



Technical Note

Interactions of *Trametes versicolor*, *Corioloopsis rigida* and the arbuscular mycorrhizal fungus *Glomus deserticola* on the copper tolerance of *Eucalyptus globulus*C. Arriagada^{a,*}, E. Aranda^b, I. Sampedro^b, I. Garcia-Romera^b, J.A. Ocampo^b^a Departamento de Ciencias Forestales, Universidad de La Frontera, Casilla 54-D, Temuco, Chile^b Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Profesor Albareda 1, 18008 Granada-España, Spain

ARTICLE INFO

Article history:

Received 5 March 2009

Received in revised form 18 July 2009

Accepted 20 July 2009

Available online 18 August 2009

Keywords:

Arbuscular mycorrhizae

Heavy metals

Phytoremediation

Saprobe fungi

Soil contamination

ABSTRACT

The presence of high levels of Cu in soil decreases the shoot and root dry weights of *Eucalyptus globulus*. However, higher plant tolerance of Cu has been observed in the presence of the arbuscular mycorrhizal (AM) fungus *Glomus deserticola*. The hyphal length of *G. deserticola* was sensitive to low Cu concentrations, and the percentage of AM root colonisation and the metabolic activity of the AM fungus were also decreased by Cu. Therefore, a direct effect of Cu on the development of the AM fungus inside and outside the root cannot be ruled out. *E. globulus* colonised by *G. deserticola* had higher metal concentrations in the roots and shoots than do non-mycorrhizal plants; however, the absence of a higher root to shoot metal ratio in the mycorrhizal plants (1.70 ± 0.11) indicated that *G. deserticola* did not play a filtering/sequestering role against Cu. The saprobe fungi *Corioloopsis rigida* and *Trametes versicolor* were able to remove Cu ions from the asparagine–glucose growth media. However, plants inoculated with *C. rigida* and *T. versicolor* did not accumulate more Cu than non-inoculated controls, and the growth of the plant was not increased in the presence of these fungi. However, *C. rigida* increased the shoot dry weight, AM root length colonisation, and metabolic mycelial activity of plants colonised with *G. deserticola* in the presence of Cu; only this saprobe-AM fungus combination increased the tolerance of *E. globulus* to Cu. Inoculation with *G. deserticola* and *C. rigida* increased the *E. globulus* Cu uptake to levels reached by hyperaccumulative plants.

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1. Introduction

Cu is essential for plant development and growth. However, excessive Cu can lead to root elongation and cause damage to root membranes. Moreover, it may cause toxicity by interfering with photosynthesis, respiratory processes, and protein synthesis (Marschner, 1995). White-rot fungi have to cope with toxic levels of metal ions such as Cu often during their growth in soil. Relatively few studies have been done using white-rot fungi in bioremediation of Cu-contaminated soils (Baldrian, 2003). The white-rot fungi belonging to the *Trametes* and *Corioloopsis* genera have been used to detoxify metal effluents from agroindustrial wastes (Barajas-Aceves et al., 2002). These saprobe fungi are able to remove heavy metals such as Cu by adsorbing them on their mycelia, and the degree of accumulation and tolerance of Cu from soil differs in different species of these fungi (Saglam et al., 1999). It is known that white-rot fungi increase the growth of plants, especially when plants are cultivated in soils contaminated with agroindustrial wastes (Aranda et al., 2006).

The arbuscular mycorrhizal (AM) fungi are a substantial component of the soil microbial biomass. This symbiosis benefits plant growth, particularly through enhanced phosphorus, water, and mineral nutrient uptake (Smith and Read, 1997). AM fungi improve plant resistance to the presence of high quantity of heavy metals such as Cu in the soil. However, the effect of AM fungi on the uptake of metals by plants is not yet totally clear. AM isolates can increase or decrease metal uptake and accumulation in shoots or in roots or can increase or reduce heavy metal translocation from roots to shoots (Jonner and Leyval, 2001; Chen et al., 2003).

