



Contribution of the saprobic fungi *Trametes versicolor* and *Trichoderma harzianum* and the arbuscular mycorrhizal fungi *Glomus deserticola* and *G. claroideum* to arsenic tolerance of *Eucalyptus globulus*

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ABSTRACT

The presence of high concentrations of arsenic (As) decreased the shoot and root dry weight, chlorophyll and P and Mg content of *Eucalyptus globulus* colonized with the arbuscular mycorrhizal (AM) fungi *Glomus deserticola* or *G. claroideum*, but these parameters were higher than in non-AM plants. As increased the percentage of AM length colonization and succinate dehydrogenase (SDH) activity in the root of *E. globulus*. *Trichoderma harzianum*, but not *Trametes versicolor*, increased the shoot and root dry weight, chlorophyll content, the percentage of AM root length colonization and SDH activity of *E. globulus* in presence of all As concentrations applied to soil when was inoculated together with *G. claroideum*. AM fungi increased shoot As and P concentration of *E. globulus* to higher level than the non-AM inoculated controls. The contribution of the AM and saprobe fungi to the translocation of As from root to shoot of *E. globulus* is discussed.

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1. Introduction

Arsenic (As) is ubiquitous in many environments and highly toxic to all forms of life. Large areas of cultivated land in many countries have been contaminated by As due to agricultural and industrial practices (Xie et al., 2006; Verma et al., 2007). As accumulates in soil because it is only partially removed by leaching, methylation, and erosion or because it is taken up and accumulated by plants (Aguilar et al., 2007). It occurs predominantly in inorganic form as arsenate. Plants actively take up arsenate via their P transporter systems, and transfer it to their leaves and it is likely that such P transporters could contribute to As removal from the soil (Meharg and Macnair, 1994). Arsenic has been demonstrated that to cause toxicity in plants by decreasing its growth and by disabling the biosynthesis of chlorophyll which will produce an alteration in plant photosynthesis and induced chlorosis symptoms in the youngest leaves (Azizur Rahman et al., 2007; Regvar and Vogel-Mikus, 2008).

Arbuscular mycorrhizal (AM) fungi are known to benefit the P nutrition of many plant species by increasing plant P uptake through its external mycelium from the soil (Smith and Read, 1997). Because AM fungi acquire P for plants and, plants uses P

transporters to take up As from soil, the AM fungi might potentially enhance arsenate uptake. However, studies related to the relationship among host and AM mycorrhizas on As toxicity are conflicting. Some studies indicated higher concentrations of As in plants due to AM colonization (Liu et al., 2005), while in others plant AM fungal association observed reduced As concentrations in plants due to mycorrhizal colonization (Gonzalez-Chavez et al., 2002; Chen et al., 2007), or no effects exerted by AM fungi on As concentrations in plant tissue was observed (Chen et al., 2006). On the other hand, conflicting results on the effect of As on AM symbiosis have been observed and the mechanisms by which the As affect AM fungi are unknown (Gonzalez-Chavez et al., 2002; Trotta et al., 2006). The specific staining reaction for Succinate Dehydrogenase (SDH) have been suggested as an indicator for rapid and direct assessment of the part of the AM mycelium inside of roots which possesses metabolic activity (MacDonald and Lewis, 1978; Ocampo and Barea, 1985).

Some experimental results confirm the existence of synergistic effects of saprobe fungi belonging to *Trichoderma* and *Trametes* genera on plant growth and root colonization by AM fungi in soil contaminated with heavy metals (Arriagada et al., 2007). The white-rot fungi belonging to the *Trametes* genera are strong degraders of various xenobiotics to detoxify metal effluents (Barajas-Aceves et al., 2002). Some fungi of *Trichoderma* genera were able to remove As from the growth media and from the soil (Harman et al., 2004).

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Plants can behave as excluders or accumulators of As. Excluders are characterized because limits As influx into the plant whereas accumulators increase the concentration of As in the shoot (Baker, 1981). Excluders and accumulators can be distinguished by the translocation factor defined as the ratio of As concentration in shoots to that in roots. The translocation factor is usually higher than 1 in accumulators and less than 1 in excluders (McGrath and Zhao, 2003; Regvar and Vogel-Mikus, 2008). Exclusion, accumulation or both mechanisms of As in AM plants have been found (Gonzalez-Chavez et al., 2002; Liu et al., 2005; Trotta et al., 2006). *Eucalyptus* is a tree species exhibiting great environmental plasticity, with the ability to grow in impoverished or marginal soils and to accumulate high quantities of heavy metals (Arriagada et al., 2004). *Eucalyptus* species developed AM symbiosis and joint inoculation with AM and saprobe fungi increased the resistance of this

plant when grown in soil contaminated with heavy metals (Arriagada et al., 2004). Most plants used in phytoextraction of As from soils are herbaceous (Craw et al., 2007; Shoji et al., 2008). However, cultivation of highly mycorrhizal tree that produce large amounts of biomass on contaminated soil are recommended as a phyto-remediation practice that prevents food chain contamination.

