 500	NM	†	$45.5 \pm 1.8^{(a)}$	†	$866 \pm 20^{(a)}$	†	$0.05^{(defg)}$
	CC	†	$20.9 \pm 0.1^{(c)}$	†	$380 \pm 12^{(d)}$	†	$0.05^{(defg)}$
	IM	$21.2 \pm 0.09^{(c)}$	$25.0 \pm 1.92^{(b)}$	$501 \pm 8.6^{(c)}$	$671 \pm 5.3^{(b)}$	$0.04^{(efg)}$	$0.04^{(efg)}$

levels, from 56 spores per 50 g of dry soil in non-Cu-added soil to only 7 spores at the highest Cu dose (Table 4).

Glomalin-related soil protein accumulation (EE-GRSP and T-GRSP) was more dependent of the SB application than AM inoculation (Fig. 4). However, we found differences between the AM fungi strains here studied. The amount of EE-GRSP and T-GRSP increased 1.2- and 1.8- fold, respectively, in IM inoculated plants as compared with the CC strain, with SB application and measured at the highest Cu supply level (Fig. 4).

4 Discussion

Copper presence in plant tissues and its toxicity depends on complex interactions between soil and plants, as well as microbial rhizospheric activities. In this sense, AM fungi and SB application have an interactive role in protecting plant from metal toxicity, enhancing both plant establishment and nutrition in Cu-polluted soils. Firstly, the SB application produced an increase in the dry matter production and allowed the survival of the plants even at the highest Cu supply level, which could be principally due to the high P content supplied by the rock phosphate applied during the transformation process