Phytoremediation, the use of plants to remove toxic metals from soils is emerging as a potential strategy for cost-effective and environmentally friendly remediation of contaminated soils (Glass, 2000). Some plants can accumulate high concentrations of heavy metals and have been used in experimental assays for the phytoremediation of contaminated soils (McGrath et al., 2002). Many of the accumulative plants used belong to the family *Brassicaceae*; this family does not form AM symbiosis. As many hyperaccumulating plant families are herbaceous and non-mycorrhizal, considerable scepticism exists about the functional importance of AM in highly tolerant hyperaccumulating plants, but nevertheless its functionality was recently confirmed (Regvar and Vogel-Mikus, 2008). However, these herbaceous plants produce little biomass, so

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they are of less interest than plants with higher productivity, such as trees (Greger and Landberg, 1999). *Eucalyptus* is a tree species with a wide plasticity to grow in impoverished or marginal soils and is able to accumulate high quantities of heavy metals (Arriagada et al., 2004). Studies of AM fungal symbiosis in trees are scarce (Wilkinson and Dickinson, 1995), but the *Eucalyptus* species were able to develop AM symbiosis (Arriagada et al., 2004). To our knowledge, there are few reports on the effect of AM fungi on Cu phytoextraction by high-biomass crops such as maize and *Eucalyptus* from Cu-contaminated soils (Arriagada et al., 2004; Wang et al., 2007). Cultivation of non-hyperaccumulating, highly mycorrhizal plants that produce large amounts of biomass, such as *Eucalyptus* and *Populus* trees, on contaminated soil is recommended as a phytoremediation practice to prevent food chain contamination because of their capacity to accumulate heavy metals in the stem-wood (Leep and Dickinson, 1998; Arriagada et al., 2004; Komarek et al., 2008; Lingua et al., 2008).

In addition, it is known that soil microorganisms such as saprobe fungi affect AM symbiosis. Some experimental results confirm the existence of synergistic, neutral, and antagonistic effects of saprobe fungi on plant root colonisation by AM fungi (Fracchia et al., 1998).

The aim of this work is to determine if the interaction between AM and saprobe fungi increases the tolerance of *Eucalyptus* to high concentrations of Cu in soil.

2. Materials and methods

2.1. Arbuscular mycorrhizal (AM) fungi

The AM *Glomus deserticola* (Trappe, Bloss and Menge) from the Instituto de Investigaciones Agrobiológicas de Galicia was used.

2.2. Saprobe fungi

The saprobe fungi *Corioloopsis rigida* and *Trametes versicolor* were isolated by the particle washing method using a multichamber washing apparatus (Widden and Bisset, 1972). These fungi were classified as described by McAllister (1992). Strains are kept at the fungal culture collection of the Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera in Temuco, Chile. Both saprobe fungi were transferred to tubes of 39 g L⁻¹ potato dextrose agar (PDA, DIFCO) and 2% malt extract at 4 °C as stock culture.

2.3. In vitro experiments

The effect of Cu on spore germination and hyphal length of *G. deserticola* was tested in 9-cm diameter plastic Petri dishes. The spores of *G. deserticola* were surface-sterilised as described by Mosse (1962). Ten surface-sterilised spores per plate were placed 1 cm from the edge of a Petri dish with 10 mL of 10 mM 2-(N-morpholin) ethane sulphonic acid buffer (pH 7) plus 0.04 g of Gel-Gro (ICN Biochemicals, Aurora, OH, USA). The quantity of 39.6, 79.2, 118.8, 237.6 and 396.5 mg of CuSO₄·5H₂O were added to Petri dishes before the solidification medium to reach a final concentrations of 0, 10, 20, 30, 60, and 100 mg Cu L⁻¹. The plates were incubated at 25 °C in the dark for 21 d and were sealed to reduce dehydration and contamination. Hyphal length of the germinated *G. deserticola* spores was determined under a binocular stereo microscope (Olympus SZ-PT) at 40× magnification at the end of the experiment using the gridline intersect method (Marsh, 1971). Ten replicates petri dishes with 10 spores each were used and all the fungal mycelia were measured.