The aim of this work is to determine if the interaction between AM fungi *Glomus deserticola* and *G. claroideum* and the saprobe fungi *Trametes versicolor* and *Trichoderma harzianum* increases the tolerance of *Eucalyptus* to high concentrations of As in soil.

2. Methods

The saprobe fungi *T. versicolor* and *T. harzianum* were isolated by the particle washing method using a multichamber washing appa-

Table 1

Significance of the main factors and their interactions based on factorial ANOVA.

	AM	SF	As	AM × T.v	AM × T.h	AM × As	T.v × As	T.h × As	AM × T.v × As	AM × T.h × As
<i>F</i> -values										
Shoot dry weight	81.47**	2.07 n.s.	103.41 n.s.	14.54*	15.67*	8.26*	0.87 n.s.	0.78 n.s.	16.78*	17.63*
Chlorophyll content	133.47***	2.23 n.s.	93.13 n.s.	16.81*	17.65*	4.68 n.s.	0.96 n.s.	1.12 n.s.	14.43*	15.01*
As in shoot	79.12**	2.11 n.s.	88.95 n.s.	16.09*	16.28*	9.71*	1.15 n.s.	1.07 n.s.	12.27*	12.87*
As in root	153.22***	2.89 n.s.	92.79 n.s.	20.15*	21.28*	11.04*	0.96 n.s.	0.93 n.s.	27.06**	25.88**

AM: arbuscular mycorrhiza; SF: saprobe fungi. T.v: *Trametes versicolor*. T.h: *Trichoderma harzianum*.

n.s.: Not significant.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

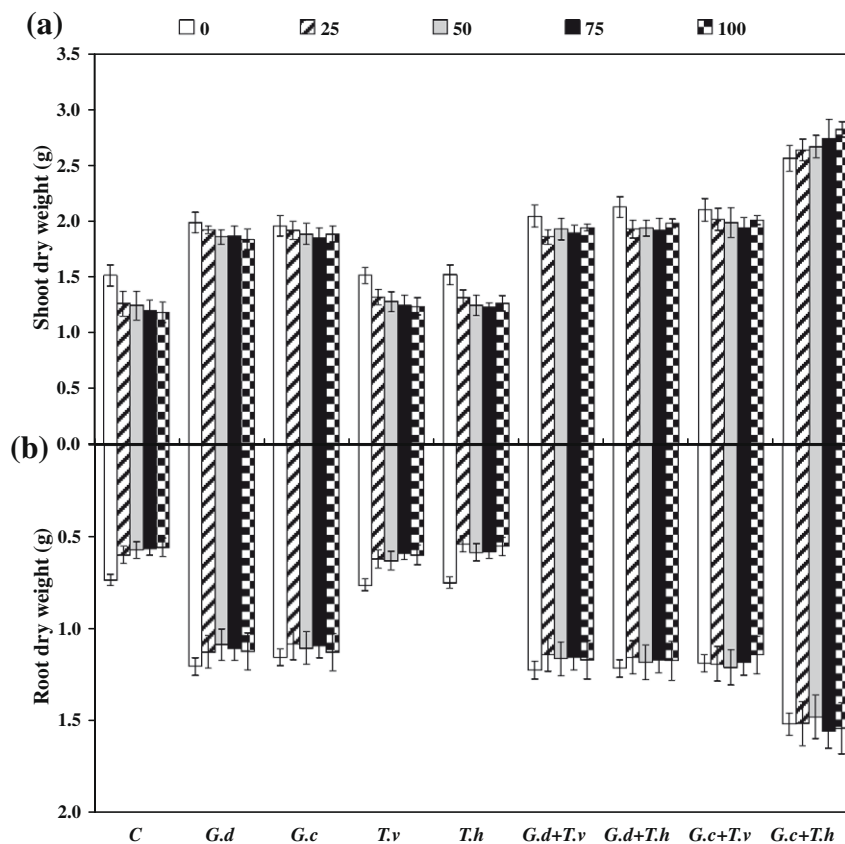


Fig. 1. Shoot (a) and root (b) dry weight of *Eucalyptus globulus* inoculated or not with AM or with the saprobe fungi in soil with different As concentrations (mg As kg⁻¹ soil). C: Control; G.d: *Glomus deserticola*; G.c: *Glomus claroideum*; T.v: *Trametes versicolor*; T.h: *Trichoderma harzianum*; G.d+T.v: *G. deserticola*+*T. versicolor*; G.d+T.h: *G. deserticola*+*T. harzianum*; G.c+T.v: *G. claroideum*+*T. versicolor*; G.c+T.h: *G. claroideum*+*T. harzianum*.

ratu (Widdén 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 potato dextrose agar (PDA, DIFCO) and 2% malt extract at 4 °C as stock culture. An aqueous suspension

in sterile distilled water containing mycelium of saprobe fungus was prepared from cultures grown in potato dextrose agar (PDA, DIFCO) for 1 week at 27 °C. Two milliliter of this suspension were inoculated in 250 mL flasks containing 125 mL of sterile AG liquid medium (Galvagno, 1976) in a shaker at 28 °C. The AG medium consisted in 1 g glucose, 0.4 g asparagine, 0.05 g MgSO₂, 0.05 KPO₂ and