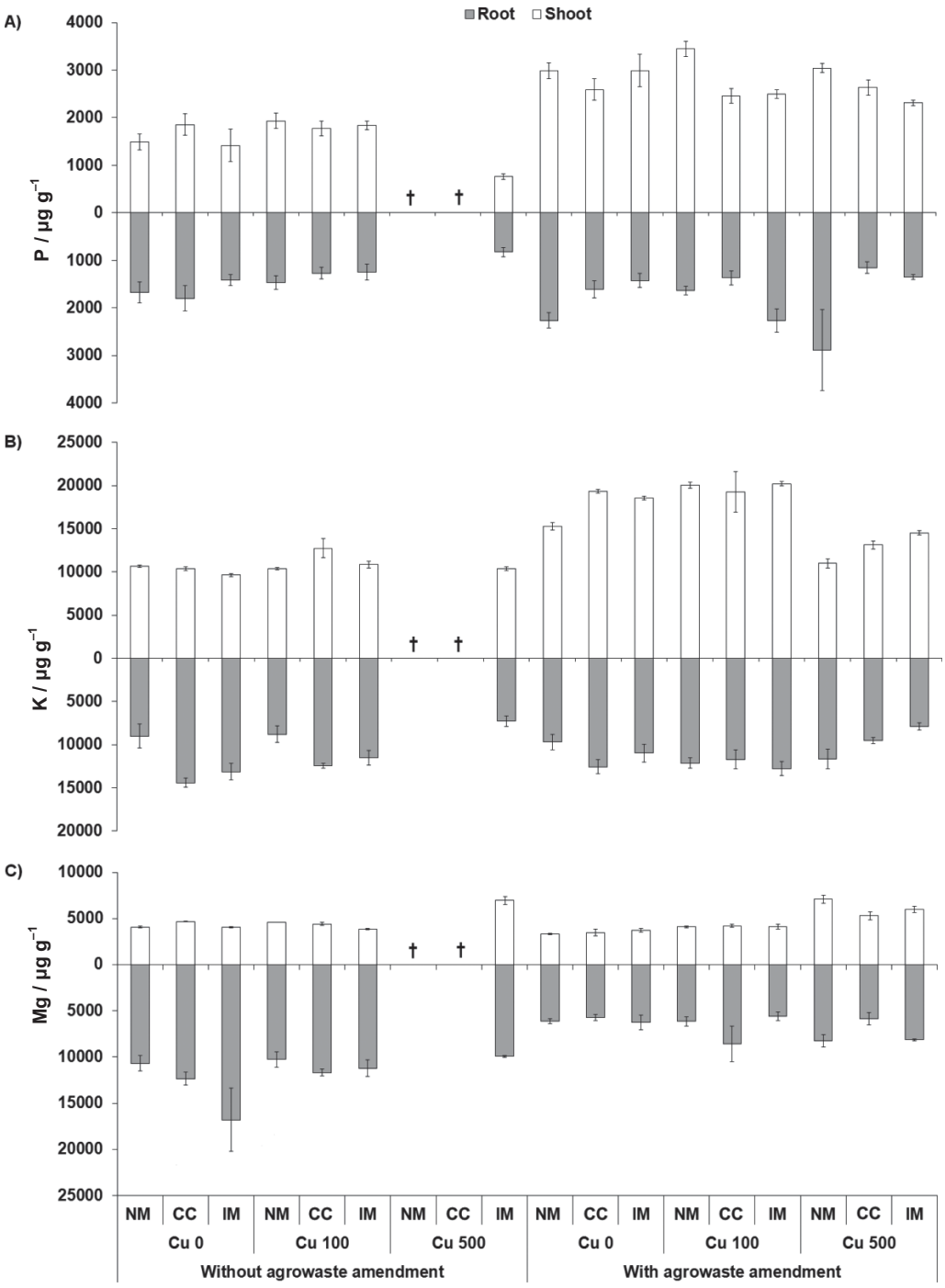

Figure 1: Effect of biotreated sugar beet agrowaste residue application, Cu levels (0, 100, or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Claroideoglomus claroideum* (CC) or Cu adapted autochthonous fungal populations (IM) on shoot and root macronutrients extraction in *Oenothera picensis.* (A) P concentration, (B) K concentration and (C) Mg concentration NM = non-mycorrhizal plants, † = dead plants. Bars denote means ± S.E.

(*Vassilev* et al., 1998). Moreover, this finding is supported by previous reports, which conclude the crucial role of SB improving plant metal tolerance in soils contaminated with Cd (*Medina* et al., 2005), Zn (*Medina* et al., 2006), and multi-contaminated (*Azcón* et al., 2009b). The benefits of SB amendment on plant growth could be related to its polysaccharide composition, which can also bind metals into its structure (*Azcón* et al., 2009b). Thus, the metal binding capacity has been correlated with the density of polysaccharides acids capable of complexing cations (*Reddad* et al., 2002). However, our results did no support a Cu-chelating activity by the amendment (Table 3). Therefore, this agrowaste residue appears to be playing principally a nutritional role, increasing Cu tolerance through a stimulating effect on plant growth (Figs. 1 and 2).