An aqueous suspension in sterile distilled water containing mycelium of the saprobe fungi was prepared from cultures grown in PDA for 1 week at 27 °C. Two ml of this suspension were added

to 250-mL flasks containing 125 mL of sterile AG liquid medium (Galvagno, 1976) in a shaker at 28 °C. The AG medium consisted of 1 g glucose, 0.4 g asparagine, 0.05 g MgSO₄, 0.05 g KH₂PO₄ and 100 mL distilled water. The quantity of 0.039, 0.39, 1.98 and 3.96 g of CuSO₄·5H₂O were added to AG medium to reach a final concentration of 0, 10, 100, 500, and 1000 mg Cu L⁻¹. The concentration of Cu was analysed in the AG medium after 2 week culture of *C. rigida* and *T. versicolor* by atomic absorption spectroscopy (Perkin-Elmer 5380, Norwalk, CT, USA) after microwave digestion with a mixture of H₂SO₄ and H₂O₂ (Mingorance, 2002). Ten replicates were used in these experiments.

2.4. Greenhouse experiments

The experiments were carried out using *Eucalyptus* (*Eucalyptus globulus* Labill) as test plants. Seeds were surface-sterilised with HgCl₂ for 10 min and thoroughly rinsed with sterilised water and sown in moistened sand. After germination, uniform seedlings were planted in 0.3-L pots (One seedling per pot), filled with a mixture of sterilised sand:soil at a proportion of 1:1 (v:v). The soil, classified as an Andisol (Acruoxic Hapludands), is moderately acidic (pH 5.5) with good drainage and water infiltration. Plants were grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 E m⁻² s⁻¹, 400–700 nm, with a 16/8 h day/night cycle at 25/19 °C and 50% relative humidity. Plants were watered from below and fertilised every week with 10 mL of a nutrient solution plus 50 mg L⁻¹ of P (Hewitt, 1966). The AM fungal inoculum was a root-and-soil inoculum consisting of rhizosphere soil containing spores (approximately 1000 spores per 100 g⁻¹) and colonised root fragments of *Medicago sativa*. The inoculation plants were in amounts of 8 g of soil inoculum per pot, an amount that was predetermined to enable high levels of root colonisation. In order to restore the microbial population present in the soil inoculum, uninoculated plants were given a filtrate (Whatman No. 1 paper) of the inoculum containing common soil microflora that was free of AM fungal propagules.

We inoculated *E. globulus* pots with: (1) *G. deserticola*, (2) *C. rigida* or *T. versicolor*, and (3) *C. rigida* or *T. versicolor* plus *G. deserticola*, as well as we kept some seedlings as controls. Plants were inoculated at the time of transplanting (after 3 week of growth). The saprobe fungi were inoculated at the same time as was *G. deserticola*. The quantity of 0.039, 0.39, 1.98, 3.96 and 7.93 g of CuSO₄·5H₂O were applied to *E. globulus* pots to reach the concentrations of 0, 10, 100, 1000, up to 2000 mg Cu kg⁻¹ of sand:soil. Ten replicate pots per treatment and Cu concentration were used.

Plants were harvested after 12 week and dry mass was determined. After the harvest, two samples of fresh weight were taken from the entire root system at random. One of the samples was cleared and stained (Phillips and Hayman, 1970), and the percentage of root length colonisation with AM fungus was measured by the gridline intersect method (Giovannetti and Mosse, 1980). In the other sample, succinate dehydrogenase (EC 1.3.99.1) (SDH) activity was measured in fungal mycelia by the reduction of tetrazolium salts (Natrium blue tetrazolium from Sigma Chemicals) at the expense of added succinate (Succinic acid disodium salt from Fluka Analytical), (MacDonald and Lewis, 1978); the percentage of AM fungal mycelia with SDH activity was determined under a compound microscope (Ocampo and Barea, 1985).