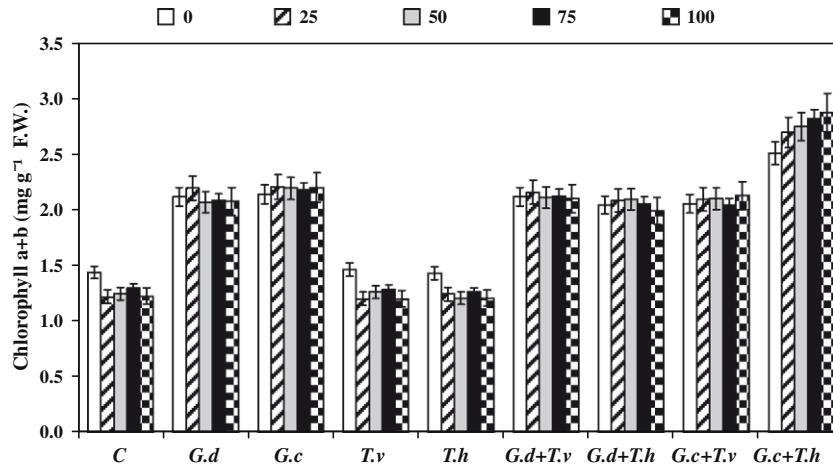


Fig. 2. Chlorophyll content of *Eucalyptus globulus* inoculated or not with AM or with the saprobe fungi in soil with different As concentrations (mg As kg⁻¹ soil). C: Control; G.d: *Glomus deserticola*; G.c: *Glomus claroideum*; T.v: *Trametes versicolor*; T.h: *Trichoderma harzianum*; G.d+T.v: *G. deserticola* + *T. versicolor*; G.d+T.h: *G. deserticola* + *T. harzianum*; G.c+T.v: *G. claroideum* + *T. versicolor*; G.c+T.h: *G. claroideum* + *T. harzianum*.

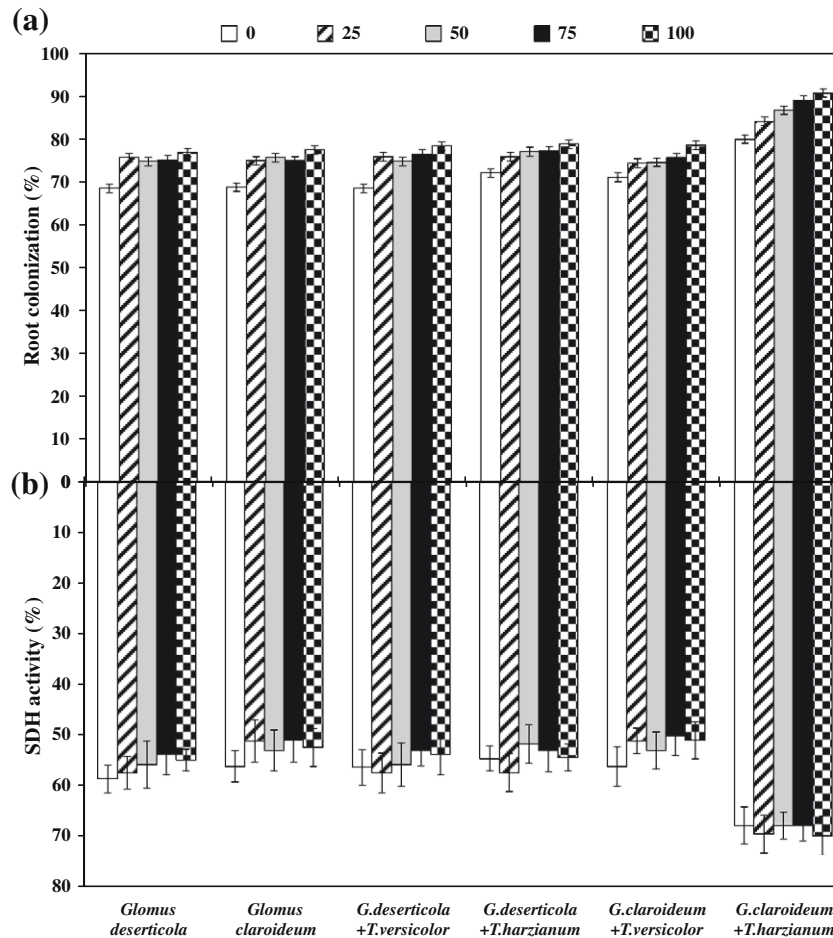


Fig. 3. Effect of AM (a) and Saprobe fungi on root length colonization and percentage of AM mycelium with SDH activity (b) of *Eucalyptus globulus* in soil with different As concentrations (mg As kg⁻¹ soil).

100 mL distilled water. $\text{Na}_2\text{HAsO}_4 \cdot 7 \text{H}_2\text{O}$ was added to AG medium to a final concentration of 0, 25, and 50 mg L^{-1} As. The culture medium was filtered through a disk of filter paper, dried at 80 °C for 72 h and the dry mycelium of the saprobe fungi was weighted (McAllister, 1992). In 25 and 50 mg L^{-1} As treatment the concentration of As was analyzed in the AG medium after 2 weeks culture of *T. versicolor* and *T. harzianum* (Mingorance, 2002). AG medium with 500 mg L^{-1} As but without fungal culture was used as control. Ten replicates were used in these experiments.