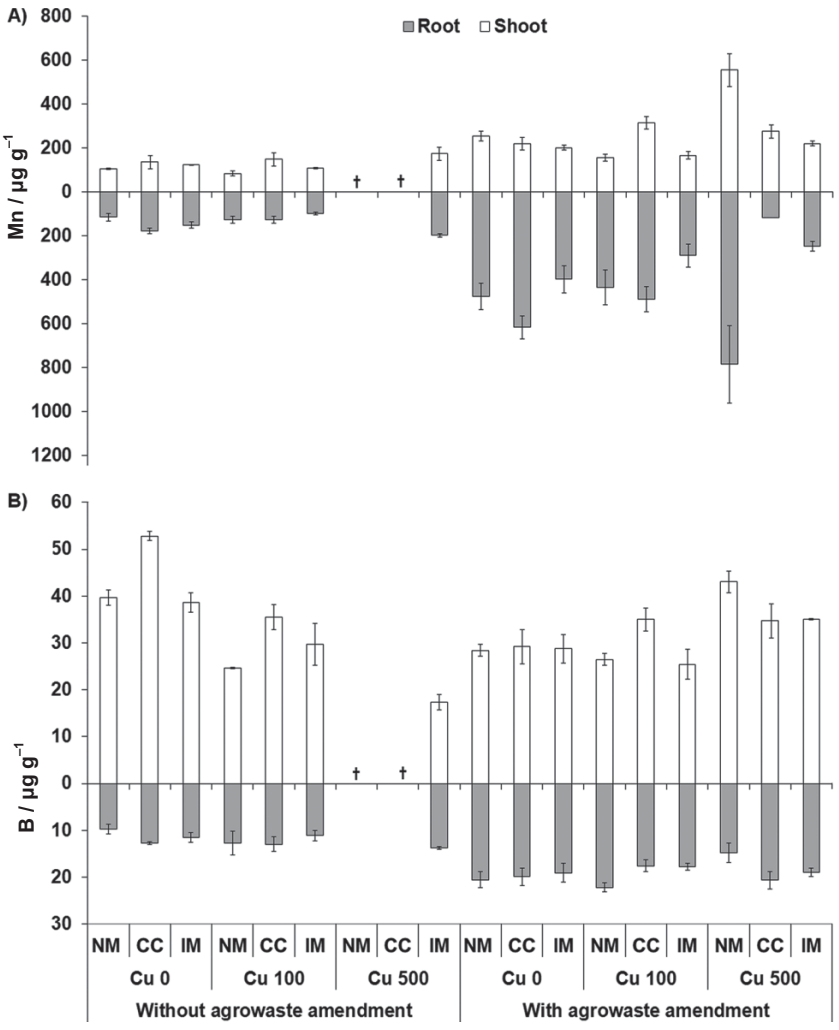

Figure 2: Effect of biotreated sugar beet agrowaste residue application, Cu levels (0, 100, or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Claroideoglomus claroideum* (CC) or Cu-adapted autochthonous fungal populations (IM) on shoot and root micronutrients concentration in *Oenothera picensis.* (A) Mn concentration and (B) B concentration NM = non-mycorrhizal plants, † = dead plants. Bars denote means ± S.E.

Secondly, AM fungal inoculation promoted the plant survival in Cu-polluted soil (Table 2). Nevertheless, this response was strictly dependent of AM fungal strain, which suggests the presence of metal adaptation mechanisms (*Pawlowska* et al., 2000; *Cornejo* et al., 2013). In fact, according to our results, the most important effect of the Cu adapted IM was observed at the highest soil Cu concentration (500 mg Cu kg^{-1}). Under these conditions, only the plants colonized by IM were able to survive and grow when no SB residue was added (Table 2). Such protective effect has been previously observed in plants

Table 4: Effect of agrowaste residue application (+SB with application; –SB without application of the amendment), Cu levels (0, 100, or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Claroideoglomus claroideum* (CC) or Cu adapted autochthonous fungal populations (IM) on root colonization and spore number in *Oenothera picensis.* Values are mean ± standard error. † = dead plants. Different letters on each row indicate significant differences ($P < 0.05$) using Tukey test ($n = 5$).

Cu levels	AM Inoculation	Colonization / %		Spore number	
		–SB	+SB	–SB	+SB
Control	CC	$54.6 \pm 2.7^{(ab)}$	$26.0 \pm 0.6^{(fg)}$	$14.7 \pm 0.3^{(d)}$	$56.0 \pm 2.5^{(b)}$
	IM	$35.3 \pm 1.5^{(de)}$	$42.7 \pm 1.5^{(cd)}$	$46.3 \pm 2.4^{(bc)}$	$50 \pm 0.5^{(b)}$
Cu 100	CC	$53.6 \pm 4.3^{(ab)}$	$29.3 \pm 0.9^{(ef)}$	$35.3 \pm 4.4^{(c)}$	$16.7 \pm 2.5^{(d)}$
	IM	$50.3 \pm 0.9^{(bc)}$	$45.6 \pm 0.9^{(bc)}$	$57.3 \pm 2.4^{(b)}$	$58.0 \pm 5.9^{(b)}$
Cu 500	CC	†	$16.3 \pm 0.3^{(g)}$	†	$7.2 \pm 0.6^{(d)}$
	IM	$62.0 \pm 0.6^{(a)}$	$52.7 \pm 2.1^{(b)}$	$92.3 \pm 1.2^{(ab)}$	$94.7 \pm 4.3^{(a)}$