We measured the following response variables; total Cu content in the root and shoot of 10 *E. globulus* seedlings per treatment. Cu concentrations were measured by atomic absorption spectroscopy (Perkin-Elmer 5380, Norwalk, CT, USA) after microwave digestion with a mixture of H₂SO₄ and H₂O₂ according to the procedure of Mingorance (2002).

We studied the following three main factors and their respective levels as follows AM fungal (control and *G. deserticola*), Sap-

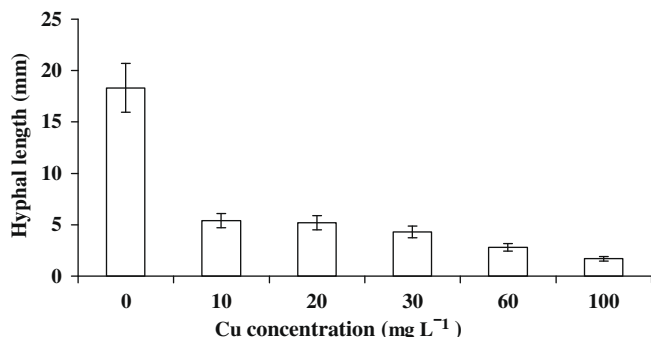


Fig. 1. Effect of Cu on the hyphal length *Glomus deserticola* spores. The data are shown as mean ± standard error of the mean (n = 10).

robe fungi (control, *C. rigida* and *T. versicolor*), and Cu supply (in sand:soil pot at 0, 10, 100, 1000 and 2000 mg Cu kg⁻¹). We also analysed the interaction among the main factors using a factorial analysis of variance. Statistical analyses were conducted in SPSS software, version 11.0 (SPSS Inc., 1989–2001).

3. Results

The length of AM fungal mycelia was strongly reduced by Cu presence, even at the lowest concentration (10 mg L⁻¹) to a

5 mm (Fig. 1). The concentration of 1000–10 mg mL⁻¹ Cu in the AG growth medium decreased between 53% and 66% after culture of *C. rigida* and between 26% and 47% after culture of *T. versicolor*.

Factorial analyses results were as follows. We found significant differences in the population means of all the response variables (shoot dry weight, Cu in shoot, and Cu in root) to the main factors AM fungal (P < 0.01) and Cu supply (P < 0.001), but not to the factor saprobe fungi. We conducted contrasts between a main factor and levels of some factors in order to assess their interactions. The following contrasts between; the entire factors AM fungal and Cu supply, *C. rigida* and the factor AM fungal, as well as between *T. versicolor* and the factor AM fungal, were found statistically significant (P < 0.01, and P < 0.05 for the last ones, respectively) for all the response variables. Nevertheless, we did not find significant interactions between any of the levels of saprobe fungi (*C. rigida* and *T. versicolor*) and the factor Cu supply. Finally, we further explore the interaction of *T. versicolor* and *C. rigida* by themselves with the factors AM fungal and Cu supply. Both saprobe fungi shown significant interactions with those factors (P < 0.05), although *T. versicolor* shown a stronger difference in the population mean of the variable Cu in root (P < 0.01).

The shoot dry weight average for each factor and their interactions in Fig. 2a illustrate that saprobe fungi did not provide an additional Cu tolerance to *E. globulus*. However, the mycorrhizal fungus *G. deserticola* inoculated alone increased the shoot dry weight of *E. globulus* such that it was significantly higher, even at

