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Contribution of Mycorrhizae in Phytoremediation of Lead Contaminated Soils by *Eucalyptus rostrata* Plants

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Abstract: In a greenhouse trials, the effects of arbuscular mycorrhizae fungi (AMF) on growth and uptake of N, P, K and Pb by *Eucalyptus rostrata* L., grown with or without *Phaseolus vulgaris*, in lead (Pb) contaminated soil were investigated. Inoculation of the host plants with AMF, *Glomus deserticola* spores, significantly increased the dry weight, shoot length, total N, P and K as well as chlorophyll concentration in *Eucalyptus*. The inoculation with mycorrhizal fungi enhanced the amount of Pb absorbed and accumulated by *Eucalyptus*. The results showed that inoculation of the host plants with AMF protects them from the potential toxicity caused by increased uptake of Pb. At all cases, *E. rostrata* growth and other parameters performed better at the presence of *P. vulgaris* plants. It seems that arbuscular mycorrhizae has a potential in phytoremediation of the heavy metal contaminated soils, particularly in the presence of legumes.

Key words: Mycorrhizae · *Eucalyptus* · Heavy metal · Legumes · Contaminated soil

INTRODUCTION

Heavy metals can remain in the soil for a long time, with consequent problems for the human population. Besides, numerous heavy metals were found to be toxic to plant growth and production [1]. Soil microorganisms are important in the recovery of the potentially toxic environments and might be used in agriculture to remediate the polluted soil and improve nutrient availability to plants [2]. 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 [3, 4].

Many reports cleared that the use of heavy metal tolerant plants, to extract metals from the soil, for subsequent processing is technically efficient [3]. Unfortunately, most of the accumulative plants used belong to the family Brassicaceae which rarely form AM symbiosis, besides, these plants produce little biomass. Plants with higher biomass production such as trees are of more interest in soil phytoremediation [5]. Besides, many tree species growing on metal-contaminated soils possess mycorrhizae, Chaudhary [6], indicating that these organisms have evolved a tolerance to heavy metals and can play an important role in the phytoremediation of contaminated soils [3, 7].

Eucalyptus rostrata trees have a high capacity to grow in poor or marginal soils [8]. This species is able to develop mycorrhizal symbiosis and has a great tolerance to heavy metals [9]. AM fungi were able to increase *Phaseolus vulgaris* growth in heavy metal contaminated soils and found to reduce their harmful effect on plants [10]. Under field conditions, different plant species live together and hyphae of AM fungi interconnect the root systems of adjacent plants and can mediate nutrient transfer between plants [11].

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

MATERIALS AND METHODS

The experiments were carried out during 2006/2007 to investigate the effect of lead (Pb) and mycorrhyzae on the growth and chemical contents of *Eucalyptus* trees grown with *Phaseolus vulgaris* in sandy soil. The soil was low in the organic matter content as indicated from the physical and chemical analysis results (Table 1).

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Table 1: Chemical and physical analyses of the experimental soil

Chemical properties									
		Soluble cations ($meqL^{-1}$)			Soluble anions ($meq.L^{-1}$)				
pH*	Ece** (ms)	Na^+	Ca^{2+}	Mg^{2+}	HCO_3^-	SO_4^{2-}	Cl^- (%)	$CaCO_3$	O.M
8.2	2.06	11.0	4.35	2.5	2.99	11.7	7.6	4.0	0.23
Physical properties									
Fractions (%)									
Sand		Silt			Clay			Texture	
95.30		3.60			1.10			Sandy soil	

* pH of H_2O (soil : water = 2.5 : 1).

** ECe = Electric conductivity of the extract

Soil Preparation: The soil was sieved (4 mm) and steam sterilized (100°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 15 days with frequent mixing (once every 3 days) to allow thorough equilibration [12].

Isolation of AM Fungi: *G. deserticola* was 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) and colonized root fragments (80% root length) of *Phaseolus vulgaris* L. in amounts of 1 g per kg soil, which were predetermined to have achieved high levels of root colonization [13]. 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. Surface sterilized seeds of *P. vulgaris* were sown in 30 cm pots filled with the previously prepared soils and left to grow for 2 weeks.