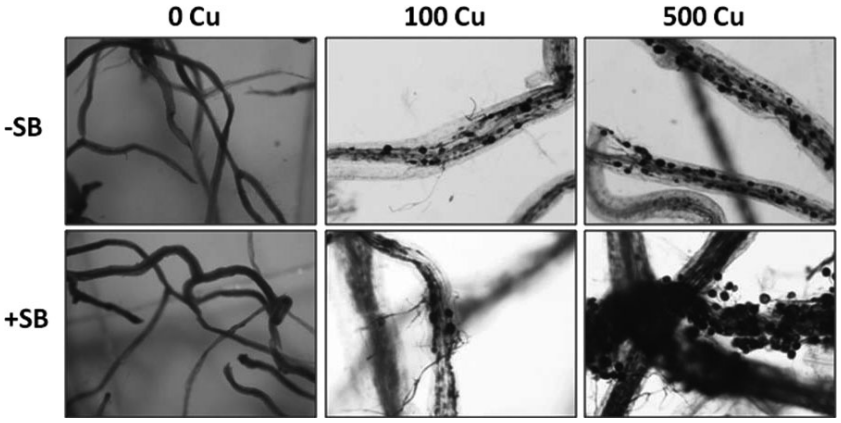

Figure 3: Effect of biotreated sugar beet agrowaste residue application (+SB with application; –SB without application of the amendment), Cu levels (0, 100, or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with Cu adapted autochthonous fungal populations (IM) on root colonized plants of *Oenothera picensis.*

colonized by other strains of indigenous AM fungi in soils polluted with Zn (*Hildebrandt* et al., 2007), Pb (*Sudová* and *Vosátka*, 2007), or As (*Leung* et al., 2006).

In addition, different mechanisms seem to be functioning in mycorrhizal plants, stimulating a protective effect to cope with the Cu accumulation in plant tissues (*Meier* et al., 2012b). In fact, we found a substantial reduction of Cu concentration in shoots and roots in AM colonized plants and such reduction became more evident under the highest Cu level. Under this stressful condition, IM was able to concentrate 29% and 82% lesser Cu in roots and shoots than control plants (NM), respectively (Table 3). Moreover, in a related report *Meier* et al. (2012c) found a decrease in the Cu uptake by *O. picensis* plants inoculated with IM together with the SB application. The above was related with the decrease on its antioxidative enzyme activity, suggesting that IM and SB enhanced plant Cu tolerance as a consequence of adaptive physiological mechanisms provided by the interaction between both components here studied. In addition, the impact of AM colonization on metal uptake by host plants has been earlier proved for several metals like Pb (*Vivas* et al., 2003a; *Zhang* et al., 2010), Cd (*Vivas* et al., 2003b), Ni (*Vivas* et al., 2006), and Zn (*Vivas* et al., 2005), suggesting the presence of metal exclusion mechanisms for IM against high Cu concentration in soils.

