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Improved zinc tolerance in *Eucalyptus globulus* inoculated with *Glomus deserticola* and *Trametes versicolor* or *Coriolopsis rigida*

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ABSTRACT

The potential of interactions between saprophytic and arbuscular mycorrhizal (AM) fungi to improve *Eucalyptus globulus* grown in soil contaminated with Zn were investigated. The presence of 100 mg kg $^{-1}$ Zn decreased the shoot and root dry weight of *E. globulus* colonized with *Glomus deserticola* less than in plants not colonized with AM. Zn also decreased the extent of root length colonization by AM and the AM fungus metabolic activity, measured as succinate dehydrogenase (SDH) activity of the fungal mycelium inside the *E. globulus* root. The saprophytic fungi *Trametes versicolor* and *Coriolopsis rigida* increased the shoot dry weight and the tolerance of *E. globulus* to Zn when these plants were AM-colonized. Both saprophytic fungi increased the percentage of AM root length colonization and elevated *G. deserticola* SDH activity in the presence of all Zn concentrations applied to the soil. In the presence of 500 and 1000 mg kg^{-1} Zn, there were higher metal concentrations in roots and shoots of AM than in non-AM plants; furthermore, both saprophytic fungi increased Zn uptake by *E. globulus* colonized by *G. deserticola*. The higher root to shoot metal ratio observed in mycorrhizal *E. globulus* plants indicates that *G. deserticola* enhanced Zn uptake and accumulation in the root system, playing a filtering/sequestering role in the presence of Zn. However, saprophytic fungi did not increase the root to shoot Zn ratio in mycorrhizal *E. globulus* plants. The effect of the saprophytic fungi on the tolerance and the accumulation of Zn in *E. globulus* was mediated by its effect on the colonization and metabolic activity of the AM fungi.

1. Introduction

Zinc is a micronutrient essential for plant development and growth and is involved in a number of enzymatic reactions catalyzing nucleic acid metabolism. However, excess of Zn can inhibit many plant metabolic functions, resulting in retarded growth and senescence (Marschner, 1995). It is recognized that soil microorganisms such as some saprophytic and arbuscular mycorrhizal (AM) fungi play an important role in plant health, nutrient uptake and tolerance against heavy metals (Heggo et al., 1990; Haselwandter and Berreck, 1994; Arriagada et al., 2005).

The arbuscular mycorrhizal (AM) fungi are a primary component of the microbial biomass in soil. This symbiosis can benefit plant growth, particularly through enhanced phosphorus, water and mineral uptake (Smith and Read, 2008). Mycorrhizal fungi enhance nutrient availability and plant tolerance to the presence of high quantities of heavy metals such as Zn in soil (Gaur and Adholeya, 2004). However, the effect of AM fungi on the uptake of metals by plants is not yet clear. It is rather well established that AM accumulate Zn; hence, they increase Zn absorption and accumulation in the roots (Chen et al., 2003).

Saprophytic fungi are important and abundant components of rhizosphere soil, where they obtain great nutritional benefit from organic and inorganic compounds released from living roots and sloughed cells (Dix and Webster, 1995). Some experimental results confirm the existence of synergistic effects of some saprophytic fungi on plant growth and root colonization by AM fungi in soil contaminated with heavy metals (Arriagada et al., 2004, 2005, 2007). Saprophytic fungi of *Trametes* and *Coriolopsis* genera were found to be tolerant to Zn; they can concentrate this heavy metal in their mycelia (Barajas-Aceves et al., 2002). Although these white-rot fungi effectively degrade various xenobiotics, relatively few studies have been done using these fungi for soil remediation (Baldrian, 2003; Bayramoglu et al., 2003).

Eucalyptus is a tree species exhibiting great environmental plasticity, with the ability to grow in impoverished or marginal soils

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and to accumulate high quantities of heavy metals (Arriagada et al., 2004). *Eucalyptus* species are able to develop AM symbiosis. The joint inoculation of this plant with AM and saprophytic fungi increased its capacity of accumulation and tolerance to some heavy metals such as Cd, Al and Pb. The inoculation with both fungi also increased the capacity of *Eucalyptus* to remove these heavy metals from contaminated soils (Arriagada, 2001).

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). Most plants used in phytoremediation of soil contaminated with heavy metals are herbaceous. However, cultivation of non-hyperaccumulating but highly mycorrhizal plants that produce large amounts of biomass on contaminated soil are recommended as a phytoremediation practice.