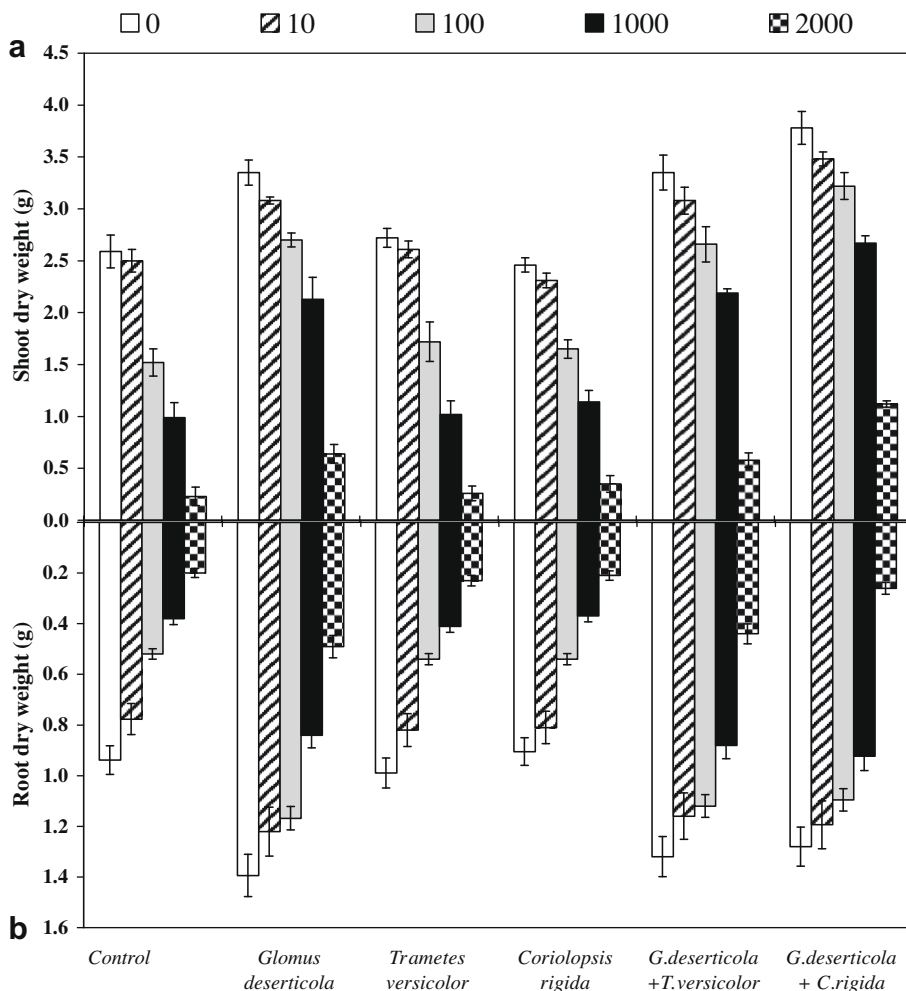


Fig. 2. Shoot (a) and root (b) dry weight of *Eucalyptus globulus* inoculated or not with AM or with the saprobe fungi in soil contaminated with different Cu concentrations (0, 10, 100, 1000 and 2000 mg Cu kg⁻¹ sand:soil). The data are shown as mean ± standard error of the mean (n = 10).

the application rate of 100 mg Cu kg⁻¹ sand:soil. The application of doses higher than 100 mg Cu kg⁻¹ sand:soil decreased the shoot dry weight of plants in all treatments tested, except when *C. rigida* was inoculated together with *G. deserticola*, which decreased only at 2000 mg Cu kg⁻¹ sand:soil. In our experiments, *E. globulus* did not show any visible sign or symptom of copper toxicity from each treatment.

The root dry weight means for each factor and their interactions, illustrated in Fig. 2b, show that the root dry weight of plants inoculated with *G. deserticola* in either the absence or presence of all Cu doses was higher than the non-AM-inoculated plants. Inoculation with *C. rigida* or *T. versicolor* did not increase the root dry weight of plants colonised by *G. deserticola* (Fig. 2b).

The effect of Cu treatment on mycorrhizal root colonisation and SDH activity of *E. globulus* decreased in the presence of 100 mg Cu kg⁻¹ sand:soil (Fig. 3a and 3b). Plants inoculated with *T. versicolor* did not have increased AM root colonisation or SDH activity of *E. globulus* colonised by *G. deserticola* under any treatment. However, at all Cu doses, the percentage of root colonisation and the SDH activity of *G. deserticola* were both higher in the presence than in the absence of *C. rigida*.

