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The use of *Acacia saligna* inoculated with mycorrhizae in phytoremediation of lead-contaminated soils in the Kingdom of Saudi Arabia

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Acacia, Contamination; heavy metals, Arbuscular mycorrhiza

A B S T R A C T

In a greenhouse experiments, *Acacia saligna* seedlings inoculated with arbuscular mycorrhizae fungi (AMF) on growth and uptake of N, P, K, and Pb, grown in lead (Pb) contaminated soil were investigated. Inoculation of the host plants with AMF, *Glomus mosseae* and *G. deserticola* spores, significantly increased the dry weight, shoot length, total N, P and K as well as chlorophyll concentration in the trees. The protection of *Acacia* by AMF against the toxic action of Pb was more evident when seedlings were Inoculated with *G. deserticola* than with *G. mosseae*. The inoculation of *Acacia* with mycorrhizal fungi enhanced the amount of Pb absorbed and accumulated by *Acacia*. The results showed that inoculation of the host plants with AMF protects them from the potential toxicity caused by increased uptake of Pb. It seems that arbuscular mycorrhizae has a potential in phytoremediation of the heavy metal contaminated soils.

Introduction

Contamination of soil, water and food plants with toxic heavy metals due to mining activities in mining towns is still a major environmental and human health problem (Dzomba *et al*., 2012). While methods such as excavation and burial of contaminated soil at designated waste sites have been suggested such methods are not popular due to huge costs (Arriagada *et al*., 2005). There is still a need for researches in effective and affordable methods of counteracting this challenge (Antonious *et al*., 2012). Phytoremediation offers attractive options. It takes advantage of the fact that living plants can act as solar driven pumps that can extract and concentrate particular elements from the environment (Griffin *et al*., 2011). Harvested plant tissue that would have accumulated heavy metal contaminants may be easily and safely processed by drying, ashing or composting. Metals can then be reclaimed from the ashes. This generates recycling avenues and reduces the generation of hazardous waste (Gupta *et al*., 2008). Major sources of heavy metal pollution in the environment of the Kingdom are mostly anthropogenic, including mining activities, effluent discharges and waste disposal (Akan *et al*., 2005). In trace concentrations, many metals are essential to life and have several vital functions in biological processes but in excess the same metals can be toxic. It has been observed that even long after mining activities have ceased heavy metals continue to persist in the environment (Mupangwa, 2004). They can enter the food chain when taken up by plants during farming and eventually affect human health (Bhargavi and Sudha, 2011). Heavy metals poison animals and humans by disrupting cellular enzymes, which use nutritional minerals such as magnesium, zinc and selenium for their function. It is well known that these toxic metals replace these nutrients and bind their receptor sites, causing growth disturbance.

Many studies showed that, soil microorganisms are important in the recovery of disturbed and potentially toxic environments because they produce plant growth stimulating substances such as hormones and vitamins, immobilize heavy metals in the soil, bind soil particles into stable aggregates which improve soil structure, reduce erosion potential and can contribute to nutrient availability to plants (Antonious, 2012; Shetty *et al*., 1994). Therefore, metal-tolerant mycorrhizal inoculants are promising for the phytoremediation of metal-contaminated soils. Arbuscular mycorrhizal fungi (AMF) are known to improve plant growth on nutrient-poor soils and enhance their uptake of P, Cu, Ni, Pb, and Zn (Khan *et al*. 2000; Zhu *et al*. 2001). Despite the importance of the role that AMF play in plant interactions with the soil environment in general, and heavy metals in particular, relatively few studies have focused on their effect on metal-remediation efforts. In addition, many tree species growing on metal-contaminated soils possess mycorrhizae indicating that these organisms have evolved a tolerance to heavy metals and can play an important role in the phytoremediation of contaminated soils (Khan *et al*. 2000; Khan 2001).

