Ability of *Glomus mosseae*-Alfalfa (*Medicago sativa L.*) Association for Heavy Metal Phytoextraction from Soil

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Abstract

A pot experiment was conducted to determine the phytoextractive ability of alfalfa plants both inoculated (I) and non-inoculated (I0) with *Glomus mosseae* under different combinations of heavy metal pollution. Alfalfa inoculated and non-inoculated plants were exposed to Cadmium (Cd), Lead (Pb), Cobalt (Co), Cd*Pb, Pb*Co and Cd*Pb*Co in a factorial experiment. The heavy metal concentrations in the leaves, stems, shoots and roots were measured. In inoculated and non-inoculated plants, contamination concentration in shoots was higher than in root. Findings indicated that in the triple metal treatment (Cd*Pb*Co) inoculated plants were preferred. This showed that *G. mosseae* tolerated intensive contamination and transferred contaminants to alfalfa shoots. These results suggest that alfalfa inoculated plants are potentially suitable for phytoextraction of heavy metals in multiple heavy metal stress.

Keywords: Alfalfa, Heavy metals, Mycorrhizal fungus, Phytoextraction.

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Introduction

Heavy metal contaminated soils due to human activity act on human health as the contaminants elements can enter into the food chain (Naidu et al., 1996). Cd is one of the nonessential and most phototoxic trace element contaminants (Das et al., 1997). Pb is the most persistent metal that can remain in the soil for up to 150-500 years (Nanda-kumar et al., 1995). Co in nature has a very slight concentration (ATSDR, 2000). Whereas low amounts of these elements are required for humans and animals, higher Co concentrations are toxic for organisms (Nordberg, 1999) and can affect on heart health (ATSDR, 2000).

Mycorrhizal fungi are one of the most important symbiosis micro organisms which can help plants in nutrient uptake and under heavy metal stress conditions (Smith and Read, 1997). Mycorrhizae stimulate the roots’ potential for nutrient and metal ion uptake (Khan et al., 2000). Several genotypes of arbuscular mycorrhizal fungi (AMF) are adapted to heavy metal stress and are able to survive in the contaminated soils (Del Val et al., 1999). Investigations show that AMF improves tolerance to heavy metals and increases plant growth in the soil (Liao et al., 2003; Vivas et al., 2003). Scientists’ findings have indicated that AM can increase heavy metal concentration (Joner and Leyval, 1997; Weissenhorn and Leyval, 1995; Liao et al., 2003), while other researchers found that AM decreased Cu, Zn (Heggo et al., 1990; Xiong, 1993) and Mn in plants (Xiong, 1993). Generally, the role of AM in the uptake of metals by roots is not clear and depends on the type of metal and the plant species (Li et al., 2004). Galli et al., (1994) suggested mycorrhizae protect root plants against heavy metal toxicity. This role is confirmed by Marschner (1995) and Leyval et al., (1997).

The role of AM in Cd stress reduction was investigated by Rivera-Becerril et al. (2002). These researchers have revealed G. intraradices were not exposed the heavy metal contaminants, but reduced Cd toxicity in Pisum sativum L. Also, Joner and Leyval (1997) reported extra radical hyphae in G. mosseae that translocated Cd from the soil into subterranean clover roots. Use of G. mosseae and G. macrocarpum in land contaminated with Zn and Pb showed that mycorrhizal plants of Anthyllis cytisoides and Lygeum spartum under low concentration conditions had Pb and Zn concentrations equal to or more than non-mycorrhizal ones. But, at higher concentrations, plants inoculated with G. macrocarpum had equal or more Pb and Zn concentration than control (Diaz et al., 1996).

As the behaviour of mycorrhizal fungi in polluted habitats is unpredictable and there is huge complexity in the plant-fungi relationship and increment of contaminations, more information and investigation are needed. In the present study, we investigated the ability of alfalfa inoculated plants with Glomus mosseae in the phytoextraction of heavy metals (Cd, Co, Pb) and the role of G. mosseae in distribution of these metals in the plant.