The aim of this work was to determine whether there were interactions between the AM fungus *Glomus deserticola* and the saprophytic fungi *Trametes versicolor* and *Coriolopsis rigida* and whether this increased the tolerance and accumulation of Zn in *Eucalyptus globulus*.

2. Materials and methods

2.1. In vitro experiments

The effect of Zn on spore germination and hyphal length in *G. deserticola* (Trappe, Bloss and Menge) from the Instituto de Investigaciones Agrobiológicas de Galicia (CSIC) was tested in 9-cm-diameter plastic Petri dishes. Spores of *G. deserticola* were isolated by wet-sieving the soil (Gerdemann, 1955) from alfalfa plant pot cultures; spores were then stored in water at 4 °C. Ten surface-sterilized spores (Mosse, 1962) 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 (MES) buffer (pH 7) plus 0.04 g of Gel-Gro (ICN Biochemicals, Aurora, OH, USA). $ZnSO_4 \cdot 7H_2O$ was added to Petri dishes to a final concentration of 0, 10, 20, 50, 100 or 200 mg l^{-1}. Ten replicates were used. The plates were incubated at 25 °C in the dark for 21 days and were sealed to reduce dehydration and contamination. Hyphal length of the germinated *G. deserticola* spores was determined under a binocular microscope at 40× magnification at the end of the experiment, using the gridline intersect method (Marsh, 1971). All fungal mycelia were measured. In the 100 and 200 mg l^{-1} Zn treatment conditions, the concentration of Zn was analysed in the Gel-Gro medium after 21 days of AM spore culture (Mingorance, 2002). Gel-Gro medium with 200 mg l^{-1} Zn but without spore culture was used as control. Ten replicates were used in these experiments.

The saprophytic fungi *C. rigida* and *T. versicolor* were isolated using the particle washing method and 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 saprophytic fungi were transferred to tubes of potato dextrose agar (PDA, DIFCO) and 2% malt extract at 4 °C as stock culture. An aqueous suspension in sterile distilled water containing saprophytic fungus mycelia was prepared from cultures grown in PDA for 1 wk at 28 °C. Two millilitres of this suspension were inoculated in 250 ml flasks containing 125 ml of sterile AG liquid medium in a shaker at 28 °C. The AG medium comprised 1 g glucose, 0.4 g asparagine, 0.05 g $MgSO_2$, 0.05 KPO_2 and 100 ml distilled water (Galvagno, 1976). $ZnSO_4 \cdot 7H_2O$ was added to AG medium to a final concentration of 0, 500 and 1000 mg l^{-1} Zn. The culture medium was filtered through a disk of filter paper, dried at 80 °C for 72 h and the dry mycelia of the saprophytic fungi were weighed (McAllister, 1992). In the 500 and 1000 mg l^{-1} Zn treatment conditions, the concentration of Zn was analysed in the AG medium after 2 weeks of culture with *C. rigida* and *T. versicolor* (Mingorance, 2002). AG medium with 500 mg l^{-1} Zn but without fungal culture was used as control. Ten replicates were used in these experiments.

2.2. Greenhouse experiments

The experiments were carried out using eucalyptus (*E. globulus* Labill) as test plants. The seeds were supplied by the Centro de semillas forestales, Departamento de Ciencias Forestales, Universidad de La Frontera (Temuco, Chile). Seeds were surface-sterilized with $HgCl_2$ for 10 min, thoroughly rinsed with sterilized water, and then sown in moistened sand. After germination, uniform seedlings were planted in 0.3 l pots filled with a mixture of sterilized sand:soil at a proportion of 1:1 (V:V). The soil, classified as an Andisol (Acrudoxic Hapludands), with low P content (7.3 mg kg^{-1}, $NaHCO_3$-extractable), is moderately acidic (pH 5.4) with good drainage and water infiltration. The 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. The plants were watered from below and fed every week with 10 ml of a nutrient solution plus 50 mg l^{-1} of P (Hewitt, 1952), which did not affect negatively the AM colonization of *E. globulus* root (Arriagada, 2001).

The *G. deserticola* inoculum was a root-and-soil inoculum consisting of rhizosphere soil containing spores and colonized root fragments of *Medicago sativa* L. Each pot was inoculated with 8 g, an amount determined to achieve high levels of root colonization. Uninoculated plants were given a filtrate (Whatman no. 1 paper) of the inoculum containing the common soil microflora, but free of AM fungal propagules.

