Contribution of inoculation with arbuscular mycorrhizal fungi to the bioremediation of a copper contaminated soil using *Oenothera picensis*

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Abstract

The Bradford-reactive soil protein (BRSP) fraction includes glomalin, a glycoprotein produced by arbuscular mycorrhizal (AM) fungi able to bind some metals, such as copper (Cu), which could promote the bioremediation of Cu-polluted soils. This study aimed to analyze the Cu-binding capacity of BRSP in *Oenothera picensis* that was inoculated or not inoculated with AM fungi. *O. picensis* plants were established in a Cu contaminated sterilized soil and treated with the following: *i*) uninoculated (-M); *ii*) inoculated with native AM fungal propagules (+M); or *iii*) inoculated with a *Claroideoglomus claroideum* (CC) strain isolated from non-contaminated soil. In each case, five Cu levels were applied to the soil (basal level 497.3 mg Cu kg⁻¹): 0 (T1); 75 (T2); 150 (T3); 225 (T4); and 300 mg Cu kg⁻¹ (T5). A high BRSP accumulation in AM inoculated treatments, especially with CC, was observed. A higher Cu-bound-to-BRSP content was found with increasing Cu concentrations, representing up to 20-22% of the total Cu in the soil. Moreover, a higher root Cu concentration in +M was observed. These results suggest a high Cu binding capacity by BRSP, which is a relevant aspect to consider in the design of bioremediation programs together with the selection of endemic metallophytes and AM fungal strains, which are able to produce glomalin at high quantities.

Keywords: Arbuscular mycorrhizal fungi, bioremediation, glomalin, metallophyte

1. Introduction

Copper production is Central to Chile's economy, but is responsible for a series of deleterious effects on ecosystems, producing irreversible losses of soil and plants (Meier *et al.*, 2012b). For example, in the Mediterranean ecosystems near the Ventanas smelter (Puchuncaví Valley, Valparaíso Region), numerous negative effects have been generated by Cu-enriched particulate matter deposits and the acid rain generated by the smelter (Ginocchio *et al.*, 2004; Cornejo *et al.*, 2008; González *et al.*, 2008). At present, it is possible to identify a low diversity of plant species that are, in general, tolerant to Cu pollution and, in some cases, that are also adapted to soil acidity (Ginocchio and Baker, 2004; González *et al.*, 2008). Among these plants, *Oenothera picensis* is a prime example because it is the metallophyte with the highest Cu accumulation capability described to date in Chile (González *et al.*, 2008; Meier *et al.*, 2012a).

Although there is interest in using living organisms to remediate metal contaminated soils, plant symbioses have been scarcely studied regarding their use for optimizing phytoremediation processes (Meier *et al.*, 2012b). However, the use of arbuscular mycorrhizal (AM) fungi has garnered increasing interest (Khan, 2006). AM fungi contribute to plant growth and establishment, especially under conditions of limited water, fertility or high metal toxicity (Meier *et al.*, 2011; 2012c; Seguel *et al.*, 2013; Pigna *et al.*, 2014; Barea, 2015; Aguilera *et al.*, 2015). Therefore, the manipulation and use of AM fungi would be an important tool to remediate Cu-contaminated soils (Meier *et al.*, 2012b; 2015).

Previous studies have shown that glomalin, a glycoprotein produced by AM fungi (Wright *et al.*, 1996; Gadkar and Rillig, 2006; Rillig and Mummey, 2006), can binds metals, including Cu (González-Chávez *et al.*, 2004; Cornejo *et al.*, 2008; Meier *et al.*, 2012c).