Material and Methods

A pot experiment was set up in a 2×8 factorial completely randomised design, with four replicates in 2007 at the Agricultural, Medical and Industrial Research School of the Nuclear Energy Organization, Karaj, Iran. The first factor was inoculation (I) or non-inoculation (I0) with a G. mosseae inoculum and the second factor consisted of seven levels of contamination (Co =50 mg/kg dried soil, Cd =8 mg/kg dried soil, Pb =400 mg/kg dried soil, Co*Cd, Cd*Pb, Pb*Co and Pb*Co*Cd) plus a control treatment (C) which was uncontaminated. A sample of clay loamy soil from the surface of the soil horizon (0-20 cm) was used. The physical and chemical traits of the soil were: pH =7.91, organic carbon =1.48%, total nitrogen=0.15%, total Co content =51.91 mg/kg dried soil, total Cd content =8.5 mg/kg dried soil and total Pb content =436 mg/kg dried soil.

The soil was air dried, sieved and filled into pots of 30 cm height and 30 cm diameter (10 kg soil/pot).
The heavy metal salts used in this study included CoSO$_4$ for Co, CdCl$_2$ for Cd and Pb(NO$_3$)$_2$ for Pb. The soil contamination was performed before planting by adding the calculated amounts of salt formed of heavy metals dissolved in distilled water and mixed throughout the soil profile. They were allowed to stabilise for 15 days. Mycorrhizal treatments were inoculated with 50 g per pot $G$. mosseae inoculum in sandy substrate. In our pre-test, $G$. mosseae revealed maximum symbiosis with alfalfa that four inoculums such as $G$. intraradices, $G$. mosseae, $G$. etunicatum, and mixed inoculums ($G$. mosseae, $G$. fusciculatum and Gigaspora margarita) were evaluated (Rezvani et al., 2007). Inoculums were mixed with the 5 cm upper surface of pot soil. Alfalfa seeds treated with Sinorhizobium meliloti before planting.

After emerging, plants were thinned to reach a plant density of five plants per pot. During the trial, tap water was used as water source for the plants. Plants were harvested about five months after germination at the early stage of flowering and were cut from the soil surface of pots. Roots were removed from pots and washed with tap water and then sub samples were taken for evaluation of mycorrhizal colonization. Aboveground materials separated into stems and leaves and washed with distilled water. Total plant material was placed in the oven at 70 $^\circ$C for 48 hours. Dried plant samples were then ground.

**Chemical analyses of plant samples**

Ground samples were digested in 10 ml nitric acid according to the microwave technique until clear and diluted to 25 ml with deionised water (Brooks, 2002). For heavy metal analysis, an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Variant-Liberty 150AX Turbo) was used (Brooks, 2002).

**Mycorrhizal Colonization Index**

The colonisation of each plant was evaluated on fifty root samples. The roots were stained according to the modified method of Phillips and Haymann, (1970) and then evaluated by the Grid Line Intersect Method (Giovanetti and Mosse, 1980).

**Statistical Analysis**

The data were statistically analysed using the General Linear Models procedure of SAS program (Version 8, SAS institute Inc.). All the data were subjected to two-way analysis of variance and differences were considered to be significant at $P<0.01$. Significant differences between means were separated by the Duncan’s Multiple Range Test ($P<0.01$).

**Results**

**Shoot and Root Biomass**

Alfalfa plants inoculated with $G$. mosseae and grown in metal contaminated soil produced significantly higher shoot and root biomass than corresponding non-inoculated ones (Table 1; Figures 1a and 1b). Enhancement of plant biomass allocation to shoot was induced by $G$. mosseae in alfalfa grown in contaminated soil.

**Heavy Metal Concentrations**

**Leaf**

Results of Duncan’s multiple range test revealed that leaf Cd concentration in the I0CoCd treatment was highest (Table 1; Figure 2a). Also, Figure 1a showed in three metal contaminated pots $G$. mosseae–association with alfalfa had a higher concentration of Cd in leaf than non-inoculated plants. I0Co treatment showed the greatest Co concentration in the leaf (78.17 mg/kg leaf DM) (Table 1; Figure 2b). In the Co contaminated pots, Co concentration in non-inoculated plants’ leaf was more than in inoculated plants’ leaf in dual metal pots. But in the triple metal contaminated pot (Co*Cd*Pb treatment), mycorrhizal plants had a higher leaf Co concentration than in non-mycorrhizal ones (Figure 2b).

A significant interaction between inoculation and contaminants was observed (Table 1). The maximum
amount of Pb accumulated in the I0PbCd treatment (Figure 2c). When the pots were contaminated with all three metals, namely Pb*Co*Cd, mycorrhizal plants had better ability in the accumulation of heavy metals in their leaves than non-inoculated alfalfa ones. We can conclude that mycorrhizae benefited alfalfa plants in three metal contamination (Pb*Co*Cd) due to their better nutritional state under conditions of high Pb availability.