The Cu concentration in shoots and roots of *E. globulus* plants did not show any differences at 10 and 100 mg Cu kg⁻¹ sand:soil, and in plants growing at such concentrations, the shoot and root concentrations of Cu are not affected either by AM fungi or by sap-

robe fungi inoculation (Fig. 4). However, at 1000 and 2000 mg Cu kg⁻¹ sand:soil, *G. deserticola* produced a significant increase in shoot and root Cu concentration; this increase was only reinforced by the inoculation of *G. deserticola* together with *C. rigida* at 2000 mg Cu kg⁻¹ sand:soil.

Root to shoot metal content ratios of *E. globulus* were not affected by the presence of either AM (1.70 ± 0.11), saprobe fungi (1.69 ± 0.15) or non inoculated controls (1.75 ± 0.09) and non significant differences between all treatments were observed (data not shown).

4. Discussion

The growth of *E. globulus*, like other plants, was affected by the presence of a high level of Cu in the soil (Marschner, 1995). Cu concentrations inhibiting shoot dry weight were higher in AM than in non-AM *E. globulus* plants, thus indicating higher plant tolerance to Cu soil contamination as a result of AM colonisation. Colonisations by AM fungi are characterised by uptake of Cu to the colonised plant. However, in contrast with other essential metals, Cu is toxic to most fungi even at very low concentrations (Baldrian, 2003). The hyphal length of the AM fungus *G. deserticola* was sensitive to low concentrations of Cu, indicating that this metal will affect the development of this fungus outside the root. Cu also decreased the percentage of AM root length colonisation and the metabolic