Acacia saligna trees have a high capacity to grow in poor or marginal soils (Masvodza *et al*., 2013). This species are able to develop mycorrhizal symbiosis and they have a great tolerance to heavy metals (Arriagada *et al*., 2004). AM fungi were able to increase plants growth in heavy metal contaminated soils and found to reduce their harmful effect on plants (Khan, 2006 and Griffin *et al*., 2011).

The objective of the present work was to study the effect of soil Pb concentrations on the growth and mycorrhization of *Acacia saligna* seedlings inoculated with *G*. *mosseae* or *G*. *deserticola*; and to evaluate the influence of AM infection on the uptake of nutrients and Pb by mycorrhizal *Acacia* trees.

Materials and Methods

Experiments were carried out during the summer seasons of 2013/2014. The aim of the study was to investigate the potential effect of *Acacia* tree seedlings with or without MF in phytoremediation of lead contaminated soils. The growth and chemical constituents of *Acacia* trees were also studied. The soil was sandy and low in the organic matter content as indicated from the physical and chemical analysis results (Table 1).

Soil preparation

The soil was sieved (4 mm) and steam sterilized (100^{o}C for 1 hour for 3 consecutive days) to eliminate naturally occurring AMF. Appropriate amounts of lead nitrate aqueous solutions were added to obtain the lead (Pb) concentrations to 10, 100, and 1,000 mg Pb/kg soil. Control soil was left without metal addition. After mixing the soil with the added chemical solutions, soil moisture was adjusted to a field capacity by adding deionized water. The soils were then stored in a plastic boxes for 10 days with frequent mixing (once every 3 days) to allow thorough equilibration (Arriagada *et al*., 2005).

Isolation of AM Fungi

G. mosseae and *G. deserticola* were the AM fungi used. The AM fungal inoculum was a root and soil inoculum, consisting of rhizosphere soil containing spores (45 spores g^{-1} of soil) in amounts of 1 g per kg soil, which were predetermined to have achieved high levels of colonization (Wu *et al*., 2005). Soil was inoculated by spreading and mixing the inoculum thoroughly with the adjacent soil. Non-AM-inoculated plants were grown in soils free of AM fungus.

Plant culture and experimental design

Seeds of Acacia were germinated on May 2012 and 2013, in a greenhouse. The seeds were pretreated by immersing them in water at 100 °C and leaving them to soak overnight as the water cooled. Seeds were then surface sterilized with HgCl2 for 10 min and thoroughly rinsed with sterilized water. The imbibed seeds were sown in moist vermiculite and germinated at 27 °C. Most seeds germinated on the third day after sowing. The seedlings were transplanted to plastic pots (4 × 4 × 6 cm) and grown for 1 month, with regular watering. Uniform seedlings were then inoculated or not with MF and transferred to larger pots (30 cm high and 25 cm in diameter) containing the Pb polluted soils. The pots were randomly arranged on a greenhouse in two blocks.

Plants were grown in the greenhouse with supplementary light 400 E m-2 s-1, 400–700 nm, with a 16/8 h day/night cycle at 25/19oC. The temperature was controlled and 60% relative humidity was established and regulated by a humidifier. Plants were watered when needed and fed every week with Hoagland nutrient solution.

The experiment consisted of mycorrhizal and nonmycorrhizal treatments for all levels of Pb, in a factorial completely randomized block design (RCBD), with 6 replicates. Treatments used were: (1) Pb treatments: 0, 10, 100 or 1000 mg/kg (2) AMF inoculation with *G. mosseae*, AMF inoculation with *G. deserticola* or no AMF inoculation. Thus the whole experiment consisted of 72 pots, one seedling per pot.

Measurements and analyses

Mycorrhizae

At harvest (Six months after transplanting), the *Acacia* trees were separated from the soil. The *Acacia* root segments from each treatment were washed with deionized water to remove all particles adhering to the root surface, cleared for 20 min. in 10% KOH, and stained with lactophenol cotton blue (Phillip and Hayman, 1970). The stained root segments were mounted on glass slides (five pieces of 1-cm root per slide) for examination under a compound microscope with an eyepiece equipped with a crosshair that could be moved to randomly select positions. Mycorrhizal colonization was estimated for each sample by examining the stained pieces of the roots (Brundrett *et al*. 1996).