**Stem**

There was a significant interaction between inoculation and contaminants (Table 1). Cd concentration of stem in the treatment of I0Cd was the highest (Figure 2d). The investigation indicated that interaction between inoculation and contaminants was significant (Table 1). The ICo treatment produced the highest amount of Co in the stem (Figure 2e). Non-inoculated plants that grew in soil contaminated with Pb (mean treatment of I0Pb) had highest amount of Pb in the stem (Table 1; Figure 2f). In three heavy metal contaminated treatments, inoculated plants with G. mosseae had higher concentrations of Cd, Co and Pb than non-inoculated ones (Figure 2d, 2e and 2f).

**Shoot**

The treatment of I0CoCd had the highest concentration of Cd (Table 2; Figure 3a). Co concentration in non-inoculated plants was higher than in plants inoculated with G. mosseae, (Table 2; Figure 3b). In Co contaminated pots, there was the maximum concentration of Co in the non-inoculated plants shoot but, in the dual contamination condition, there was less. In triple metal contaminated soil, inoculated plants had a higher concentration of Co than non-inoculated ones (Figure 3b). The I0PbCd treatment had the highest concentration of Pb in shoot (Table 2; Figure 3c). Inoculated plants with G. mosseae, in three metal contaminated pots had a greater concentration of Pb, Cd and Co in the shoot than other contaminated pots. Under field conditions, plants are often affected by more than one metal.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Shoot biomass</th>
<th>Root biomass</th>
<th>Leaf Cd</th>
<th>Leaf Co</th>
<th>Leaf Pb</th>
<th>Stem Cd</th>
<th>Stem Co</th>
<th>Stem Pb</th>
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<td>87.68</td>
<td>34.3</td>
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<td>44.77</td>
<td>249017</td>
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<td>2126</td>
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<td>Inoculation *Contaminants</td>
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<td>219131</td>
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<td>22669.7</td>
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<td>113.32</td>
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<td>48</td>
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<td>0.185</td>
<td>0.265</td>
<td>0.448</td>
<td>2.04</td>
<td>0.0043</td>
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<td>0.027</td>
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<td>C.V</td>
<td>5.99</td>
<td>6.6</td>
<td>0.55</td>
<td>4.08</td>
<td>1.16</td>
<td>7.09</td>
<td>16.62</td>
<td>4.29</td>
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n.s: non-significant.

**: Statistically significant at P<0.01.

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<th>df</th>
<th>Shoot Cd</th>
<th>Shoot Co</th>
<th>Shoot Pb</th>
<th>Root Cd</th>
<th>Root Co</th>
<th>Root Pb</th>
<th>Mycorrhizal colonization index</th>
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<td>0.879</td>
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n.s: non-significant.

**: Statistically significant at P<0.01.
Root
Root Cd concentration was influenced by different contaminants and inoculation (Table 2). I0CoCd treatment had the highest concentration of Cd in root (Figure 3d), with the highest concentration of cobalt in the M0PbCo treatment (Figure 3e). There is not so much investigation on the Co accumulator plant. The maximum concentration of Pb was produced in the I0Pb treatment (Table 2; Figure 3f). The root of inoculated plants had a higher concentration of Cd, Co and Pb than non-inoculated plants grown in the triple contaminated pots (Figure 3d, 3e and 3f). This is important for natural conditions and has a application for the clean-up of contaminated land since, in natural contaminated land, various contaminants usually occur together.

Mycorrhizal Colonization Index
The highest mycorrhizal colonization was established in the control treatment. In all the contaminated treatments, *G. mosseae* colonised alfalfa plants. The differences between the mean percentages were statistically significant (Figure 4). In highly contaminated soil (Pb*Cd*Co treatment) in this experiment, *G. mosseae* formed a symbiosis with alfalfa roots about 42%. It show the used inoculum tolerated hard condition and toxicity of Pb*Cd*Co.

Heavy Metal Distribution in Plant Organs
Heavy metal accumulation and distribution indicated that non-inoculated and inoculated plants translocated the greatest amounts of Cd, Co and Pb into the shoot. But, non-inoculated alfalfa plants had a higher concentration of heavy metals than inoculated plants, while heavy metal concentration in the mycorrhizal roots was higher (Figure 5).