Sterilized barleys seeds were used as saprophytic fungal inoculums carriers. The seeds were inoculated with a thin slice of PDA (1 × 1 cm) with mycelia of a 14 days old culture of the saprophytic fungi *C. rigida* or *T. versicolor* grown at 28 °C. Soil pots were inoculated with 10 barleys seeds grown with the saprophytic fungi under static incubation at 28 °C for 20 weeks. Pots with 10 barley seeds but without fungal culture were used as control.

A 6 × 5 full factorial randomized experimental design was used. There were six treatments: (1) uninoculated controls, (2) soil pot inoculated with *C. rigida*, (3) soil pot inoculated with *T. versicolor*, (4) soil pot inoculated with *G. deserticola*, (5) soil pot inoculated with *C. rigida* and *G. deserticola*, and (6) soil pot inoculated with *T. versicolor* and *G. deserticola*. Zn was applied to *E. globulus* pots at the concentration of 0, 10, 100, 500 and 1000 mg Zn kg^{-1} of soil. Five replicate pots per treatment and Zn concentration were used. A total of 150 pots (Six treatments × five Zn doses × five replicates), were used. Plants were inoculated at the time of transplant (After three weeks of growth). The saprophytic fungi were inoculated at the same time as *G. deserticola*.

The plants were harvested after 12 weeks and the dry mass was determined. After the harvest, two fresh weight samples were taken at random from the root system. One of the samples was cleared and stained (Phillips and Hayman, 1970), and the percentage of root length colonization was measured (Giovannetti and Mosse, 1980). In the second sample, succinate dehydrogenase (EC 1.3.99.1) (SDH) activity was measured in fungal mycelia violet stained with the formazan deposits formed by the reduction of tetrazolium salts at the expense of added succinate (MacDonald and Lewis, 1978). The percentage of AM fungal mycelia with SDH activity was determined in 30 root segments per plant that were mounted on slides and examined at ×160 magnification under a compound microscope (Ocampo and Barea, 1985).

Table 1
Significance of the main treatment effects and their interactions based on factorial ANOVA.

	F-values						
	AM	SF	Zn	AM × SF	AM × Zn	SF × Zn	AM × SF × Zn
Shoot dry weight	73.31**	2.53 n.s.	526.53*	13.27*	11.56**	0.75 n.s.	12.36**
Zn in shoot	72.25**	2.58 n.s.	191.39***	9.05 n.s.	12.13**	0.69 n.s.	9.17**
Zn in root	191.73***	2.49 n.s.	263.92***	8.98 n.s.	14.27**	0.87 n.s.	9.93**

AM: Arbuscular mycorrhizae; SF: Saprophytic fungi.
ns: not significant.
*: $p < 0.05$.
**: $p < 0.01$.
***: $p < 0.001$.

Total Zn content in the root and shoot of 150 plants of *E. globulus* was measured by atomic absorption spectroscopy (Perkin–Elmer 5380, Norwalk, Conn.) after microwave digestion with a mixture of H_2SO_4 and H_2O_2 according to the procedure of Mingorance (2002).

2.3. Statistical analyses

We studied the three main factors and their respective levels: AM fungus (Control and *G. deserticola*), saprophytic fungi (Control, *C. rigida* and *T. versicolor*) and Zn doses (0, 10, 100, 500 and 1000 mg Zn kg^{-1}). We also analysed the interaction among the main factors using a three-way ANOVA. Statistical analyses were conducted in SPSS software, version 11.0 (SPSS Inc., 1989–2001). The percentage values were arcsine transformed for statistical analyses. Statistical significance was determined at $p < 0.05$.

3. Results

Table 1 shows the results of the factorial ANOVA. We found significant differences in the means of all the response variables (shoot dry weight with $p < 0.01$, Zn in shoot with $p < 0.01$, and Zn in root with $p < 0.001$), related to the AM fungal and Zn supply factors (shoot dry weight with $p < 0.05$, Zn in shoot and root with $p < 0.001$), but not those related to saprophytic fungi factor. The contrasts between the AM fungus and the Zn supply factors were found statistically significant ($p < 0.01$) for all variables. The contrasts between the AM fungus and the saprophytic fungi factors were found statistically significant only in the shoot dry weight ($p < 0.05$). Nevertheless, we did not find significant interactions between any of the levels saprophytic fungi and the Zn supply factor. Finally, the interaction between AM fungus, saprophytic fungi and Zn supply factors were significant ($p < 0.01$).