Dry weight

The harvested *Acacia* seedlings were dried at 70^{o}C until constant weight and the dry weights of roots and shoots were recorded. N, P and K contents of *Acacia* shoots were analyzed after digestion of samples with H_2SO_4+H_2O_2. Total N (Kjeldahal method) and P (molybdenum blue method) were determined and K was analyzed by flame photometry (Sparks *et al*., 1996). Pb concentration was measured after digestion of the air-dried plant samples with HNO_3+H_2O_2, followed by inductively coupled plasma atomic emission spectrometry (ICP-AES), as described by Mikanova *et al*. (2001).

Chlorophyll

Total chlorophyll of *Acacia* leaves was extracted with 80% acetone, 4 months after transplanting, and estimated as described by Wettestein (1957).

Statistical analyses

Data analyses were performed using SPSS statistical program (SPSS, Inc., Chicago, IL, U.S.A.). Analysis of variance was used to test whether treatment effects existed, followed by Duncan's multiple range test to identify means which differed significantly (at the 5% level) in mycorrhizal treatments and nonmycorrhizal control at each metal concentration (Little and Hills, 1978). The correlation coefficient between mycorrhizal dependency and Pb concentration was also analyzed.

Results and Discussion

Mycorrhizal colonization

At the end of the experiment, visual symptoms of chlorosis and necrosis in non-mycorrhizal plants were observed. *Acacia* reached a higher percentage of root colonization when inoculated with *G. deserticola* than with *G. mosseae* (Fig 1).

For *Acacia* growing in different Pb treatments, on inoculation with *G. mosseae*, the infection increased from 30% at 0 Pb to 55% at 100 mg/kg Pb but dropped to 25% at 1000 mg/kg Pb. While, on inoculation with *G. deserticola*, the infection increased from 37 to 65% at 100 mg/kg Pb but dropped to 30% at 1000 mg/kg Pb.

Plant analyses

Dry weights

Shoot dry weights of the nonmycorrhizal *Acacia* plants were decreased by Pb treatments (Fig 2). Generally, shoot dry weights decreased as metal concentrations increased. At high Pb concentrations (100 and 1000 mg/kg), shoot dry weights were significantly lower ($p < 0.05$) in nonmycorrhizal than in mycorrhizal plants.

When plants were not colonized by AM, there were no significant differences between the shoot dry weight of *Acacia* either when cultivated at low or middle concentration of Pb. The shoot dry weight of *Acacia* was increased in the presence of *G. mosseae* or *G. deserticola*. A significantly positive correlation ($r = 0.85$) between the shoot dry weight of *Acacia* and the percentage of AM root colonization was found.

Unexpectedly, the dry weights of mycorrhizal *Acacia* roots were significantly lower than those of the nonmycorrhizal trees (Fig 3). Therefore, root/shoot (R/S) ratios of mycorrhizal plants were lower than those of nonmycorrhizal ones (Fig 4).

Chlorophyll

Pb treatments decreased the chlorophyll content of the trees (Table 2). But, the AM-colonized plants had higher chlorophyll contents than the non-colonized plants, and a strong correlation between AM root colonization and chlorophyll content of *Acacia* leaves was found (*r* = 0.92). There were no significant differences in the chlorophyll content between the *Acacia* trees cultivated alone or in combination with *M. sativa*, although the later trees were slightly higher in their chlorophyll contents.