Eucalyptus rostrata seeds were surface sterilized with $HgCl_2$ for 10 min and thoroughly rinsed with sterilized water and sown in moistened sand. After germination, uniform seedlings of 4-week old were transferred to the pots which were previously planted with *P. vulgaris* (2 weeks old). Plants were grown in a greenhouse with supplementary light 400 E m^{-2} s^{-1}, 400-700 nm, with a 16/8 h day/night cycle at 25/19°C. 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 four different levels of Pb concentrations, in a factorial completely randomized block design (RCBD), with five replicates. The plants used were: (1) *Eucalyptus rostrata* alone (one plant per pot) and (2) *E. rostrata* (one seedling) + *Phaseolus vulgaris* (3 plants) per pot. Treatments used were: (1) Pb treatments: 0, 10, 100 or 1000 mg/kg (2) AMF inoculation: non-inoculated pots, or inoculated with *G. deserticola*. Plants were inoculated with the AM fungi, once more, at the time of transplanting.

MATERIALS AND METHODS

Mycorrhizae: At the end of experiments (three months after transplanting), the *Eucalyptus* trees were separated from *P. vulgaris* plants. The *Eucalyptus* 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 [14]. 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 [15].

Dry Weight: The harvested *Eucalyptus* seedlings were dried at 70°C until constant weight and the dry weights of roots and shoots were recorded. N, P and K contents of *Eucalyptus* 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 [16]. 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*. [17].

Chlorophyll: Total chlorophyll of *Eucalyptus* leaves was extracted with 80% acetone and estimated as described by Wettestein [18].

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 Pb concentration [19]. The correlation coefficient between mycorrhizal dependency and Pb concentration was also analyzed.

RESULTS

Mycorrhizal Colonization: At the end of the experiment, visual symptoms of chlorosis and necrosis in non-mycorrhizal plants were observed, particularly when monocultured without *P. vulgaris*. *E. rostrata* reached a higher percentage of root colonization when inoculated with *G. deserticola* than without inoculation (Fig. 1). Generally, the trees cultivated together with *P. vulgaris* had a higher AM root colonization than those cultivated in the absence of *P. vulgaris*.

For *Eucalyptus* growing alone in different Pb treatments, 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. For *Eucalyptus* growing with *P. vulgaris*, it is clear that mycorrhization was much higher and Pb was less negative on mycorrhizal infection, than when grown alone. On inoculation with *G. deserticola*, mycorrhizal infection increased in low pb treatments from 0 to 100 mg/kg, reaching a maximum of 70% but dropped to 35% at 1000 mg/kg Pb.

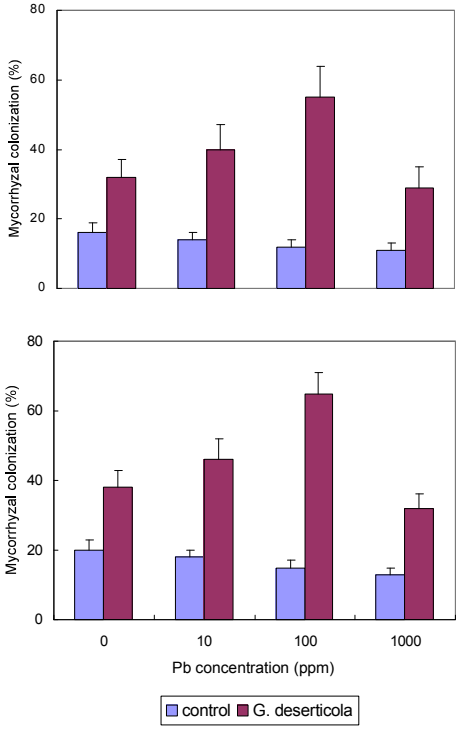

Fig. 1: Effect of different Pb concentrations on the percentageof mycorrhizal colonization on *Eucalyptus rostrata* grown alone (A) or with bean (*Phaseolus vulgaris*) plants (B)

Plant Analyses

Dry Weights: Shoot dry weights of the nonmycorrhizal *Eucalyptus* plants were decreased by Pb treatments (Fig. 2). Generally, shoot dry weights decreased as Pb 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.