The hyphal length of the mycorrhizal fungus *G. deserticola* was strongly decreased by Zn, even at the lowest concentration (10 mg l^{-1}) (Fig. 1). However, non-significant decrease of Zn concentration in the spore growth medium was observed.

The concentration of Zn in the growth medium decreased 51–67% after culture with *C. rigida* and 54–66% after culture with *T. versicolor* (Fig. 2).

Fig. 3A shows that applying doses higher than 100 mg kg^{-1} of Zn decreased the shoot dry weight of plants in all treatments tested. *G. deserticola* inoculated alone increased the shoot dry weight of *E. globulus*, even at an application rate of 100 mg kg^{-1} Zn. No differences were observed at 500 and 1000 mg kg^{-1} between the inoculation of *G. deserticola* and non-inoculated control. The shoot dry weight of *E. globulus* inoculated with *G. deserticola* in the presence of *C. rigida* or *T. versicolor* was higher than in plants inoculated with *G. deserticola* alone in the presence of doses lower than 500 mg kg^{-1} Zn. As Fig. 3B shows, *G. deserticola* either alone or together with *C. rigida* or *T. versicolor* increased the root dry weight of *E. globulus* in the presence of doses lower than 500 mg kg^{-1} Zn.

The percentage of AM root length colonization and the percentage of AM mycelium with SDH activity in *E. globulus* inoculated with *G. deserticola* alone were reduced in the presence of 10 mg kg^{-1} Zn. However, the percentage of root length colonization and the percentage of AM mycelia with SDH activity in *G. deserticola* inoculated with each of the saprophytic fungi were higher than those of *G. deserticola* inoculated alone, at all Zn doses examined (Fig. 4).

The saprophytic fungi *T. versicolor* and *C. rigida* did not increase shoot and root Zn concentrations (Fig. 5). *G. deserticola* inoculation either alone or together with the saprophytic fungi *C. rigida* and *T. versicolor* increased shoot Zn concentration of *E. globulus* in the presence of Zn doses higher than 10 mg kg^{-1} (Fig. 5). However, at all doses of Zn applied, the AM fungus increased root Zn concentration. This increase was higher when *G. deserticola* was inoculated together with *T. versicolor* or *C. rigida*.

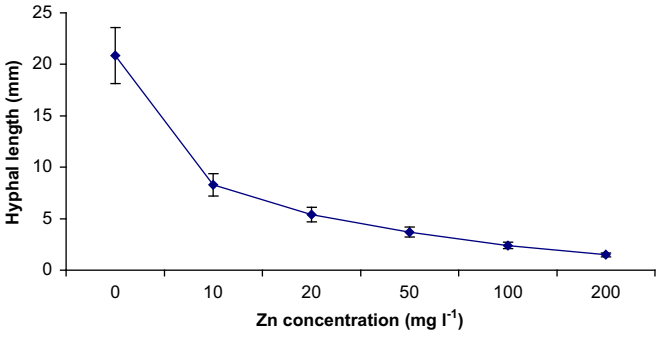

Fig. 1. Effect of incremental concentration of Zn on the hyphal length densities of *Glomus deserticola* spores, in the Gel-Gro medium after 21 days culture. The data are the means ± standard errors of means ($n = 10$).

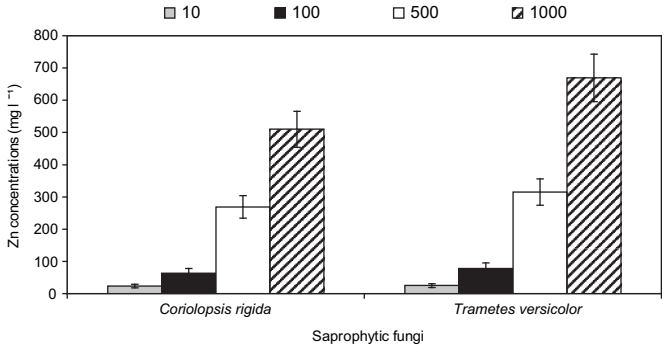

Fig. 2. Zn concentration in the growth medium after the culture of the saprophytic fungi *Coriolopsis rigida* and *Trametes versicolor* with different Zn concentrations. The data are the means ± standard errors of means ($n = 10$).