Nutrient contents

Data in Table (2) show the N, P and K concentrations in the shoot of *Acacia* grown under different Pb treatments with or without AM inoculation. Additions of Pb resulted in substantial decreases of nutrient concentrations in *Acacia* shoots in all treatments, particularly in nonmycorrhizal, compared with the mycorrhizal plants. It is clear that in the absence of Pb treatments, there were no significant differences in P or K concentrations between the nonmycorrhizal *Acacia* trees. Furthermore, both AM fungi increased the total N concentration in *Acacia* shoots. *G. deserticola* increased significantly the shoot P concentration in the trees (0.99%). The concentration of K was increased only in *Acacia* trees inoculated with *G. deserticola* (2.11%).

Pb concentration

Results obtained in Table (3) show that the inoculation with G. mosseae or G. deserticola enhanced the accumulation of Pb in *Acacia* plants and a significant correlation between both was found (r= 0.85).

At low levels of soil Pb (0 and 10 mg/kg), root Pb concentrations of the mycorrhizal and the nonmicorrhizal *Acacia* trees were more or less the same. At higher levels of soil Pb (100 and 1,000 mg/kg), Pb concentrations in mycorrhizal roots were significantly higher than those in the noninoculated trees. In the case of shoots, Pb concentrations in mycorrhizal *Acacai* were higher than those recorded in nonmycorrhizal plants at all levels of Pb. When mycorrhizal with *G. mosseae*, Pb contents were significantly lower in shoots, and higher in roots, compared with the *G. deserticola*-inoculated *Acacia* trees.

Colonization

The percentage of Am infection was significantly reduced at high levels of heavy metals in the soil. Nevertheless, Am fungi still function at low levels of heavy metal polluted soil. These results indicated that the low concentrations of heavy metals in the soil were not harmful to Am fungus *G. mosseae* and *G. deserticola*. This finding is in line with results of Vogel-Mikus *et al*. (2005) who proved the sensitivity of Am symbionts to heavy metal contaminated soil expressed as a reduction in spore germination, hyphal growth or root colonization.

The data of the present study clearly showed that mycorrhizal infection in the presence of alfalfa with *Acacia* increased more than that in the absence of alfalfa in heavy metal polluted soil. The percentage of AM colonization of *Acacia* and the beneficial action of the AM fungi was increased by the presence of alfalfa plants. The occurrence of AM fungi was confirmed in alfalfa roots growing in heavy metal–contaminated soils as reported by Gaur and Adholeya (2004). Alfalfa is yet another plant that has been found to be mycorrhizal with AMF in heavy metal–contaminated soils and showed dependency on mycorrhizae when soil is contaminated with Pb and other heavy metals (Chaudhry *et al*., 1998). Several other investigations have shown such synergistic effects of these co-culture methods on the dry weight and on heavy metal resistance of plants colonized by AM fungi (Arriagada *et al*., 2004). The presence of the alfalfa also contributed to the enhancement of shoot dry weight and N, P and K content of AM *Acacia* grown together with alfalfa. However, it is not possible to determine if it was a direct beneficial action of the alfalfa on the AM fungi or if alfalfa plants benefit AM fungi through their effect on *Acacia* roots or through the modification of its root exudates (Arriagada *et al*., 2005).

One of the questions posed in this study is whether mycorrhizae of different AMF would behave differently in the presence of heavy metals in soil. The sensitivity of AM endophytes to high amounts of heavy metals, expressed as a reduction or delay in its colonizing ability, has been observed (Karagiannidis and Nikolaou, 2000). This does not seem to be the case for the fungi used in this study because their ability to colonize increased when soil Pb concentration was increased.

Dry weights

The results of our study show that *Acacia* plants developed chlorosis and necrosis when were grown in heavy-metal-contaminated soil not inoculated with AM fungi but these plants resisted the adverse soil conditions when they were inoculated with AM fungi. High amounts of heavy metals in soil can decrease plant growth and nutrient uptake and it is known that AM fungi protect plants against toxic actions of heavy metals (Rabie, 2005). A correlation between the AM root length colonization and the shoot dry weight of *Acacia* was found. *G. mosseae* and *G. deserticola* contributed to a better development of the plants grown in contaminated soil since they increased the total N and other nutrients as well as chlorophyll concentration in *Acacia* shoots.