The greenhouse experiments were carried out using eucalyptus (*Eucalyptus globulus* Labill) as test plants. Seeds were surface-sterilised with HgCl_2 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 filled with a mixture of sterilized sand:soil at a proportion of 1:1 (V:V). The soil, classified as an Andisol (Acruodiox Hapludands), is moderately acidic (pH 5.4) with a good drainage and water infiltration. Plants were grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 $\text{E m}^{-2} \text{ s}^{-1}$, 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 fed every week with 10 mL of a nutrient solution plus 50 mg L^{-1} of P (Hewitt, 1952). *G. deserticola* (Trappe, Bloss, and Menge) from the Instituto de Investigaciones Agrobiológicas de Galicia (CSIC) and *G. claroideum* Schenck and Smith from the Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera in Temuco (Chile), were the AM fungi used in the experiments. The AM inoculum was a root-and-soil inoculum consisting of rhizosphere soil containing spores and colonized root fragments of *Medicago sativa* L. in amounts of 8 g per pot, which were predetermined to have achieved high levels of root colonization. Uninoculated were given a filtrate (Whatman No. 1 paper) of the inoculum containing the common soil microflora, but free of AM fungal propagules.

Four treatments were used: (1) Uninoculated controls, (2) Soil pot inoculated with *T. versicolor* or *T. harzianum*, (3) Soil pot inoculated with *G. deserticola* or *G. claroideum*, and (4) Soil pot inoculated with *T. versicolor* or *T. harzianum* and *G. deserticola* or *G. claroideum*. Plants were inoculated at the time of transplanting (after 3 weeks of growth). The saprobe fungi were inoculated at the same time as *G. deserticola* and *G. claroideum*. Five replicate pots per treatment were used. As was applied to eucalyptus pots at the concentration of 0, 25, 50, 75, and 100 mg As kg^{-1} of soil pot.

Plants were harvested after 12 weeks 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 colonization was measured (Giovannetti and Mosse, 1980). In the second sample SDH (EC 1.3.99.1) activity was measured in fungal mycelia 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 under a compound microscope (Ocampo and Barrea, 1985). Phosphorous (P), nitrogen (N), magnesium (Mg), and iron (Fe) contents in the shoot and As content in the root and shoot of *Eucalyptus* were determined as described by Mingorance (2002). To determine total chlorophyll, *chlorophyll a* and *chlorophyll b*, the leaves were extracted with 80% (V:V) acetone at the same developmental stage (after 16 weeks transplanting) and measured according Lichtenthaler (1987).

The percentage values were arcsine transformed for statistical analyses. We studied the following three main factors and their respective levels as follows AM fungal (Control, *G. deserticola* and *G. claroideum*), Saprobe fungi (Control, *T. versicolor* and *T. harzianum*), As supply (in sand:soil pot at 0, 25, 50, 75, and 100 mg As kg^{-1}). We also analyzed the interaction among the main factors using a factorial analysis of variance (Sokal and Rohlf, 1981). Statis-

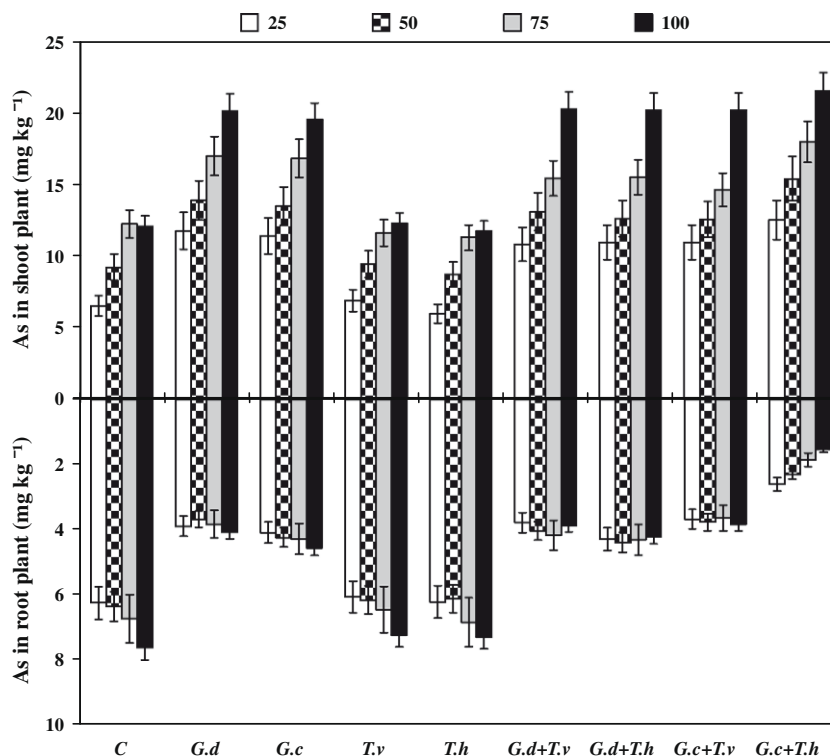


Fig. 4. As concentration in root and shoot of *Eucalyptus globulus* inoculated or not with AM or with the saprobe fungi in soil with different As concentrations (mg As kg^{-1} soil). C: Control; G.d: *Glomus claroideum*; G.c: *Glomus deserticola*; T.v: *Trametes versicolor*; T.h: *Trichoderma harzianum*; G.d+T.v: *G. deserticola*+*T. versicolor*; G.d+T.h: *G. deserticola*+*T. harzianum*; G.c+T.v: *G. claroideum*+*T. versicolor*; G.c+T.h: *G. claroideum*+*T. harzianum*.