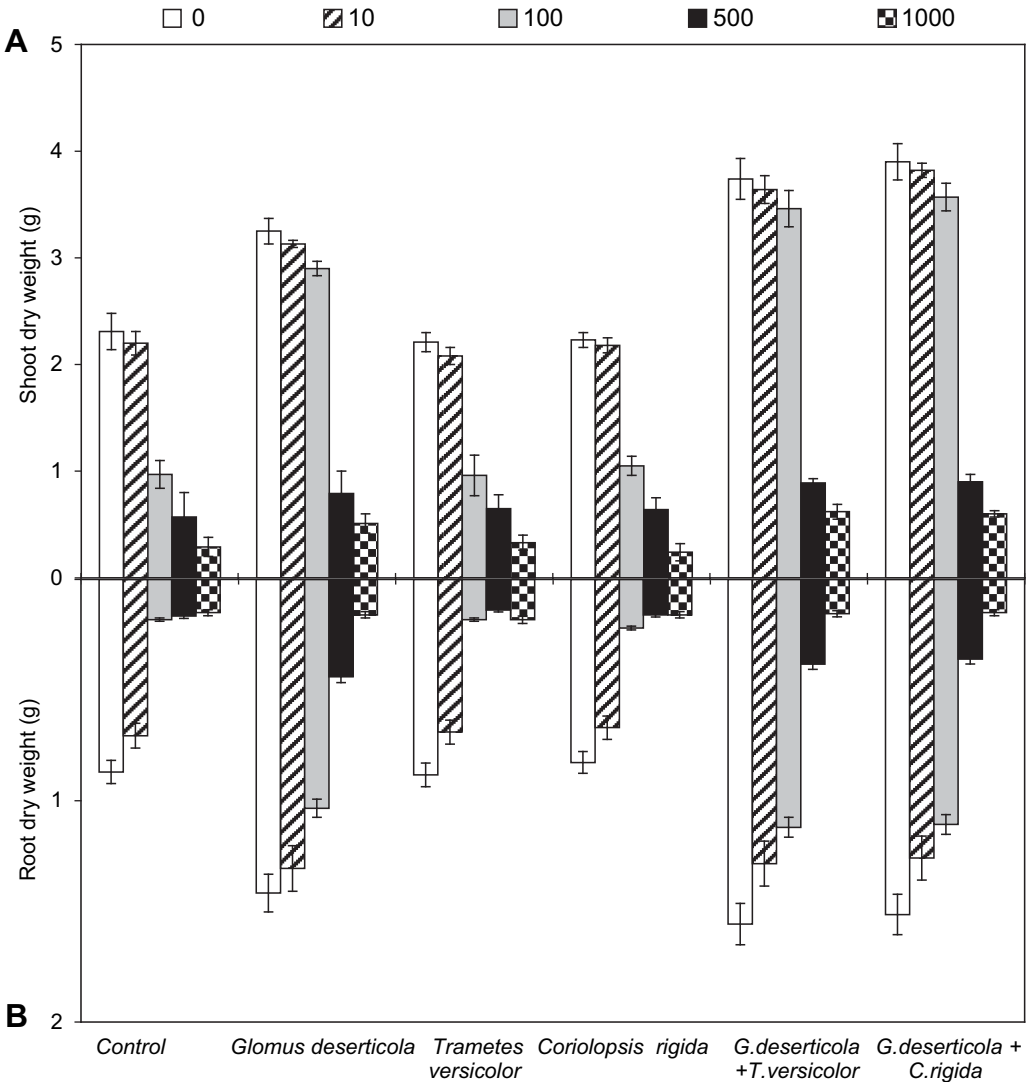

Fig. 3. Shoot (A) and root (B) dry weight of *Eucalyptus globulus* (non-inoculated, inoculated with AM or inoculated with saprophytic fungi) grown in soil with different Zn concentrations (mg kg^{-1}). The data are the means ± standard errors of means ($n = 5$).

Root to shoot (R/S) metal content ratios in *E. globulus* were enhanced by the presence of the AM fungus at all Zn concentrations tested. The saprophytic fungi did not affect the S/R metal content ratios of any plant tested (Fig. 6).