Discussion
Symbiosis with *G. mosseae* increased plant biomass production in contaminated soil. The known beneficial effect of plant mycorrhization on biomass production has been observed (Smith and Read, 1997; Khan, 2005; Khan, 2006). AMF can improve plants growth in the contaminated land by induction of indole-3-acetic acid (IAA) production. Enhancement of P contents in plant tissues is attributed to its active uptake from soil and its translocation to plants by the AMF mycelium, with arbuscules being the main sites of host fungus transfer (Smith and Read, 1997).

Colonised *Thlaspi praecox* Wulfen. (Brassicaceae) plants with mycorrhizae showed significantly improved nutrients and a decreased Cd and Zn uptake, thus confirming the functionality of the symbiosis (Vogel-Mikus *et al.*, 2005). The major advantage of
arbuscular mycorrhiza is an enhanced supply of essential nutrients from the soil by extraradical mycelium. At the same time, colonisation by AMF frequently reduces plant uptake and/or the phytotoxic effects of soil heavy metals (Gildon and Tinker, 1983; Hetrick et al., 1994; Hildebrandt et al., 1999; Chen et al., 2003), although in some cases enhanced uptake of toxic metals may be observed (Killham and Firestone, 1983; Weissenhorn et al., 1995; Guo et al., 1996).

There are few reports on the effects of mycorrhizal fungi on Co uptake by plants. Mosse, (1973) reported VAM fungi assist the plants to absorb mineral nutrients from the soil, particularly low available elements like phosphorus (P), molybdenum (Mo) and cobalt (Co). One of the main objectives of our investigation was to verify whether mycorrhizal plants were protected or affected by excessive levels of Co in the soil. We recorded less Co in the leaf of mycorrhizal plants as compared with non-mycorrhizal plants growing at the mono (Co treatment) and dual (Co*Cd and Pb*Cd treatments) metal contaminated pots.

Mycorrhizae benefited alfalfa plants in three metal contamination (Pb*Co*Cd) due to their better nutritional state under conditions of high Pb availability. Such conclusions have been confirmed by Smith and Read (1997), Joner and Leyval (1997) and Anderade et al. (2004).

The ICo, IPb treatment produced the highest amount of Co and Pb in the stem. In three heavy metal contaminated treatments, inoculated plants had higher concentrations of Cd, Co and Pb than non-inoculated ones. Our result is similar to previous findings (Diaz et al., 1996; Galli et al., 1994; Leyval and Joner, 2001; Rivera-Becerril et al., 2002). An AMF contribution to metal tolerance mechanisms of host plants is not well understood and documented. A protection mechanism suggested by Galli et al., (1994) is the immobilisation of metals by intra and extraradical mycelium, preventing the translocation of metals to shoots. Joner and Leyval (1997) reported that metal transfer from fungi to plant is restricted by fungal immobilisation. This agrees with results of studies of element localisation, using EELS (electron energy loss spectroscopy) and ESI (electron spectroscopy imaging), in mycorrhizal roots of Pteridium aquilinum from soils with large doses of heavy metals, showing accumulation of metals within intracellular hyphae, mainly in phosphate-rich materials in the vacuoles (Turnau et al., 1993). Thus, the benefits of mycorrhizae may be associated with metal tolerance, and also with better plant nutrition. Thus in degraded and contaminated soils, which are often poor in nutrients and with low water holding capacities, mycorrhizae formation would be of great importance.

The I0CoCd treatment had the highest concentration of Cd in shoot. AM decreased the Cd uptake of the tobacco plants per unit of shoot biomass in both experiments and decreased the Cd accumulation in the shoots of the transgenic tobacco relatively to the non-transgenic tobacco (Janouskova et al., 2005). Arbuscular mycorrhiza represents an almost ubiquitous relationship between soil microflora and plants. The fungal symbiont increases its host’s uptake of nutrients and can improve its growth and resistance to environmental stresses (Smith and Read, 1997). Arbuscular mycorrhiza symbiosis can also modify the response of plants to excess heavy metal in soil (Leyval et al., 1997), e.g. increase the heavy metal tolerance of plants (Hildebrandt et al., 1999), increase (Rivera-Becerril et al., 2002) or decrease (Heggo and Angle, 1990) their heavy metal uptake per unit of biomass or reduce their heavy metal translocation from root to shoot (Loth and Hofner, 1994).