tical analyses were conducted in SPSS software, version 11.0 (SPSS Inc., 1989–2001). Each experiment was repeated at least twice.

3. Results

The concentration of 25 and 50 mg L⁻¹ of As in the AG growth medium decreased to 13.2 ± 2.5 and 17.6 ± 4.1 mg L⁻¹ after culture of *T. harzianum* but no significant decrease in the concentration of As in the growth medium after culture of *T. versicolor* was observed.

The results of factorial analyses can be seen in Table 1. The saprobe fungi *T. versicolor* and *T. harzianum* were not significant on all analyzed variables. The shoots and root dry weight and chlorophyll content means for each factor and their interaction, illustrated in Figs. 1a, b and 2, respectively, shows that all doses of As decreased the shoots and root dry weight and chlorophyll content of the non-AM inoculated plants. *G. deserticola* and *G. claroideum* increased the shoots and root dry weight and chlorophyll content of *E. globulus* in all treatments. The inoculation of *T. versicolor* did not increase the shoots and root dry weight and chlorophyll content of plants colonized with *G. deserticola* and *G. claroideum*. However, shoots and root dry weight and chlorophyll content of *E. globulus* inoculated with *T. harzianum* together with *G. claroideum* were significantly higher than the other treatments.

As Fig. 3a shows the application of As increased the AM root length colonization of *E. globulus* by *G. deserticola* and *G. claroideum*. When *G. claroideum* was inoculated together *T. harzianum* the AM root length colonization was higher than the other inoculation treatments and was increasing as the concentration of As applied was increased. The percentage of AM mycelium with SDH activity of *E. globulus* inoculated with *G. deserticola* and *G. claroideum* was similar in all treatments tested except when *G. claroideum* was inoculated together *T. harzianum* which were higher than the other treatments (Fig. 3b). No significant effect of the different doses of As applied on the AM mycelial SDH activity were found.

The Fig. 4 shows that the concentration of As in shoots and roots of *E. globulus* increased as the doses of As in soil increased in all treatments. Similar As concentration either in shoot or in root of plants inoculated with *T. versicolor* or *T. harzianum* and the non inoculated controls were found. Higher concentration of As in root than in shoot of the non inoculated controls or plants inoculated with *T. versicolor* or *T. harzianum* were found. Lower As concentration in shoot and higher As concentration in root of AM colonized plants compared with those of non inoculated controls were found. The inoculation of the saprobe fungus *T. versicolor* did not increase the As concentration of shoot or root of AM colonized plants. However, *T. harzianum* decreased the concentration of As in the root of plants colonized with *G. claroideum*, being this concentration lower as the concentration of As applied to soil was higher.