4. Discussion

4.1. Reciprocal effects of Zn, E. globulus and fungi

Although AM fungi may increase plant Zn uptake in Zn-deficient soils (Mathur et al., 2007), this heavy metal is toxic to most fungi, even at low concentrations (Baldrian, 2003). In fact, we found that the hyphal length of the AM fungus *G deserticola* (Arriagada et al., 2005), was decreased by low concentrations of Zn (Equivalent to 16 mg Zn kg^{-1} soil). The presence of high concentrations of Zn (500 mg Zn kg^{-1} soil) decreased the shoot dry weight of AM-colonized plants, but the shoot dry weight of AM was higher than in non-AM *E. globulus* plants in the presence of moderate concentrations of Zn, indicating a higher plant tolerance to Zn-contaminated soil following AM colonization.

The root length colonization and the metabolic activity of the AM fungi, measured as SDH activity of the fungal mycelia inside the *E. globulus* root, were also decreased by exposure to Zn. The negative effect of Zn on AM symbiosis may be partly due to the toxic effect that Zn has on the plants and partly the inhibition of AM fungi development. Nevertheless, due to the reduced metabolic activity of AM fungi, the direct effect of Zn on the development of the AM fungi inside the root cannot be ruled out. Moreover, the fact that Zn inhibited the hyphal length of *G. deserticola* spores suggests that this metal also affects the development of AM fungi external to the root. However, mycorrhizal *E. globulus* had higher biomass despite the toxic effect of Zn. It has been demonstrated that plants colonized with AM fungi enhanced both metal concentrations and plant biomass (Jamal et al., 2002). The mechanisms why mycorrhizal plants had higher biomass despite toxic effect of Zn are unknown, but the increase in P uptake by mycorrhizal plants has been suggested as a possible mechanism (Khan et al., 2000).

On the other hand, the saprophytic fungi *C. rigida* and *T. versicolor* were able to remove Zn ions from the growth media, possibly by adsorbing them on their mycelia (Bayramoglu et al., 2003). However, plants inoculated with *C. rigida* and *T. versicolor* did not accumulate more Zn than the non-inoculated controls, and the shoot dry weight of *E. globulus* was not increased in the presence of these fungi. Nevertheless, both saprophytic fungi increased the