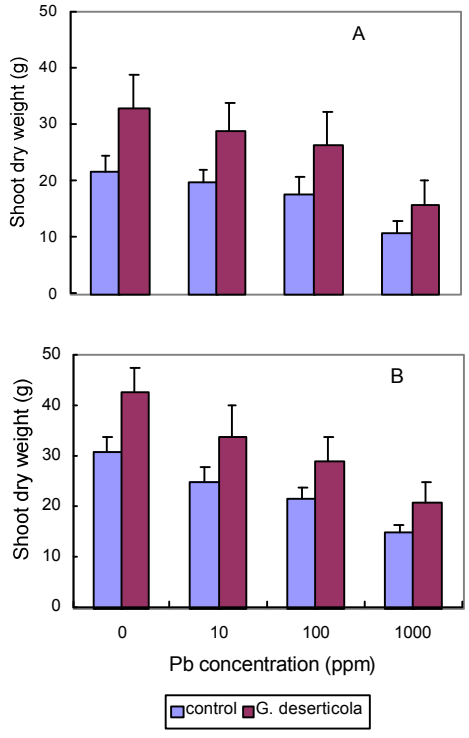

Fig. 2: Effect of different Pb concentrations on shoot dry weight of *Eucalyptus rostrata* grown alone (A) or with bean (*Phaseolus vulgaris*) plants (B)

Table 2: Shoot N, P, K and total chlorophyll content of *Eucalyptus rostrata* grown alone or as co-cultured with bean (*Phaseolus vulgaris*) grown in Pb contaminated soil and inoculated or non-inoculated with mycorrhizal fungi

Pb (mg/kg)	Mycorrhizae	N%	P%	K%	Chlorophyll (mg/gdwt)
Eucalyptus rostrata					
0	Control	2.95b	0.74b	1.55b	1.14b
	G. deserticola	3.98a	0.99a	1.92a	2.08a
10	Control	2.11b	0.75b	1.35b	1.06b
	G. deserticola	2.99a	0.85a	1.85a	1.85a
100	Control	1.83c	0.54c	1.07b	0.72c
	G. deserticola	2.26b	0.75b	1.73a	1.63a
1000	Control	1.05c	0.23c	0.85c	0.56d
	G. deserticola	2.00b	0.69b	1.08b	0.84c
Eucalyptus rostrata + *Phaseolus vulgaris*					
0	Control	3.55a	0.88a	1.78a	1.62a
	G. deserticola	4.88a	1.85a	2.11a	2.18a
10	Control	3.05a	0.84a	1.73a	1.30b
	G. deserticola	3.50a	1.62a	2.03a	1.91a
100	Control	2.32b	0.65b	1.51b	1.05b
	G. deserticola	3.11a	1.03a	1.85a	1.63a
1000	Control	1.55c	0.44c	1.04c	0.75c
	G. deserticola	2.52b	0.88a	1.45b	0.96b

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

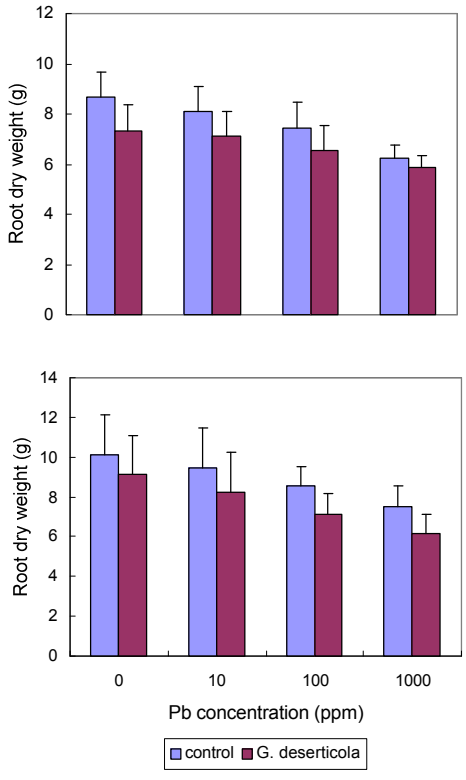

Fig. 3: Effect of different Pb concentrations on root dry weight of *Eucalyptus rostrata* grown alone (A) or with bean (*Phaseolus vulgaris*) plants (B)

When plants were not colonized by AM, there were no significant differences between the shoot dry weights of *Eucalyptus* either when cultivated alone or cultivated with *P. vulgaris* at the same concentration of Pb. The shoot dry weight of *Eucalyptus* cultivated alone or in the presence of *P. vulgaris* was increased in the presence of *G. deserticola*. A significantly positive correlation (r = 0.85) between the shoot dry weight of *Eucalyptus* and the percentage of AM root colonization was found. However, the positive effect of AM on the shoot dry weights of the trees was higher when cultivated together with *P. vulgaris* than when grew alone.

