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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*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.

توانائی یونجه (*Medicago sativa* L.) و *Glomus mosseae* در برداشت گیاهی فلزات سنگین از خاک

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چکیده

جهت تعیین توانائی یونجه (*Medicago sativa* L.) در برداشت عناصر سنگین از خاک در شرایط تلقیح (I) و بدون تلقیح (I0) با میکوریزا (*Glomus mosseae*) در خاک آلوده به فلزات سنگین شامل کبالت (Co)، کادمیوم (Cd) ، سرب(Pb) و ترکیب دوتایی (Cd*Co، Pb*Co و Pb*Cd) و سه‌تایی (Co*Cd*Pb) در یک آزمایش فاکتوریل در قالب طرح کاملاً تصادفی در آزمایش گلدانی انجام شد. شاخص کلونی‌زایی میکوریزایی، بیوماس گیاه، غلظت و توزیع فلزات سنگین در ریشه، ساقه، برگ و اندام هوائی گیاه اندازه‌گیری شد. نتایج نشان داد در شرایطی که خاک در معرض آلاینده‌های سه‌گانه فلزات سنگین قرار داشت، گیاهان میکوریزایی یونجه دارای برتری نسبت به گیاهان تلقیح نشده در جذب فلزات سنگین بودند. این موضوع نشان می‌دهد که سویه *Glomus mosseae* آلودگی شدید خاک را تحمل نموده و توانست انتقال آلاینده‌ها به اندام هوائی گیاه را افزایش دهد. نتایج پیشنهاد می‌کند که تلقیح یونجه با *Glomus mosseae* ممکن است رهیافت مناسبی برای افزایش برداشت گیاهی فلزات سنگین در شرایط آلودگی چندگانه خاک باشد.

کلید واژه‌ها: یونجه، فلزات سنگین، قارچ‌های میکوریزا، برداشت گیاهی.

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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 cytisoiles* 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%, P =27.8 mg/kg, K =461 mg/kg, Fe =2.54 mg/kg, 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 pof 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. fasciculatum* 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° 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 Pd 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 1. Analysis of variance of some parameters of alfalfa.

Source of variation	df	MS							
		Shoot biomass	Root biomass	Leaf Cd	Leaf Co	Leaf Pb	Stem Cd	Stem Co	Stem Pb
Inoculation	1	87.68**	34.3**	220326**	1513.5 **	53799.0 **	2179 **	0.005 ns	15.25 **
Contaminants	7	15.03**	44.77 **	240017**	2462.8**	128012.75**	2126 **	1.61 **	218.82**
Inoculation *Contaminants	7	8.12 **	0.44**	219131**	923.23**	22669.7 **	2.41**	0.058 **	113.52**
Error	48	0.237	0.185	0.265	0.448	2.04	0.0043	0.018	0.027
C.V		5.99	6.6	0.55	4.08	1.16	7.09	16.62	4.29

n.s: non-significant.

**: Statistically significant at $P<0.01$.

Table 2- Analysis of variance of some parameters of alfalfa.

Source of variation	df	MS						
		Shoot Cd	Shoot Co	Shoot Pb	Root Cd	Root Co	Root Pb	Mycorrhizal colonization index
Inoculation	1	221898.7 **	1507.87 *	55625.81 **	341.56 **	16.51 **	0.879 **	0.054 **
Contaminants	7	240793.97 **	2562.99 **	132350.61 **	1333.15 **	79.94 **	2758.11 **	0.063 **
Inoculation *Contaminants	7	219759.81**	916.25 **	24779.46 **	172.98 **	16.16 **	248.79 **	0.049 **
Error	48	0.284	0.476	2.12	0.202	0.055	0.191	0.06
C.V		0.564	4.01	1.15	3.7	7.82	2.68	4.68

n.s: non-significant.