The dry weight of noninoculated *Acacia* roots was higher than that of the mycorrhizal plants. The reason may be found in a finer root system which can improve the absorption of water and mineral nutrients. Less photosynthesis products were likely to be needed to maintain the mycorrhizal root system, with a consequent benefit to the plant shoot growth, which should result in lower R/S ratios (Arriagada *et al*., 2005). We may speculate, also, that the mycorrhizal association could enhance the root to shoot metal translocation which may result in reducing root dry weight.

Chlorophyll

It has been found that the production of chlorophyll can significantly be reduced in plants when cultivated in soils contaminated by heavy metals (Ouzounidou, 1995). In agreement with results of Rabie (2005), the present study showed that the total chlorophyll production increased when the plant was colonized with AM fungi and both were strongly correlated. But, the chlorophyll content did not increase further when *Acacia* was cultivated together with *M. sativa*. The high total chlorophyll content of mycorrhizal plants apparently resulted directly in an increased photosynthetic efficiency of the plants. Earlier research suggested that some heavy metals disable the biosynthesis of chlorophyll (Ouzounidou, 1995).

Nutrient elements

It seems that the soil nutrient concentration and its availability are affected by Pb concentration. This, in turn, affected nutrient uptake by plants as reflected by shoot nutrient concentrations. For instance, results showed that P concentration decreased according to the increase in soil Pb concentration, which could be attributed to the possible P precipitation with added metals (Ma *et al*., 1997). Mycorrhizae increased the total N, P and K uptake in plant shoots. As compared with nonmycorizal trees, the two AMF, *G. deserticola* and *G. mosseae*, stimulated the nutrient uptake by *Acacia*. However, the former AMF had a better performance under Pb stress than the latter. This implies that *G. deserticola* could endure higher Pb, but in contrary, *G. mosseae* seems to be more sensitive to Pb. The protection of *Acacia* by AM fungi against heavy metals was more evident when this plant was grown as an intercrop with alfalfa than as a monoculture. It has been found that the AM fungus can mediate nutrient transfer between two plants through direct hyphal connection from root to root and, a competitive effect of recipient to donor plant has been observed (Bethlenfalvay *et al*., 1996). Although mycorrhizal shoot have nutrient concentrations significantly higher than those in nonmycorrhizal controls, it is generally believed that high metal concentrations would lower the availability of some nutrients to host plant (Tang *et al*., 2001), thus favoring the colonization of the roots.

The enhancement of shoot dry weight, and total P and K content of mycorrhizal *Acacia* when grown together with alfalfa and the lack of such enhancements in non-mycorrhizal *Acacia* plants suggest that there were no antagonistic or competitive effects by alfalfa on *Acacia*.

Table.1 Physical and chemical analyses of the soil used in the experiment

Physical properties	Chemical properties	
Particle size distribution:	$CaCO_3$ (%) 0.41	Soluble anions (meq/l)
Sand (%) 92.3	OM (%) 0.26	CO_3^{2-} 0.22
Silt (%) 6.2	Ec (dsm^{-1}) 0.53	HCO_3^- 0.86
Clay (%) 1.5	Soluble cations (meq/l)	Cl^- 1.83
Soil texture (Sandy)	Ca^{2+} 2.96	Avail. elements (mg/kg)
	Mg^{2+} 1.68	N 19.2
pH 8.01	Na^+ 2.04	P 8.3
	K^+ 0.21	Fe 2.4
Soil suspension (1 soil : 2.5 water)		

Table.2 Shoot N, P, K and total chlorophyll content of Acacia saligna grown in Pb contaminated soil and inoculated or non-inoculated with mycorrhizal fungi