Plants inoculated with G. mosseae, in three metal contaminated pots, had higher concentrations of Pb, Cd and Co in the shoot than other contaminated pots. Under field conditions, plants are often affected by more than one metal. Baker et al., (1990), reported that cadmium (Cd) never occurs alone in the natural environment, but mostly as a “guest” metal in Plumbum (Pb)/Zn mineralization. Various interactions
can occur when plants are exposed to unfavourable concentrations of more than one metal. Such combined effects may be synergistic, antagonistic, additive, or independent. These interactions among metals obviously complicate studies of the effects of metals on plant growth and other processes (Sun et al., 2005). The association of G. mosseae-Alfalfa probably act as a hyperaccumulator species and enhanced heavy metal translocation from root into plant shoot for sequestration. In the shoot extra concentration of metals sequester in the vacuoles of cells by metallothioneins and phytochelatins complexes (Assuncao et al., 2003).

Our findings showed that G. mosseae reduced Cd movement from root into the shoot and inoculated plants with G. mosseae had a higher Pb concentration than non-mycorrhizal plants. The decreased Cd uptake in roots and shoots and Zn in roots of inoculated plants further confirm the protective role of AMF in metal polluted soils. In addition, the results suggest that AMF colonisation of alfalfa has the potential to reduce heavy metal uptake, especially at mono (Cd) and dual (Co*Cd and Cd*Pb) metal contents, thus changing the plants metal tolerance strategy. AMF may reduce metal uptake by limiting translocation to roots, either by binding of heavy metals on fungal cell wall components such as chitin and melanins, or sequestration of heavy metals in fungal investigation infection to G. mosseae increased Cd and Zn uptake to roots, however did not effect on the shoots (Dehn and Schuepp, 1999). Galli et al., (1994) suggested a possible retention of heavy metals by fungal mycelia involving adsorption to cell walls, thereby minimising metal translocation to the shoots. This hypothesis was corroborated by Joner et al., (2000) who demonstrated that AM mycelia had a high metal sorption capacity.

G. mosseae colonised alfalfa plants in the contaminated pots. There was mycorrhizal colonisation in the non-inoculated treatments, which can be rise from mycorrhizae native population. AMF isolates differ in their tolerance to heavy metal levels and other stresses that may be present in industrial wastes (Weissenhorn et al., 1993; Bartolome-Esteban and Schenck, 1994). In addition, they should resist competition with spontaneously appearing, or native, fungal strains, that might be less effective in stimulating plant growth. Application of high concentration of heavy metals and also multiple contaminants in many researches reduced colonization percentages (Joner and Leyval, 1997; Anderade et al., 2004; Citterio et al., 2005). In this experiment, contaminants concentration in the alfalfa shoot was 3-10 folds more than root. Heavy metal concentration in the mycorrhizal roots was higher. In the mono metal and dual metal condition the G. mosseae-alfalfa association was not more successful than non-inoculated alfalfa in phytoextraction of contaminants. With respect to this research finding, infection with G. mosseae ceased movement of heavy metals to the shoot and acted as inhibitor for heavy metal transition while it helps to plant association for heavy metal stress tolerant. This result is in agreement with other research works carried out (Liao et al., 2003; Vivas et al., 2003).

Generally, it should be considered that alfalfa has a substantial ability for phytoextraction and can be used for reclamation of contaminated soils. However, mycorrhizae could not form a proper association with alfalfa for mycorrizoremediation except in triple metal treatments. Overall, in the highly contaminated soils in this experiment that polluted pots with the triple metals, namely Cd*Co*Pb, mycorrhizae translocated the higher mass of metals to the shoot and therefore can be important in phytoextraction applied aspects. In turn, G. mosseae acts as a bioprotectant of plants in intensively contaminated land and helps them to survive it. Use of G. mosseae on multiple heavy metal contaminated soil may be a suitable approach for heavy metal phytoextraction that helps us in phytorestoration of polluted land, although the G. mosseae-alfalfa relationship and its mechanisms still require more investigation.
Figure 2. Mean comparisons of interaction between inoculation and contaminants based on Duncan’s multiple range test ($P<0.01$). I0: non-inoculated plants, I: inoculated plants, C: control. Same letters indicate non-significant differences between interactions.
Figure 3. Mean comparisons of interaction between inoculation and contaminants based on Duncan’s multiple range test ($P<0.01$). I0: non-inoculated plants, I: inoculated plants, C: control. Same letters indicate non-significant differences between interactions.
Figure 4. Mean comparisons of interaction effect between inoculation and contaminations based on Duncan’s multiple range test ($P<0.01$).

I0: non-inoculated plants, I: inoculated plants, C: control. Same letters indicate non-significant differences between interactions.

Figure 5. Heavy metals distribution in alfalfa root and shoot.
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