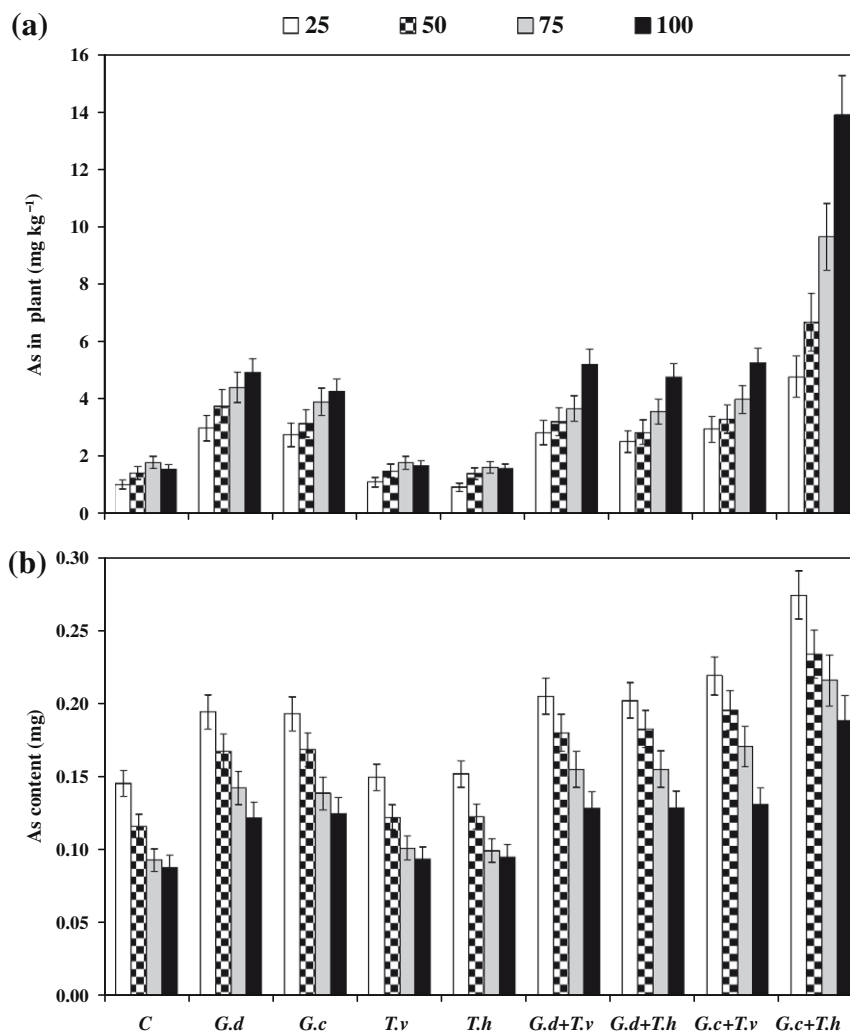


Fig. 5. As shoot/root (a) concentration ratio and As content (b) of *Eucalyptus globulus* inoculated or not with AM or with the saprobe fungi in soil with different As concentrations (mg As kg⁻¹ soil). C: Control; G.d: *Glomus deserticola*; G.c: *Glomus claroideum*; T.v: *Trametes versicolor*; T.h: *Trichoderma harzianum*; G.d + T.v: *G. deserticola* + *T. versicolor*; G.d + T.h: *G. deserticola* + *T. harzianum*; G.c + T.v: *G. claroideum* + *T. versicolor*; G.c + T.h: *G. claroideum* + *T. harzianum*.

Shoot to root (S/R) metal content ratios of *E. globulus* increased when higher doses than 25 mg kg^{-1} of As were applied to soil (Fig. 5a). Non significant differences in R/S ratios between plants inoculated with the saprobe fungi alone and the non inoculated controls were found. The S/R metal content ratios were significantly increased in AM colonized plants, being this increase higher when *G. clarioideum* was inoculated together with *T. harzianum*. The joint inoculation of AM and saprobe fungi increased the R/S ratio as the concentration of As applied to soil increased. The total As content of *E. globulus* decreased as the concentration of As applied to soil was increased, in all treatments (Fig. 5b). Non significant differences in the total As content between plants inoculated with the saprobe fungi alone and the non inoculated controls were found. The total As content was significantly increased in AM colonized plants, being this increase higher when *G. clarioideum* was inoculated together with *T. harzianum* (Fig. 5b).

All doses of As decreased the shoots P and Mg content of the non-AM inoculated plants (Fig. 6a and c). The shoot concentration of P, N, Mg, and Fe were similar in each treatment at any of the doses of As applied (Fig. 6a–d). The saprobe fungi did not increase the shoot concentration of P, N, Mg, and Fe but these elements were increased by both AM fungi. The inoculation of *T. versicolor*

together with *G. deserticola* or *G. clarioideum* did not increase the shoot Mg content of plants. When *T. harzianum* and *G. clarioideum* were inoculated together the highest shoot concentration of P, N, Mg, and Fe was observed.

4. Discussion

The presence of high concentrations of As decreased the shoot and root dry weight, chlorophyll and P and Mg content of AM colonized *E. globulus* plants, but these parameters were higher than in non-AM plants, indicating higher plant tolerance to As contaminated soil as a result of AM colonization (Chen et al., 2007). The increase in N, Mg, and Fe acquisition in AM plants could have contributed to an increase in total chlorophyll synthesis (Azizur Rahman et al., 2007). Therefore, the major production of total chlorophyll by *E. globulus* colonized with AM fungi indicates that they were more efficient at light absorption, affecting plant photosynthetic efficiency (Gil, 1995). Usually As did not affect or even increase the AM root length colonization of plants, but its mechanisms is unknown (Gonzalez-Chavez et al., 2002; Trotta et al., 2006). In our experiments As not only increased the percentage of AM root length colonization but also the metabolic activity