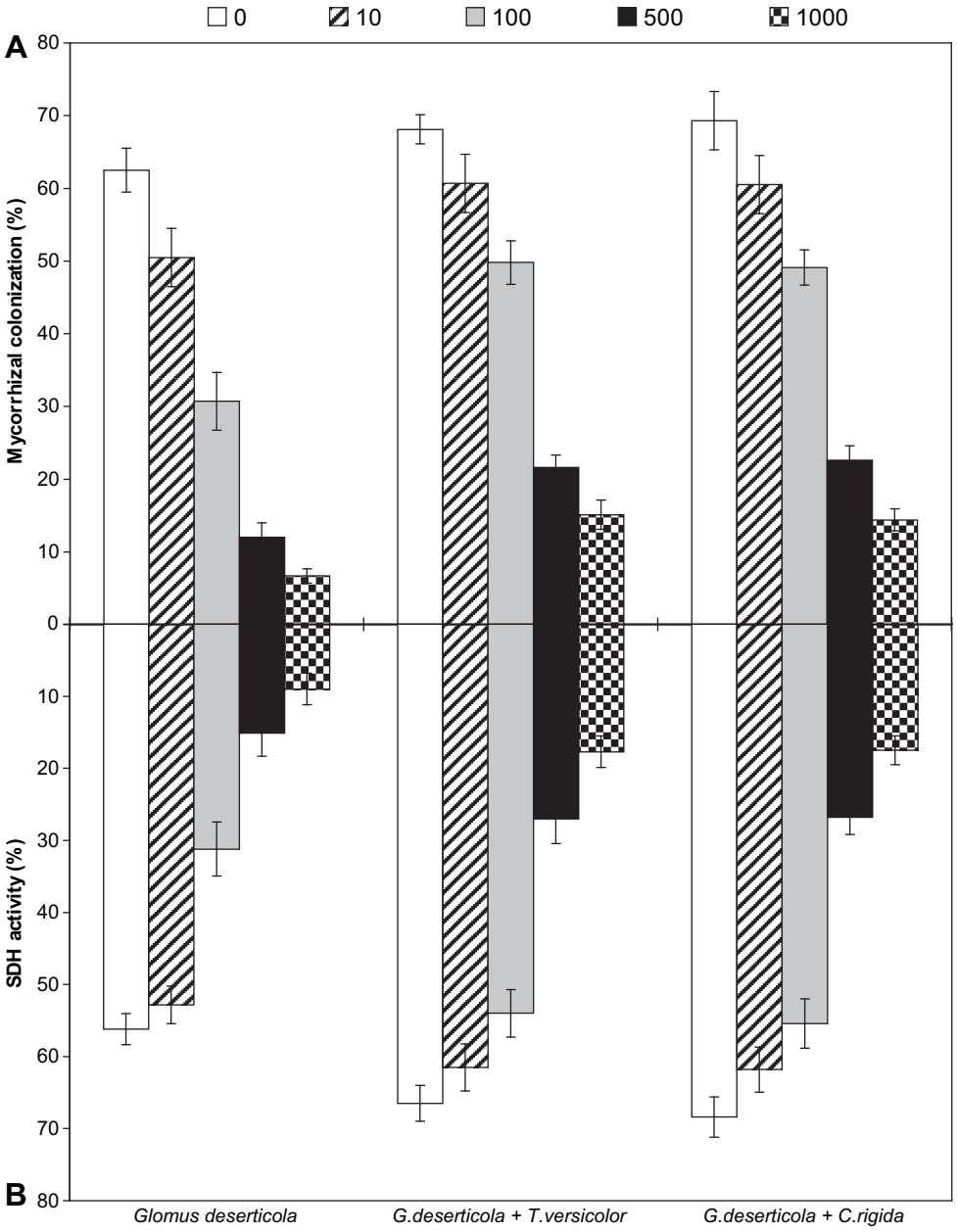

Fig. 4. Effect of AM and saprophytic fungi on root length colonization (A) and percentage of AM mycelium with SDH activity (B) of *Eucalyptus globulus* grown in soil with different Zn concentrations (mg kg^{-1}). The data are the means ± standard errors of means ($n = 5$).

percentage of AM root length colonization and SDH activity for *G. deserticola* in the presence of all Zn concentrations applied to the soil. These results indicate that the effect of the saprophytic fungi on the shoot dry weight and tolerance of *E. globulus* to Zn was mediated by it effect on the colonization and metabolic activity of the AM fungi.

4.2. Plant Zn uptake

The ability of AM fungi to increase Zn uptake by plants is well known (Chen et al., 2003; Soares and Siqueira, 2008). Although shoot and root dry weights did not increase in plants colonized by *G. deserticola* in the presence of 500 or 1000 mg kg^{-1} of Zn, the metal concentrations were higher in the roots and shoots of AM than in non-AM plants. Although plants inoculated with *C. rigida* and *T. versicolor* did not have more Zn concentration than the non-inoculated controls, both saprophytic fungi increased Zn concentration in *E. globulus* colonized with *G. deserticola*.

The effect of an increased AM root length colonization and SDH activity in *G. deserticola*, when inoculated together with *C. rigida* or *T. versicolor* in the presence of 500 or 1000 mg kg^{-1} of Zn, may have contributed to the increase in Zn absorption by the plant.

In this study, the increase on Zn uptake in plants inoculated with AM and saprophytic fungi was related to mycorrhizal fungi not to saprophytic fungi. In this way, mycorrhizal plants have the ability to make the plant more tolerant to the toxic effects of heavy metals through various heavy metal detoxification mechanisms, such as the retention of toxic metals in roots and the subsequent reduction of translocation to shoots (Kaldorf et al., 1999; Christie et al., 2004). AM fungi seem to filter out toxic heavy metals by accumulating them in different AM fungal structures such as the cell wall, electron-dense granules in the cytoplasm and vesicles to keep these