Unexpectedly, the dry weights of mycorrhizal *Eucalyptus* 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). The R/S ratio of mycorrhizal *Eucalyptus* was still lower even when cultivated together with *P. vulgaris*.

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 *Eucalyptus* leaves was found (r = 0.92). There were no significant differences in the chlorophyll content between the

Table 3: Metal (Pb) distribution in roots and shoots of *Eucalyptus rostrata* grown alone or as co-cultured with bean (*Phaseolus vulgaris*) grown in Pb contaminated soil and inoculated or non-inoculated with mycorrhizal fungi

		Pb (mg/kg)	
Pb (mg/kg)	Mycorrhizae	Root	Shoot
Eucalyptus rostrata			
0	Control	128.5c	80.3b
	G. deserticola	130.2c	60.2c
10	Control	136.8b	85.5b
	G. deserticola	139.7b	70.1c
100	Control	166.4b	95.8b
	G. deserticola	182.3b	80.5b
1000	Control	220.5a	140.6a
	G. deserticola	310.2a	105.2a
Eucalyptus rostrata + *Phaseolus vulgaris*			
0	Control	122.7c	75.2c
	G. deserticola	129.8c	60.5c
10	Control	128.7c	80.2b
	G. deserticola	134.5b	65.1c
1000	Control	151.8b	85.6b
	G. deserticola	166.2b	70.5c
1000	Control	211.4a	90.4b
	G. deserticola	260.2a	80.5b

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

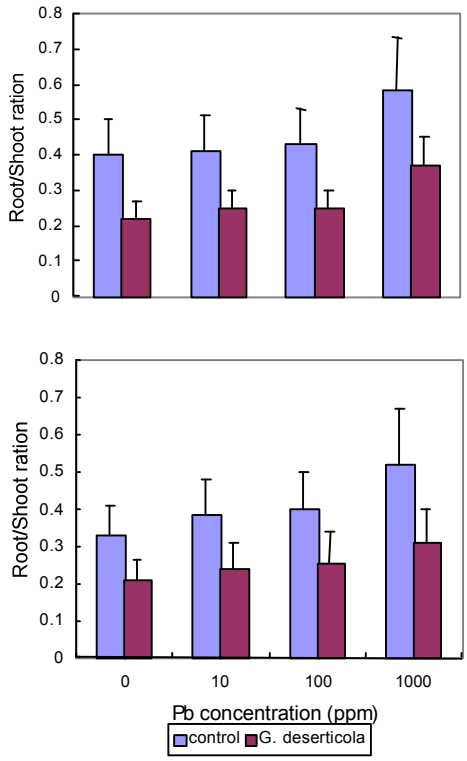

Fig. 4: Effect of different Pb concentrations on root/shoot ratio of Eucalyptus rostrata grown alone (A) with bean (Phaseolus vulgaris) plants (B)

Eucalyptus trees cultivated alone or in combination with *P. vulgaris*, although the later trees were slightly higher in their chlorophyll contents.

Nutrient Contents: Data in Table 2, show that, additions of Pb resulted in substantial decreases of N, P and K nutrient concentrations in *Eucalyptus* 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 *Eucalyptus* when cultivated alone and those cultivated in the presence of *P. vulgaris*. While N show significant increase in *Eucalyptus* trees co-cultured with *P. vulgaris*. Furthermore, AM fungi increased the total N concentration in *Eucalyptus* shoots when cultivated alone or with *P. vulgaris*. Also, *G. deserticola* increased significantly the shoot P concentration in the trees when cultivated alone (0.99%) or in combination with *P. vulgaris* plants (1.85%). The concentration of K was increased in *Eucalyptus* trees inoculated with *G. deserticola* and co-cultivated with *P. vulgaris* (2.11%).