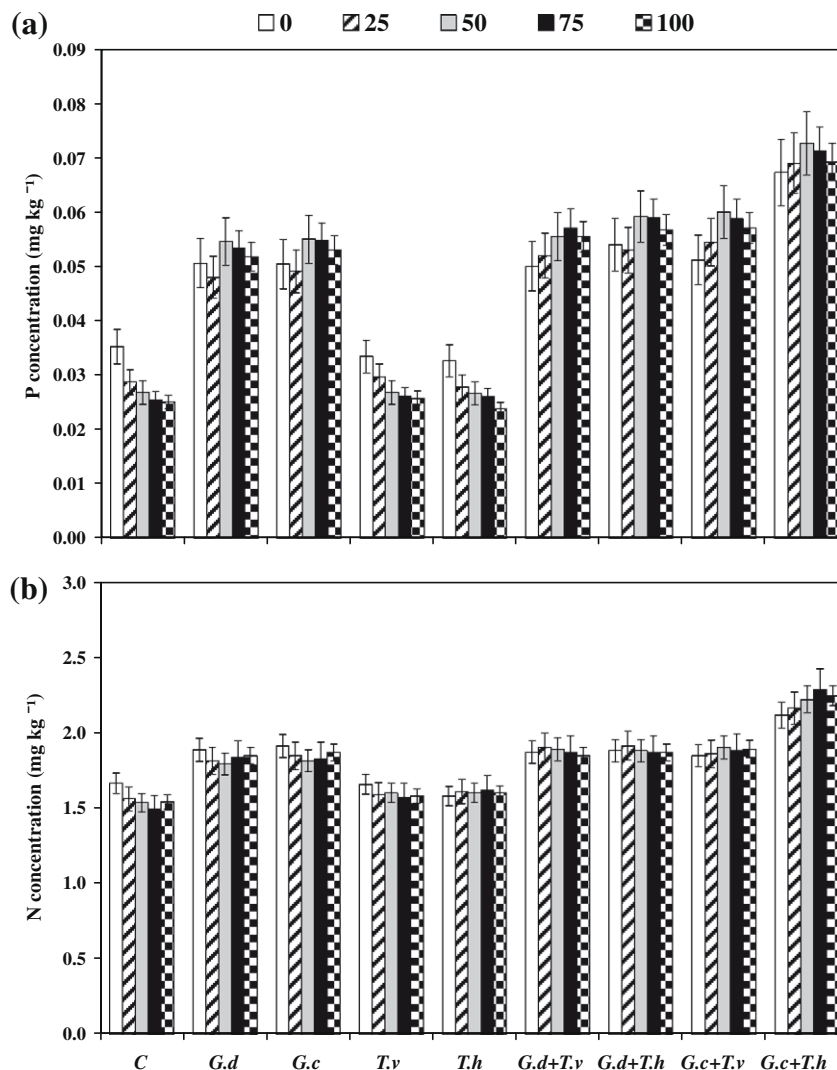


Fig. 6. P (a), N (b), Mg (c), and Fe (d) concentration of *Eucalyptus globulus* shoot inoculated or not with AM or with the saprobe fungi in soil with different As concentrations (mg As kg^{-1} soil). C: Control; G.d: *Glomus deserticola*; G.c: *Glomus clarioideum*; T.v: *Trametes versicolor*; T.h: *Trichoderma harzianum*; G.d + T.v: *G. deserticola* + *T. versicolor*; G.d + T.h: *G. deserticola* + *T. harzianum*; G.c + T.v: *G. clarioideum* + *T. versicolor*; G.c + T.h: *G. clarioideum* + *T. harzianum*.

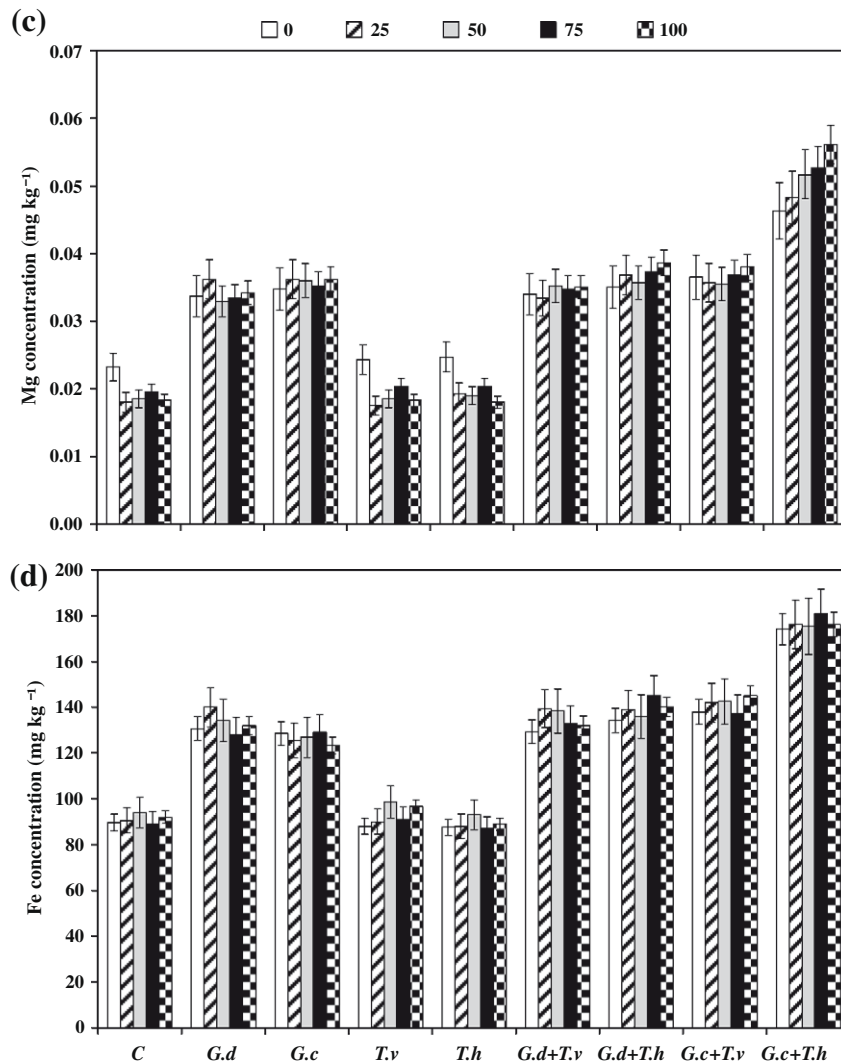


Fig. 6 (continued)

of the arbuscular fungi, measured as SDH activity of the fungal mycelium inside the root of *E. globulus*, indicating that a direct effect of As on the development of the AM fungi inside the root cannot be discarded.