This glycoprotein is distinguished by its extraction and detection methods (Wright *et al.*, 1996; 2006; Wright and Upadhyaya, 1998; Purin and Rillig, 2007) and has been quantified as a Bradford-reactive soil protein (BRSP; Rillig, 2004). Glomalin is part of the soil organic matter fraction and represents a significant amount of the total proteins in the soil due to its persistence (Preger *et al.*, 2007), helping to bind soil particles and, consequently, improving aggregate stability (Rillig and Mummey, 2006; Curaqueo *et al.*, 2011). Moreover, some molecular evidence suggests that this protein is a homolog of certain heat-shock proteins (Gadkar and Rillig, 2006), which in general, are related to unspecific environmental stresses. Therefore, the potential of BRSP in bioremediation processes in Cu-polluted soils emerges as an important consideration, especially its contribution to phytostabilization processes. Therefore, the objective of this study was to analyze and quantify the contribution of BRSP to Cu immobilization using the metallophyte *O. picensis*.

2. Material and Methods

A full factorial design was used considering the following AM treatments: *i*) without AM fungi inoculation (-M), *ii*) inoculation with native AM fungal propagules in Cu polluted soils (+M), and *iii*) inoculation with a *Claroideoglossum claroideum* (CC) strain obtained from agricultural soils from southern Chile. The Cu levels were as follows: natural soil (T1); natural soil with 75 mg Cu kg⁻¹ (T2), 150 mg Cu kg⁻¹ (T3), 225 mg Cu kg⁻¹ (T4), and 300 mg Cu kg⁻¹ (T5) added. In all cases, a CuCl₂ solution was added to the soil four weeks before the experiment was established in pots (n=4).

The soil used was collected at a 0-20 cm depth from a Mediterranean ecosystem (32° 46' 30" S; 71° 28' 17" W) located 1.5 km southeast from the Ventanas smelter (CODELCO) in the Puchuncavi Valley, Central Chile. The soil (Chilicaquén series: sandy loam texture, 497.3 mg total Cu kg⁻¹, and extractable Cu-DTPA of 50 mg kg⁻¹) was collected from bare areas at a distance of 1.5-2 m from *O. picensis* plants, sieved through a 2-mm mesh, sterilized with tyndallization for three consecutive days, air-dried and placed in 200-mL pots. In all cases, 10 mL of a filtrate of natural soil and *C. claroideum* inoculum suspension in sterile dH₂O (1/1/18; w/w/v) was applied to re-inoculate the non-mycorrhizal soil microbiota.

Seeds of *O. picensis* obtained from plants in the Puchuncavi valley were surface sterilized (2% Chloramin-T) and germinated in sterile sand, and the obtained plantlets were established in propagation trays (25 mL) with the respective AM inoculum or in a sterile substrate. A sterile sand:sepiolite mix of 1:1 w:w was used for the -M treatments; a sterile sand:sepiolite mix with natural soil collected from a grid with 53 µm diameter containing the AM fungal propagules from rhizosphere *O. picensis* soil was used in the case of +M treatments; and CC propagules obtained with the same procedure were used for the CC treatments. After 10 weeks of establishment, the shoots and roots were harvested, washed in dH₂O, dried at 65 °C in a forced-air oven and weighed. The shoots and roots were crushed, ground, and converted into ashes in a furnace at 550 °C. The ashes were acid digested and the Cu content was quantified by atomic absorption spectroscopy (-AAS-Perkin-Elmer 3110). The BRSP was determined according to the methods described by Wright and Upadhyaya (1998). To determine the BRSP-bound Cu (BRSP-Cu), the extract was precipitated by the slow addition of 2 M HCl up to pH 2.5, centrifuged at 8000 g for 20 min, re-dissolved in 0.5 M NaOH, dialyzed in deionized H₂O and freeze-dried.

The dried BRSP was acid-digested (H₂O:HCl:HNO₃; 8:1:1 v:v:v), and BRSP-Cu was determined by AAS (Perkin-Elmer 3110). The available Cu in the soil was extracted with a DTPA solution (5 mM diethylene triamine pentaacetic acid, 0.108 M triethanolamine, 10 mM CaCl₂, and pH 7.3).