**: Statistically significant at $P<0.01$.

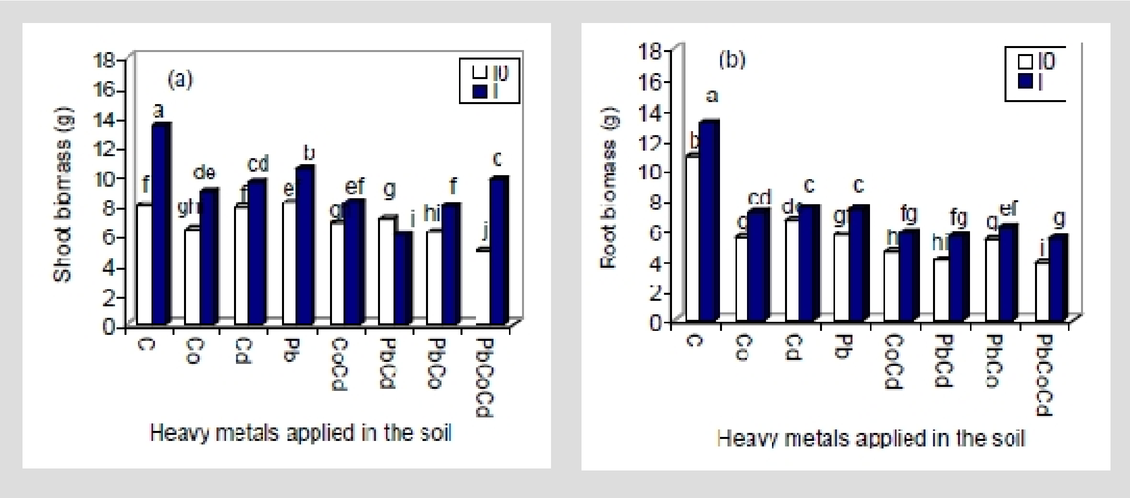

Figure 1. Mean comparisons of interaction effect between inoculation and contaminants based on Duncan's multiple range test (*P<0.01*). I0: non-inoculated plants, I: inoculated plants, C: control. Same letters indicate non-significant differences between interactions.

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).

Disscusion

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, I0Pb 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 Pd 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 metalothionieins 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, 1990). 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 phytoextaction 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 mycorrhizoremediation 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 phytoexctraction 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.

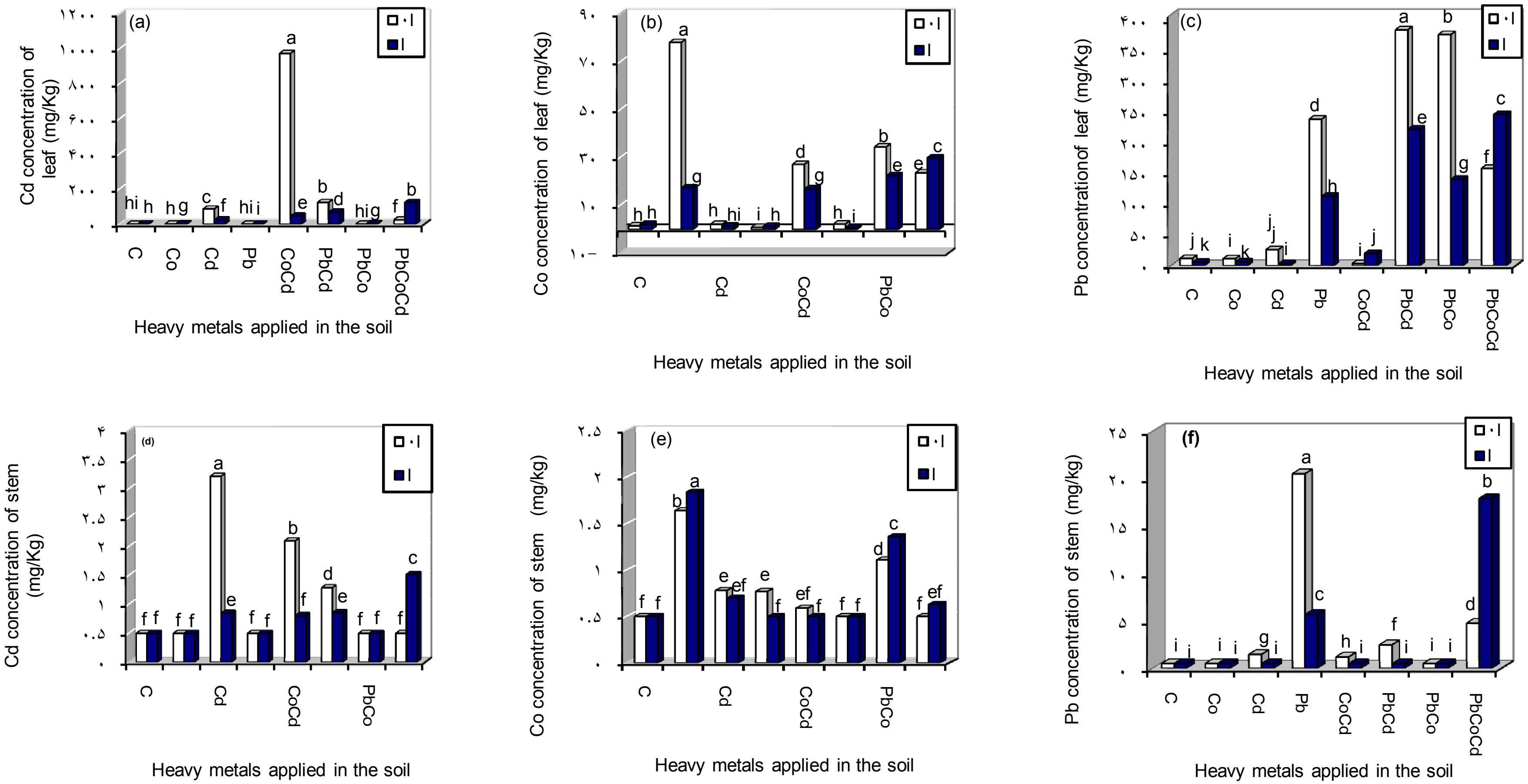

Figure 2. Mean comparisons of interaction between inoculation and contaminants based on Duncan's multiple range test (*P<0.01*). I0: non-inoculated plants, I: inoculated plants, C: control. Same letters indicate non-significant differences between interactions.