Saprobe fungi such as *T. harzianum* was able to remove As from growth media and from soil (Harman et al., 2004). We found that the saprobe fungi *T. harzianum* was able to remove As ions from the growth media possibly by adsorbing them on their mycelia but *T. versicolor* did not removed this metal. However, both *T. versicolor* nor *T. harzianum* did not increased the shoot dry and root weight and chlorophyll content of *E. globulus* neither in the presence nor in the absence of As in soil. Nevertheless, *T. harzianum* was able to increase the shoot and root dry weight, chlorophyll content, the percentage of AM root length colonization and SDH activity of *E. globulus* colonized with *G. claroideum* in presence of all As concentrations applied to soil. The increase in N, Mg and Fe acquisition in plants inoculated with *G. claroideum* together with *T. harzianum* could have contributed to an increase in total chlorophyll synthesis (Azizur Rahman et al., 2007). These results indicate that the effect of *T. harzianum* on the resistance of *E. globulus* to As was mediated by its effect on the colonization and metabolic activity of the AM fungi. It is known the differential synergistic action of some soil saprobe fungi on AM colonization of root and on the growth of AM plants by different AM fungal species in presence of heavy metals (Arriagada et al., 2004, 2007).

AM fungi increased shoot As and P concentration of *E. globulus* shoot to higher level than the corresponding uninoculated controls. It is known that As was a P analogue and the main route of As uptake in plants is through the phosphate transporters as a P analogue (Meharg and Macnair, 1994; Meharg and Hartley-Whitaker, 2002). The improvement of P concentration in AM *E. globulus* shoots indicates that AM fungus increased the capacity to plant P uptake probably by increased synthesis of P transporters which also enhanced As uptake (Tu and Ma, 2003). The higher translocation factor in mycorrhizal plants may be due to a higher As translocation to the shoot and/or to a mechanism of As exclusion in the roots (Trotta et al., 2006). We found that higher total As content in mycorrhizal *E. globulus* than in the non-AM inoculated controls was observed, therefore, there was higher As uptake in AM colonized plants. Moreover, we observed that the As concentration in shoot of mycorrhizal *E. globulus* increased as the doses of As applied to soil increased but the As concentration in the root of all mycorrhizal plants was similar at all doses of As applied to soil. On the other hand, we also observed that the acquisition of the mineral nutrients N, Mg and Fe was higher in shoot of AM colonized plants than in non-AM inoculated controls. These results indicate that the higher translocation factor in mycorrhizal plants may be due to a higher As translocation to the shoots more than the exclusion of this metal by AM colonized roots. However, we found that *E. globulus* inoculated with *T. harzianum* together with

G. claroideum in the presence of As had higher metal concentrations in shoots and lower in roots than non-mycorrhizal plants, these results might suggest that mechanisms of As translocation to the shoots and/or As exclusion in the roots might not be discarded. However, as was pointed before, *T. harzianum* increased the percentage of AM root length colonization of *E. globulus* and the functionality of plants inoculated with *G. claroideum*, as shows the highest increase of the metabolical activity of the AM fungus in *E. globulus* root and the highest increase of the acquisition of mineral nutrients by the shoot. In this treatment, the As concentration increased in the shoot as the As concentration in the root decreased, moreover, the highest increase of total As content in the plant when these saprobe and AM fungi were inoculated together was also observed. These results, together with the highest translocation factor observed in plants inoculated with *T. harzianum* together with *G. claroideum* suggest an increase of translocation of As from root to shoot more than As exclusion in the roots by the AM fungi in *E. globulus*. Plants have their own protection mechanisms against metal toxicity. It is known that *E. globulus* was able to accumulated 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 on 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 inoculation with the AM fungus *G. claroideum* and the saprobe fungus *T. harzianum* enhanced the As accumulator capacity of *E. globulus*, increasing the transference of this metal from contaminated soil to roots and contributing to the clean-up ability of this plant of As contaminated soil.

5. Conclusion

The AM fungus *G. claroideum* increased of the tolerance of *E. globulus* to As. The saprobe fungus *T. harzianum* increased the development and functionality of the AM fungus, increasing in that way, the tolerance of AM *E. globulus* to As, the concentration of As in the shoot as the As concentration in the root decreased and contributed to the highest increase of total As content in the AM plant. The saprobe fungus also contributed to reach the highest translocation factor of As from root to shoot more than As exclusion in the roots by the AM fungus in *E. globulus*.

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