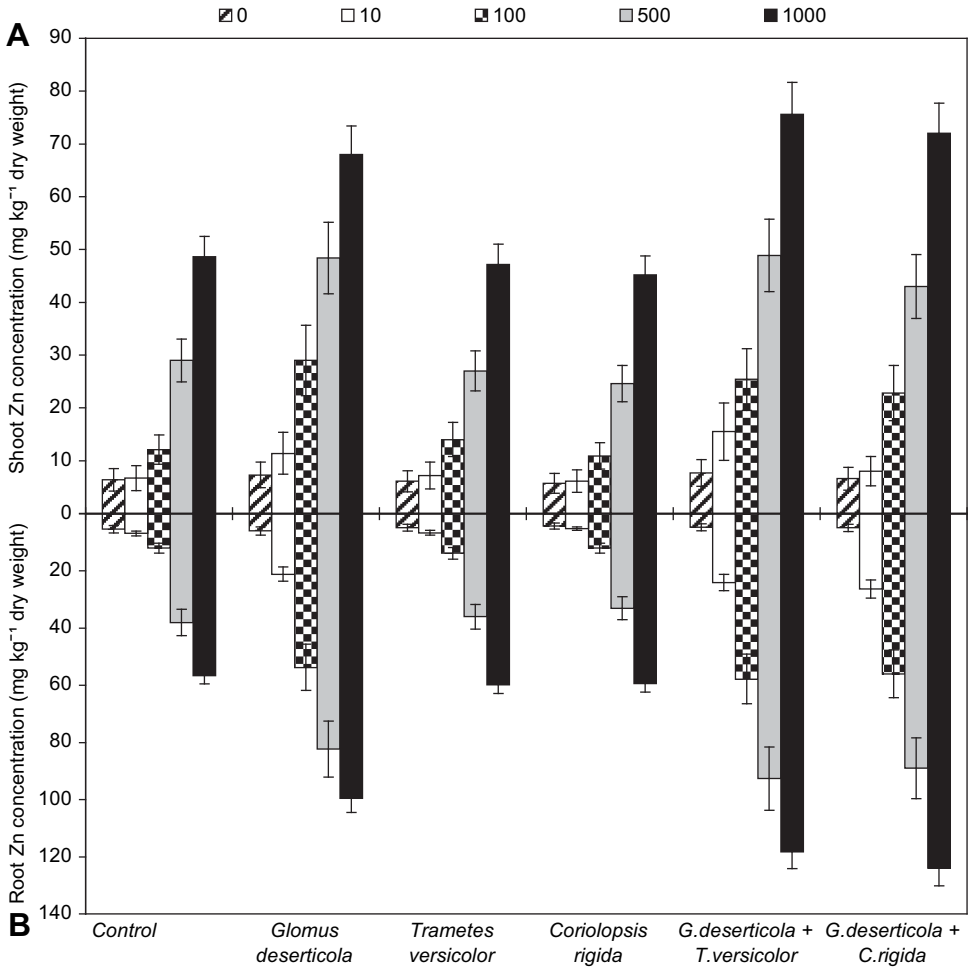

Fig. 5. Zn concentration in roots and shoots of *Eucalyptus globulus* (non-inoculated, inoculated with AM or inoculated with saprophytic fungi) grown in soil with different Zn concentrations (mg kg^{-1}). The data are the means ± standard errors of means ($n = 5$).

toxic heavy metals away from the plants (Turnau, 1998; Weiersbye et al., 1999; Hildebrandt et al., 2007).

The higher root to shoot metal ratio observed in mycorrhizal *E. globulus* plants supports the hypothesis that the AM fungus enhanced Zn uptake and accumulation in the root system compared to non-mycorrhizal plants (Carvalho et al., 2006; Orłowska et al., 2008), and that *G. deserticola* play a filtering/sequestering role with regard to Zn. The saprophytic fungi did not increase the root to shoot Zn ratio in mycorrhizal *E. globulus* plants, suggesting that the increase in the percentage of AM root length colonization and SDH activity of *G. deserticola* caused by *C. rigida* or *T. versicolor* was not enough to allow for a filtering/sequestering role of the AM fungi in the root with regard to Zn uptake.

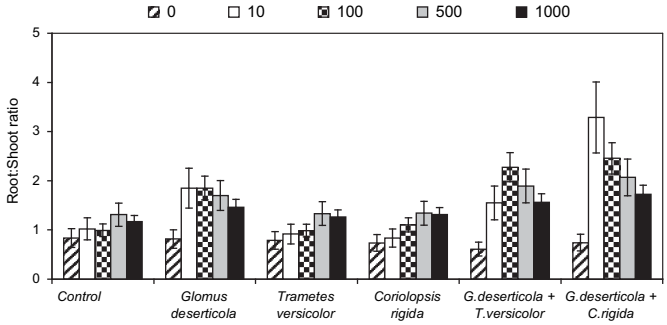

Fig. 6. Root/shoot Zn concentration ratio of *Eucalyptus globulus* (non-inoculated, inoculated with AM or inoculated with saprophytic fungi) in soil with different Zn concentrations (mg kg^{-1}). The data are the means ± standard errors of means ($n = 5$).

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