Pb (mg/kg)	Mycorrhizae	N%	P%	K%	Chlorophyll (mg/gdwt)
	Control	3.85b	0.65b	1.65b	1.21b
0	G. mosseae	4.74a	0.76a	1.75a	1.75a
	G. deserticola	5.18a	0.88a	1.82a	2.11a
	Control	3.09b	0.66b	1.45b	1.07b
10	G. mosseae	3.02b	0.73a	1.56b	1.64a
	G. deserticola	3.65a	0.76a	1.86a	1.92a
	Control	2.22c	0.45c	1.17b	0.81c
100	G. mosseae	2.85b	0.49c	1.25b	1.30b
	G. deserticola	2.15b	0.67b	1.73a	1.71a
	Control	1.65c	0.14c	0.86c	0.61d
1000	G. mosseae	1.99c	0.27c	0.92c	0.78c
	G. deserticola	1.89b	0.56b	1.11b	0.82c

The different letters in the same column of a certain Pb concentration indicate a significant difference between treatments at LSD level of 0.05 according to Duncan's multiple range test.

Table.3 Metal (Pb) distribution in roots and shoots of *Acacia saligna* grown in Pb contaminated soil and inoculated or non-inoculated with mycorrhizal fungi

Pb (mg/kg)	Mycorrhizae	Pb (mg/kg)	
		Root	Shoot
	Control	135.5c	84.3b
0	G. mosseae	130.2c	76.8c
	G. deserticola	135.5c	66.2c
	Control	138.8b	82.5b
10	G. mosseae	137.4b	81.2b
	G. deserticola	140.1b	73.1c
	Control	164.2b	92.8b
100	G. mosseae	176.4b	90.4b
	G. deserticola	185.5b	83.5b
	Control	218.3a	142.6a
1000	G. mosseae	288.2a	135.3a
	G. deserticola	312.4a	115.2a

The different letters in the same column of a certain Pb concentration indicate a significant difference between treatments at LSD level of 0.05 according to Duncan's multiple range test.

Fig.1 Effect of different Pb concentrations on the percentage of mycorrhizal colonization on Acacia saligna inoculated or not with MF

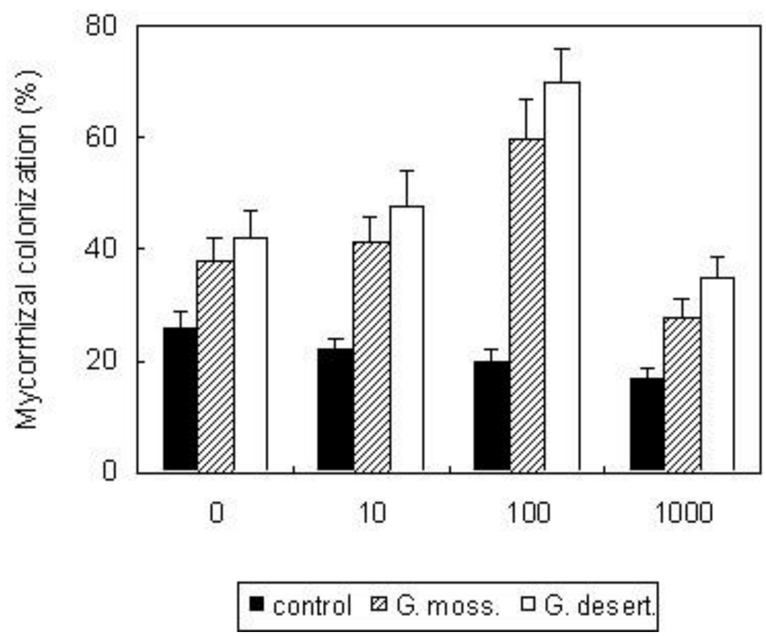


Fig.2 Effect of different Pb concentrations on shoot dry weight of Acacia saligna seedlings inoculated or not with MF