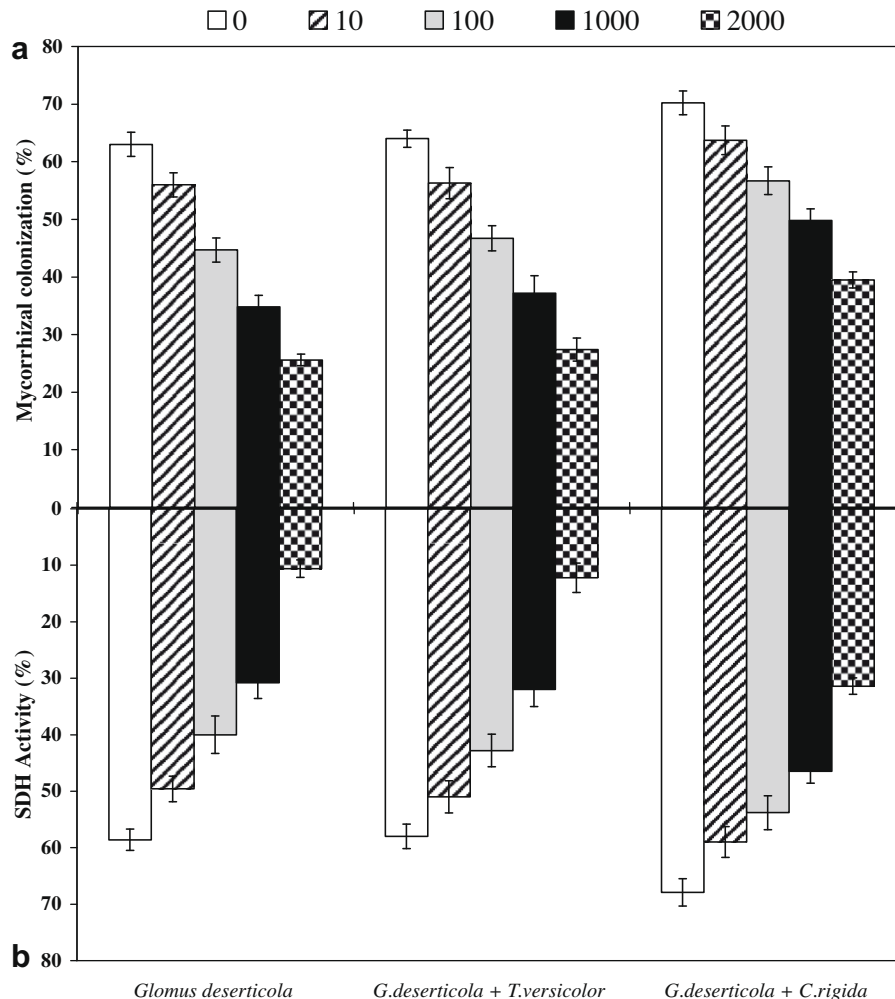


Fig. 3. Effect of AM and saprobe fungi on root length colonisation (a) and percentage of AM mycelium with SDH activity (b) of *Eucalyptus globulus* seedling in soil with different Cu concentrations (0, 10, 100, 1000 and 2000 mg Cu kg⁻¹ sand:soil). The data are shown as mean ± standard error of the mean (n = 10).

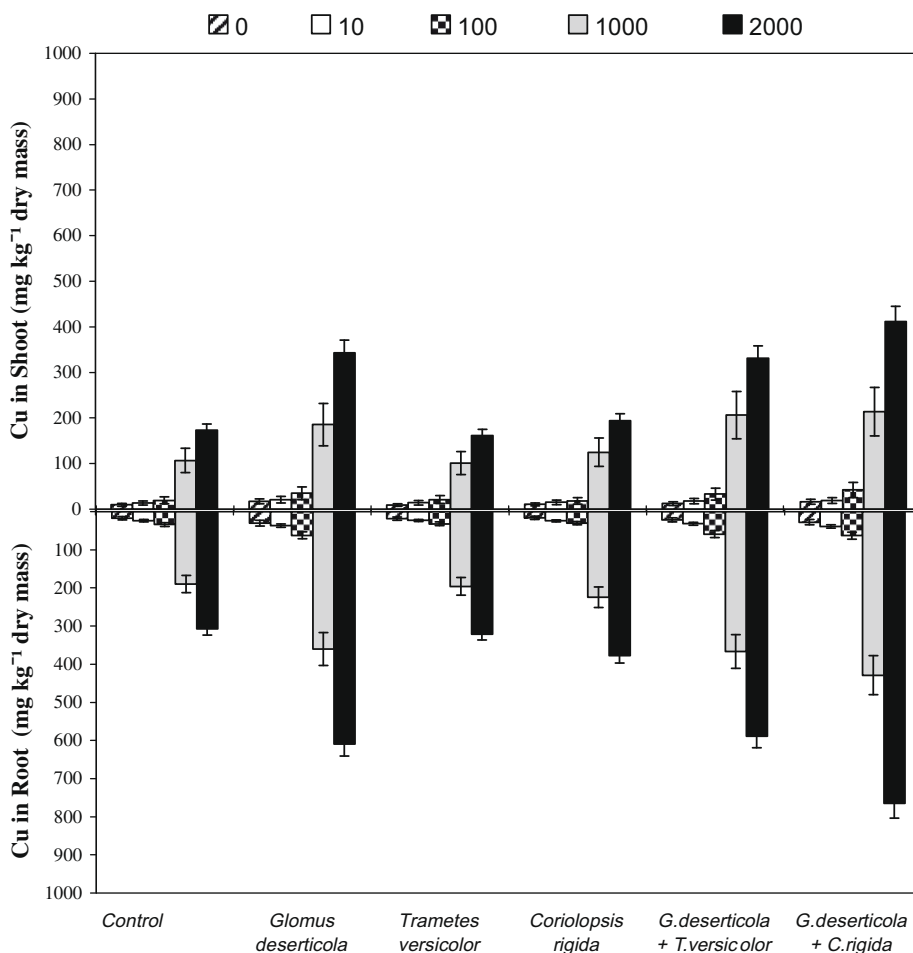


Fig. 4. Cu concentration in shoot and root of *Eucalyptus globulus* inoculated or not with AM or with the saprobe fungi in soil with different Cu concentrations (0, 10, 100, 1000 and 2000 mg Cu kg⁻¹ sand:soil). The data are shown as mean \pm standard error of the mean ($n = 10$).