The main effects of the inoculum, Cu level and their interaction were analyzed with two-way ANOVA. Where there were significant differences ($p < 0.05$), the means were compared using an orthogonal contrast test. All of the obtained data sets were subjected to a Pearson correlation analysis. Statistical analyses were performed with SPSS software v. 15.0 (SPSS, Inc., Chicago, IL).

3. Results and Discussion

All of the experimental variables were affected by the AM inoculation, applied Cu level and/or interaction of both factors (data not shown). The accumulation of T-BRSP was higher in the treatments with added Cu and those inoculated with CC, which presented levels of up to 16-17 mg T-BRSP g⁻¹ in T2 and T3 (Figure 1A). The T-BRSP accumulation was lower in the uninoculated treatments (-M), possibly representing basal levels of BRSP in the tested soil, suggesting that the small differences observed may be due to the release of fresh glomalin from *O. picensis* (Figure 1A). The procedure used here overestimated the glomalin quantity in the soil because it is well-known that the treatment co-extracts the thermo-stable compounds that interfere in spectrophotometric determination (Gadkar and Rillig, 2006); therefore, we used the term BRSP, which refers to the fraction that contains the glomalin. The CC treatments exhibited higher BRSP accumulation compared to +M. This behavior was also observed in the CC treatments under increasing Cu levels, which suggests a higher capacity of fresh glomalin production by the

non-adapted AM fungal strains. In this sense, some studies suggest that glomalin may be an indicator of stress, more produced under adverse environmental conditions (Steinberg and Rillig, 2003; Driver *et al.*, 2005; Gadkar and Rillig, 2006; Cornejo *et al.*, 2008). Considering the capability of BRSP to immobilize Cu (González-Chávez *et al.*, 2004; Cornejo *et al.*, 2008) and the high amounts of BRSP in the CC treatments, we would argue that its release into soil may be one of the most important mechanisms by which this fungus copes with high concentrations of Cu. A similar behavior was reported previously with Lead (Pb) (Vodnik *et al.*, 2008), Zn, Cu (Cornejo *et al.*, 2008), Cd (Gil-Cardeza *et al.*, 2014) and other phytotoxic elements such as Al (Seguel *et al.*, 2015; 2016a; 2016b). Moreover, high levels of Cu may increase the rate of decomposition of AM fungal structures, resulting in a higher degradation of non-metal adapted fungi (Cornejo *et al.*, 2008), which also explains the increased accumulation of BRSP (Driver *et al.*, 2005). On other hand, the BRSP exhibited an increased Cu content linked to its structure (6-12 mg Cu per g of BRSP, Figure 1B), which also resulted in significant levels of Cu bound to BRSP relative to the total amount of Cu in the soil (Figure 1C). These results suggest a direct relationship between BRSP production and the Cu content, especially when non-adapted fungi are colonizing growth roots growing in Cu-polluted environments. This is also consistent with previous studies that reported a high accumulation of glomalin-related soil protein under high metal levels (Cornejo *et al.*, 2008; Vodnik *et al.*, 2008), which could be an efficient mechanism for coping with the stress of phytotoxic metal levels. It is noticeable that the amount of Cu (as total amount) increased under high Cu levels, especially in CC treatments, which could be a good indicator of the usefulness of this strain in the bioremediation of Cu-polluted soil, even when it is isolated from soil without Cu stress. This isolate has been evaluated in other studies

for its capability to accumulate Cu in the whole spore (Cornejo *et al.*, 2013), which constitutes another valuable characteristic in Cu bioremediation applications.

Figure 1. A) Total Bradford-reactive soil protein (T-BRSP), B) concentration of Cu linked to BRSP (Cu-BRSP), and C) the ratio of Cu-BRSP with respect to the total Cu in a Cu-contaminated soil cultivated with *Oenothera affinis* inoculated or not inoculated with arbuscular mycorrhizal (AM) fungi under increasing Cu levels. (-M) without AM inoculation, (+M) inoculation with AM fungi native from Cu contaminated soils, (CC) inoculation with *Claroideoglomus claroideum* isolated from non-Cu contaminated soils. All of the soils had a total basal Cu content of 497.3 mg g⁻¹ and were supplied with a Cu concentration according to the legend in the top of figure. The bar represents the mean ± the standard error (n=4).