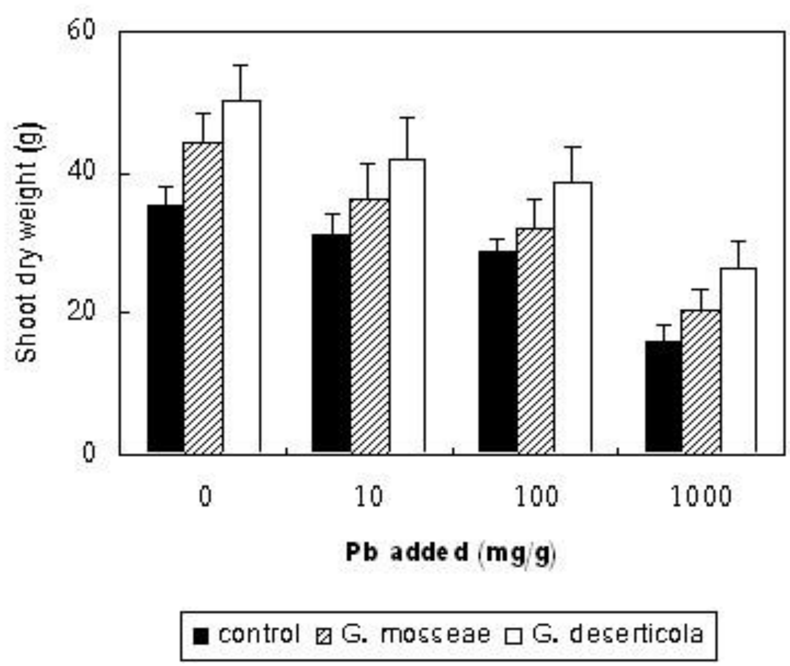


Fig.3 Effect of different Pb concentrations on root dry weight of Acacia saligna seedlings inoculated or not with MF

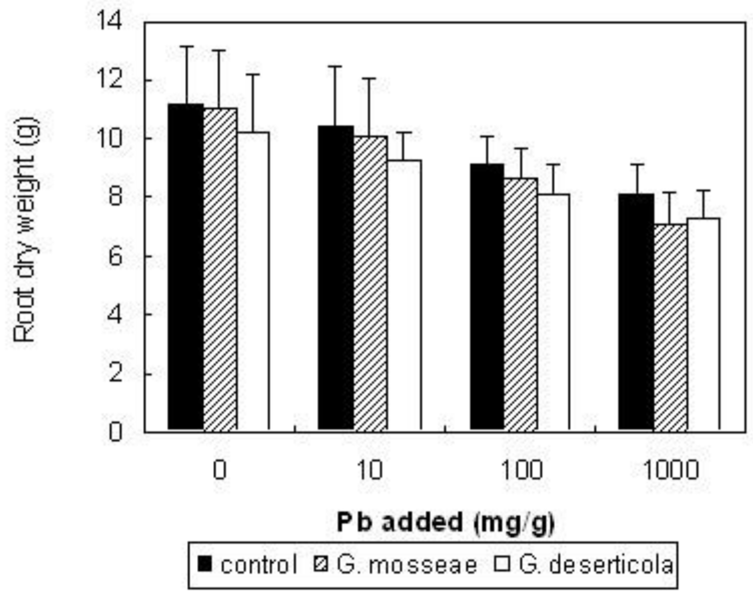
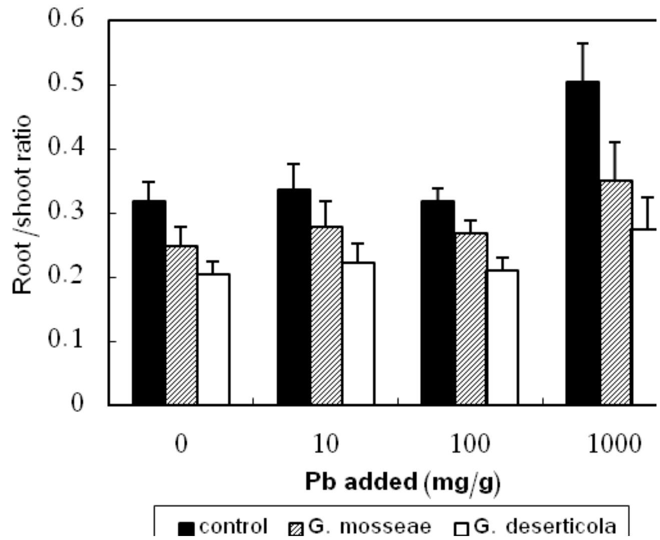