activity of the arbuscular fungi, measured as SDH activity of the fungal mycelium inside the root of *E. globulus*. It is possible that the negative effect of Cu on AM symbiosis can be due partly to the toxic effect of Cu on the plants and partly to Cu inhibition of the extraradical development of the AM fungi. Nevertheless, due to the decrease in the metabolic activity of the AM fungi, a direct effect of Cu on the development of the AM fungi inside the root cannot be discarded. However, AM fungi increased the plant Cu concentration when grown in soil with a high concentration of this metal (Carvalho et al., 2006). In fact, we found that *E. globulus* colonised by *G. deserticola* in the presence of high Cu levels had higher metal concentrations in the roots and shoots than non-mycorrhizal plants. Mycorrhizal plants have various heavy metal detoxification mechanisms, including the retention of toxic metals in roots and the subsequent reduction of translocation to shoots (Hildebrandt et al., 2007). However, the absence of a higher root to shoot metal ratio in *E. globulus* mycorrhizal plants (1.70 ± 0.11) did not support the hypothesis that the AM fungus *G. deserticola* plays a filtering/sequestering role against Cu. At present, it is not known what happens to Cu taken up by mycorrhizae and whether this metal could be transformed into unknown nontoxic compounds in *E. globulus* (Orłowska, et al., 2008). Plants have their own protection mechanisms against metal toxicity. It is known that *E. globulus* was able to accumulate heavy metals in the stem more than in the leaves, and the AM fungi seem to contribute to this redistribution of some heavy metals inside the *E. globulus* plant (Arriagada et al., 2004). This process decreased the damage caused to the physiology of

the plant more than when the heavy metals were accumulated in the metabolically active parts of the plant (Leep and Dickinson, 1998).

The white-rot fungi *C. rigida* and *T. versicolor* were able to remove Cu ions from the growth media, possibly by adsorbing them on their mycelia (Jarosz-Wilkolazka et al., 2002; Bayramoglu et al., 2003). However, plants inoculated with *C. rigida* and *T. versicolor* did not accumulate more Cu than the noninoculated controls, and the growth of the plant was not increased in the presence of these fungi. These results indicate that the effect of the white-rot fungi on the tolerance of *E. globulus* to Cu was mediated by its effect on the colonisation and metabolic activity of the AM fungi. In our experiments, *C. rigida*, but not *T. versicolor* increased the shoot dry weight, as well as the root length colonisation and metabolic activity of the AM fungus of plants colonised by *G. deserticola* in presence of Cu. Only the *C. rigida*-*G. deserticola* combination increased the tolerance of *E. globulus* to Cu. It is known that there is synergistic action of some soil saprobe fungi on the AM colonisation of root and on the tolerance of AM plants to heavy metals (Fracchia et al., 1998; Arriagada et al., 2004).

The concentration of Cu in the shoot must be higher than 1000 mg kg⁻¹ to be considered hyperaccumulative plant (Regvar and Vogel-Mikus, 2008). The Cu uptake level reached by *E. globulus* was lower than 1000 mg kg⁻¹, indicating that this plant cannot be considered a hyperaccumulative plant of this heavy metal. In the present study, *E. globulus* did not show any visible sign or symptom of copper toxicity and the higher root concentrations of Cu were

significant. In fact, some authors report an absence of symptoms of Cu toxicity in plant shoots and a higher Cu accumulation in roots rather than shoots (Marschner, 1995; Oudeh et al., 2002). Inoculation with the AM fungus *G. deserticola* and the saprobe fungus *C. rigida* increased the uptake of Cu but not to levels reached by hyperaccumulative plants (Regvar and Vogel-Mikus, 2008). However, as inoculation with this AM and saprobe fungi combination enhanced not only *E. globulus* tolerance to Cu but also the accumulation of this metal in this high-biomass plant, their use is recommended as a phytoremediation practice.

Acknowledgments

Financial support of this study was provided by the Fondo Nacional de Desarrollo Científico y Tecnológico, FONDECYT, Project No. 1060390 and Dirección de Investigación, Universidad de La Frontera. We would like to also thank Christian Salas E for statistical assistance.

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