Pb Concentration in Eucalyptus: Results obtained in Table 3, show that the inoculation with *G. deserticola* enhanced the accumulation of Pb in *Eucalyptus* 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 *Eucolyptus* trees were more or less the same, particularly when seedlings grew without *P. vulgaris*. At higher levels of soil Pb (100 and 1,000 mg/kg), Pb concentrations in mycorrhizal roots were significantly higher than those in the non-inoculated trees. Moreover, *P. vulgaris* enhanced the uptake of Pb by *Eucalyptus* roots. In the case of shoots, Pb concentrations in mycorrhizal *Eucalyptus* were higher than those recorded in nonmycorrhizal plants at all levels of Pb additions to soil. The Pb concentrations of mycorrhizal *Eucalyptus* were even higher when seedlings were co-cultivated with *P. vulgaris* plants.

DISCUSSION

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. deserticola*. This finding is in line with results of Vogel-Mikus *et al*. [20] 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 *P. vulgaris* with *Eucalyptus* increased more than that in the absence of *P. vulgaris* in heavy metal polluted soil. The percentage of AM colonization of *Eucalyptus* and the beneficial action of the AM fungi was increased by the presence of *P. vulgaris* plants. The occurrence of AM fungi was confirmed in *P. vulgaris* roots growing in heavy metal–contaminated soils as reported by Gaur and Adholeya [21]. *P. vulgaris* is yet another plant that has been found to be mycorrhizal with AM fungi in heavy metal–contaminated soils and showed dependency on mycorrhizae when soil is contaminated with Pb and other heavy metals [6]. Several other investigators 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 [9]. The presence of the *P. vulgaris* also contributed to the enhancement of shoot dry weight and N, P and K content of AM *Eucalyptus* grown together with *P. vulgaris*. However, it is not possible to determine if it was a direct beneficial action of the *P. vulgaris* on the AM fungi or if *P. vulgaris* plants benefit AM fungi through their effect on *Eucalyptus* roots or through the modification of its root exudates [22]. The sensitivity of AM endophytes to high amounts of heavy metals, expressed as a reduction or delay in its colonizing ability, has been observed [23].

Dry Weights: The results of our study show that *Eucalyptus* 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 [24]. A correlation between the AM root length colonization and the shoot dry weight of *Eucalyptus* was found. *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 *Eucalyptus* shoots.

The dry weight of noninoculated *Eucalyptus* 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 [22]. 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 [25]. In agreement with results of Rabie [24], 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 *Eucalyptus* was cultivated together with *P. vulgaris*. 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 [25].

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[26]. Mycorrhizae increased the total N, P and K uptake in plant shoots. As compared with nonmycorizal trees, *G. deserticola*, stimulated the nutrient uptake by *Eucalyptus* under Pb stress. This implies that *G. deserticola* could endure higher Pb. The protection of *Eucalyptus* by AM fungi against heavy metals was more evident when this plant was grown as an intercrop with *P. vulgaris* 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 [11]. 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 [27], thus favoring the colonization of the roots.

The enhancement of shoot dry weight and total P and K content of mycorrhizal *Eucalyptus* when grown together with *P. vulgaris* and the lack of such enhancements in non-mycorrhizal *Eucalyptus* plants suggest that there were no antagonistic or competitive effects by *P. vulgaris* on *Eucalyptus*. Moreover, the increase of mycorrhization in *Eucalyptus* when grown together with *P. vulgaris*, which is considered a high AM mycotrophic plant [9], indicated that AM colonized roots of *P. vulgaris* may act as an additional AM inoculum source to *Eucalyptus* root and this AM increase may also contribute to the *Eucalyptus* growth enhancement.

Heavy Metal: The present study indicated that Pb concentration in shoots of *Eucalyptus* 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 [28].

The obtained results showed that, *G. deserticola* is different in modulating movement of the heavy metal into the shoot [29]. It seems that the AM fungus, *G. deserticola*, a suitable AM fungus 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 *Eucalyptus* was found. In this context, Arriagada *et al*. [22] 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 *Eucalyptus* has been observed [9]. 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 *Eucalyptus*.

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 [30] could also occur. In this connection, Rabie [24] 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 [31].

CONCLUSIONS

It could be concluded that inoculation with AM fungus protects host plant from the potential toxicity caused by Pb. The obtained results indicate that *E. rostrata* is suitable to grow and rehabilitate heavy-metal-polluted soils when inoculated with *G. deserticola*. The association of *Eucalyptus* with heavy-metal-resistant legume varieties can further help to improve the resistance of *Eucalyptus* to heavy metals. It can be assumed that such legumes will also support the nitrogen nutrition of the *Eucalyptus* 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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