Fig.4 Effect of different Pb concentrations on root/shoot ratio of Acacia saligna seedlings inoculated or not with MF



Moreover, the increase of mycorrhization in *Acacia* when grown together with alfalfa, which is considered a high AM mycotrophic plant (Arriagada *et al*., 2004), indicated that AM colonized roots of alfalfa may act as an additional AM inoculum source to *Acacia* root and this AM increase may also contribute to the *Acacia* growth enhancement.

Heavy metal

The present study indicated that Pb concentration in shoots of *Acacia* can be modulated by mycorrhizae when growing in contaminated soil. The higher heavy metal concentration in mycorrhizal plants could be explained by the fact that Am infection increased plant uptake of metals by mechanisms such as enlargement of the absorbing area, volume of accessible soil, and efficient hyphal translocation (Yu *et al*., 2004).

The obtained results showed that, *G. deserticola* is different from *G. mosseae* in modulating movement of the heavy metal into the shoot, with the former being better at excluding Pb. In agreement with Joner and Leyval (2001), it seems that the AM fungus action may be species specific. Therefore, *G. deserticola* may be more suitable AM fungus than *G. mosseae* to remove high quantities of Pb from contaminated soils and a correlation between the level of AM colonization and the quantity of Pb absorbed by *Acacia* was found. In this context, Arriagada *et al*. (2005) found a positive effect of *G. deserticola* on plant growth and a higher tolerance of the mycorrhizal plants to Pb toxicity. Higher Pb accumulation in the roots than in the shoots of *Acacia* has been observed (Arriagada *et al*., 2004). This redistribution of heavy metals in the less metabolically active part of the plant might explain why AM fungi increased the content of heavy metals and enhanced the growth of *Acacia*.

Although the mechanisms of protection against heavy metals provided by mycorrhizae to their host plants are not clear, mycorrhizae appear to be protective by way of reducing the amount of the heavy metals accumulating in the shoot. The increase in plant biomass as well as growth parameters of mycorrhizal plants perhaps detoxify the potential effects of metals by dilution, precipitation or adsorption and thus limiting its delivery to shoot cells. A possible retention of heavy metals by the fungal mycelium involving adsorption to cell wall and fixation by polyphosphate granules (Galli *et al*., 1994) could also occur. In this connection, Rabie (2005) suggested that the symbioses provided the host with nutrients such as phosphorus which may be involved in plant Pb detoxification by means of molecules of phytates that can neutralize excess metals, or P can provide metabolic energy indirectly as ATP for possible compartmentalization within the cell vacuoles by means of molecules such as metallothioneins and phytochelatins (Assuncao *et al*., 2003).

Conclusions

It is concluded that inoculation with AMF protects host plant from the potential toxicity caused by Pb, but the degree of protection varies according to the fungus and host plant combination. It appears that the choice of AMF is a factor in using mycorrhiza in rehabilitation of heavy metal–contaminated sites. our results indicate that *E. rostrata* is suitable to grow and rehabilitate heavy-metal-polluted soils when inoculated specifically with *G. deserticola*. The association of *Acacia* with heavy-metal-resistant legume varieties can further help to improve the resistance of *Acacia* to heavy metals. It can be assumed that such legumes will also support the nitrogen nutrition of the *Acacia* provided that an effective Rhizobium–legume association can be established under such heavy-metal-polluted conditions. It appears that mycorrhiza will be important because it reduces the accumulation of heavy metals in mycorrhizal plants, thus offering a protective effect.

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