The concentration of DTPA extractable-Cu in the soil increased according to the Cu supply in all of the inoculation treatments (Figure 2A). The maximum level was observed in Cu T5(-M), which reached a level of 125 mg Cu kg⁻¹ soil. Nevertheless, it is observable that in the inoculated treatments, the DTPA-Cu concentrations were lower in comparison to the -M treatments, especially under higher Cu supply levels (Figure 2A). In both AM inoculated treatments, and based on the decrease of extractable Cu, it is possible to support the environmental advantage of the joint use of metallophyte and AM fungi to bioremediate Cu-polluted soils (Meier *et al.*, 2012). According to our data, the symbiosis between *O. picensis* and indigenous (presumably Cu-adapted), AM fungi preferentially enhanced Cu phytoextraction due to an increase in the Cu concentration, both in the shoots and roots (Figures 2B, 2C). Conversely, in the CC treatments, the concentration of Cu, especially in the roots, was much lower than in the other treatments (Figures 2B, 2C), presumably due to an increased capability to produce BRSP and immobilize Cu-bound-to-BRSP, thereby enhancing the phytostabilization of Cu. This behavior is well-corroborated in the grouping of CC individuals in principal component (PC) analysis, where the predominance of all Cu supply treatments (with the exception of T1) had a higher accumulation of BRSP and a proportion of Cu bound to BRSP relative to the total Cu in soil (see PC2, Figures 3A, 3B). This aspect represents different ways to cope with Cu stress that are dependent on the type of AM isolate, which need to be deeply analyzed because of their important biotechnological considerations in the use of AM inoculants in bioremediation processes.

Figure 2. A) DTPA extractable Cu in the soil, B) concentration of Cu in the shoots, and C) concentration of Cu in the roots of *Oenothera affinis* growing in a Cu-contaminated soil inoculated or not inoculated with arbuscular mycorrhizal (AM) fungi at increasing Cu levels. (-M) without AM inoculation, (+M) inoculation with AM fungi native from Cu-contaminated soils, (CC) inoculation with *Claroideoglomus claroideum* isolated from non-Cu contaminated soils. All soils had a basal total Cu content of 497.3 mg g⁻¹ and were supplied with a Cu concentration according to the legend at the top of the figure. The bar represents the mean ± standard error (n=4).

Figure 3. Principal components analysis of the results obtained for the different experimental units of *Oenothera picensis* grown in a Cu-contaminated sterilized soil and *i*) uninoculated (grey symbols) or *ii*) inoculated with native AM fungal propagules (white symbols), or *iii*) inoculated with a *Claroideoglomus claroideum* (black symbols) strain isolated from non-contaminated soil. A) Distribution and grouping of the different experimental units according to PC1 and PC2. B) Distribution of experimental variables according to PC1 and PC2. Symbols for the treatments: 0 mg Cu kg⁻¹ (○); 75 mg Cu kg⁻¹ (Δ); 150 mg Cu kg⁻¹ (◻); 225 mg Cu kg⁻¹ (◻); 300 mg Cu kg⁻¹ (◇).

4. Conclusions

According to our results, BRSP production may be one of the most important mechanisms of AM fungi for coping with high environmental levels of Cu and must be a characteristic for analyzing the functionality of AM fungal strains as well as for their selection in the bioremediation of Cu-contaminated soils. Therefore, more information about the intrinsic potential of glomalin production by AM fungal inoculants is needed. Finally, we conclude that the use of *O. picensis*, in conjunction with *Claroideoglomus claroideum*, is a good alternative for the implementation of Cu-phytostabilization in contaminated areas of Central Chile.

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