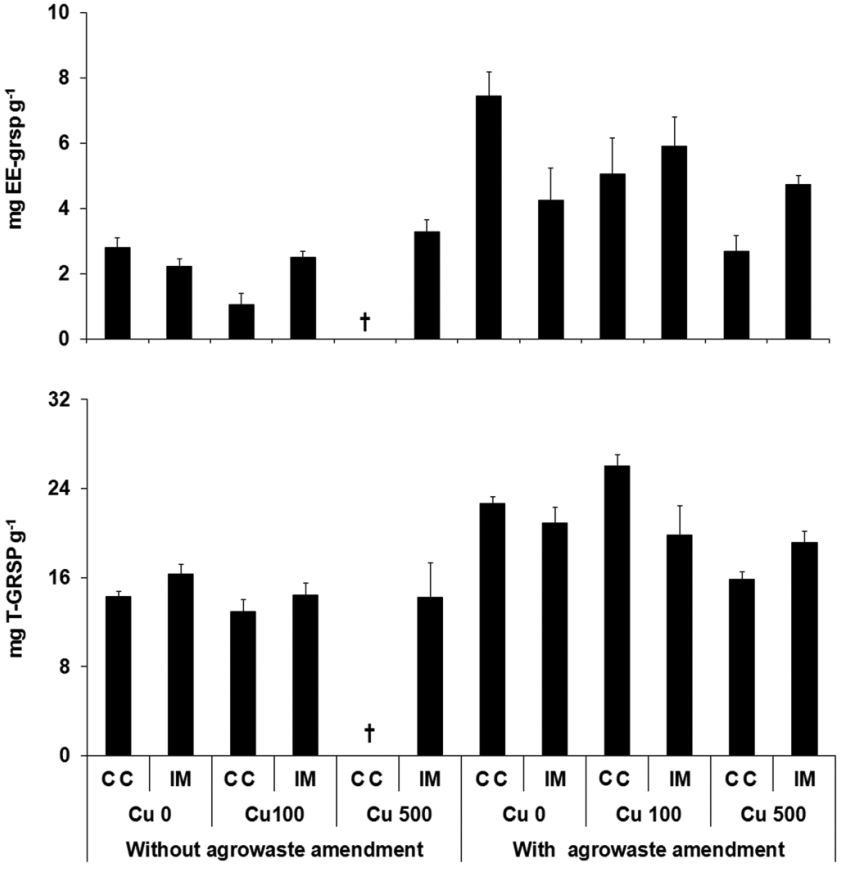

Figure 4: Effect of biotreated sugar beet agrowaste residue application, Cu levels (0, 100, or 500 mg Cu kg^{-1}) and arbuscular mycorrhizal fungal inoculation with *Claroideoglomus claroideum* (CC) or Cu-adapted autochthonous fungal populations (IM) on easily extractable glomalin and (EE-GRSP) total glomalin related soil protein (T-GRSP). † = dead plants. Bars denote means ± S.E.; $n = 5$.

On the other hand, an increase of both the AM fungal root colonization and the AM spores production occurred as a consequence of IM inoculation (Fig. 3), especially at the highest Cu supply, indicating that the autochthonous fungus was more tolerant to the Cu applied compared to the CC strain (Table 4). Moreover, the increased AM colonization and fungal structure production in IM inoculated plants together with a decrease in root Cu concentration could denote that plants may regulate the AM fungal colonization by its own benefits (*Hildebrandt* et al., 2007; *Ferrol* et al., 2009), especially when Cu-adapted AM fungi are used. These facts confirm the protective role of AM fungi in metal-polluted soils, and also allow establish tolerance differences between metal-adapted and non-adapted AM fungal strains against toxic levels of Cu in the soil (*del Val* et al., 1999). In fact, AM spore production in Cu non-adapted fungus (CC) suffered a noticeable decrease when high Cu levels were applied. Although the AM spores production did not completely disappear in CC at the highest soil Cu level, the low level of propagules presumably resulted insufficient to reach a good colonization able to support plant growth without the application of SB amendment, which probably also limits its application under field conditions.

Finally, marked differences on GRSP production were observed. Such differences seem to be more related to soil Cu-supply and SB application than the AM fungi inoculums used here (Fig. 4), which could support the hypotheses of and interactive/synergic role between SB and AM fungi. For IM colonized plants, the high GRSP production and the low Cu concentration in their roots suggest that this compound could act as a relevant exclusion mechanism developed by the autochthonous fungus to cope with toxic metal concentrations in the soil, since previous studies have demonstrated a high ability of this protein to sequester significant amounts of Cu (*Cornejo* et al., 2008; *Vodnik* et al., 2008) and other toxic metals (*Aguilera* et al., 2011; *Gil-Cardeza* et al., 2014). This aspect is especially important, since the glomalin production by different AM strains could be used as a parameter to choice the more effective ecotype to be used in remediation programs.

5 Conclusion

The interactive effect between sugar beet (SB) amendment and Cu-adapted AM fungi could be a successful biotechnological tool for improving the *Oenothera piscensis* establishment in highly Cu-polluted soils. In our study, the IM mix strain was able to control the Cu uptake by plants, allowing its survival at the highest Cu concentration, whereas SB had a direct effect on plant growth by improving plant nutrition. In addition, the use of IM produces a highest density of AM propagule, which can produce a faster plant establishment under metal stress conditions and promote a further-improved plant cover establishment in field conditions. Therefore, we conclude that the positive interaction of SB and Cu-adapted AM fungi might be of interest to improve phytoremediation strategies in Cu-polluted soils.

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