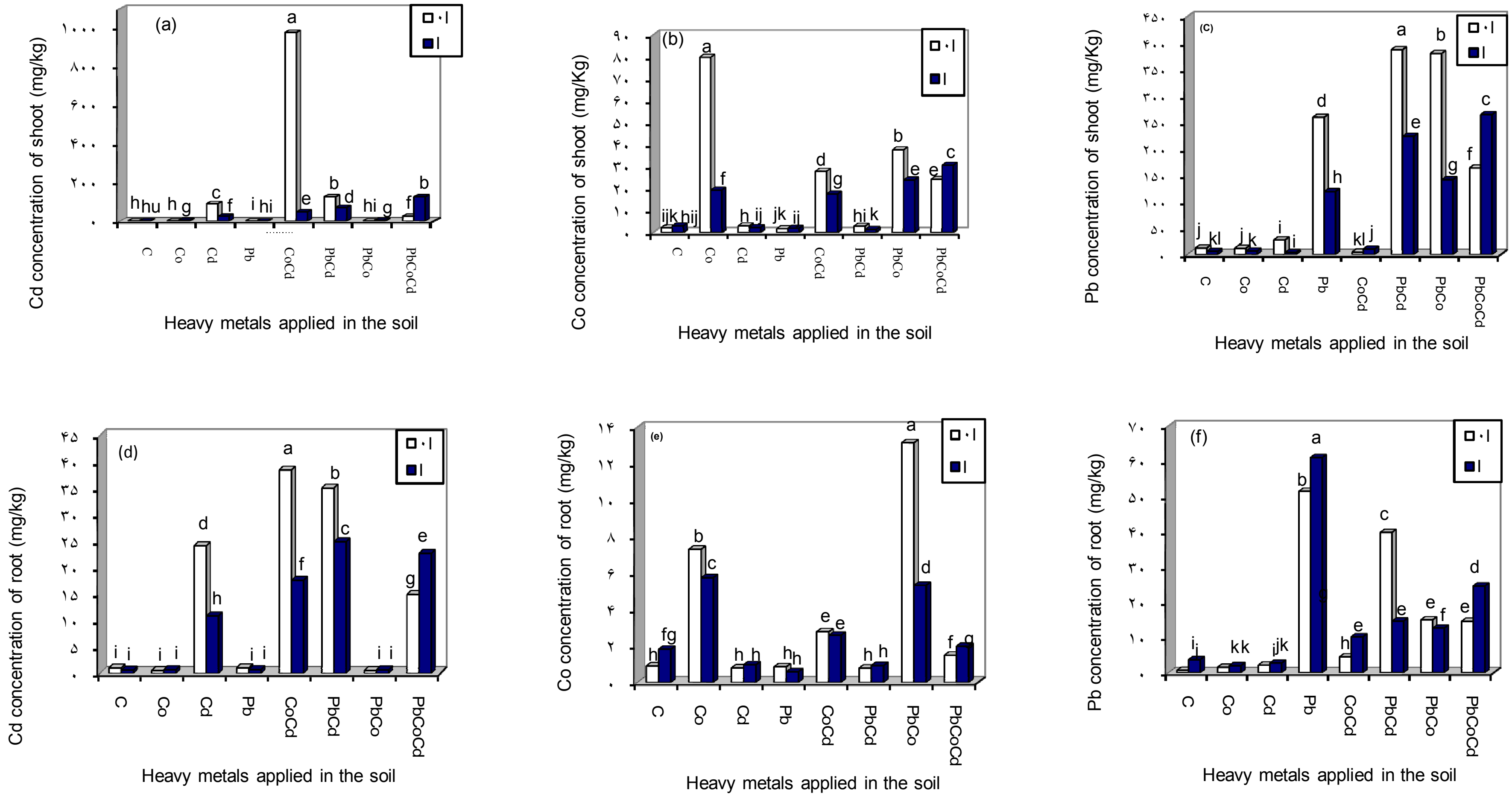

Figure 3. Mean comparisons of interaction between inoculation and contaminants based on Duncan's multiple range test (*P<0.01*). I0: non-inoculated plants, I: inoculated plants, C: control. Same letters indicate non-significant differences between interactions.

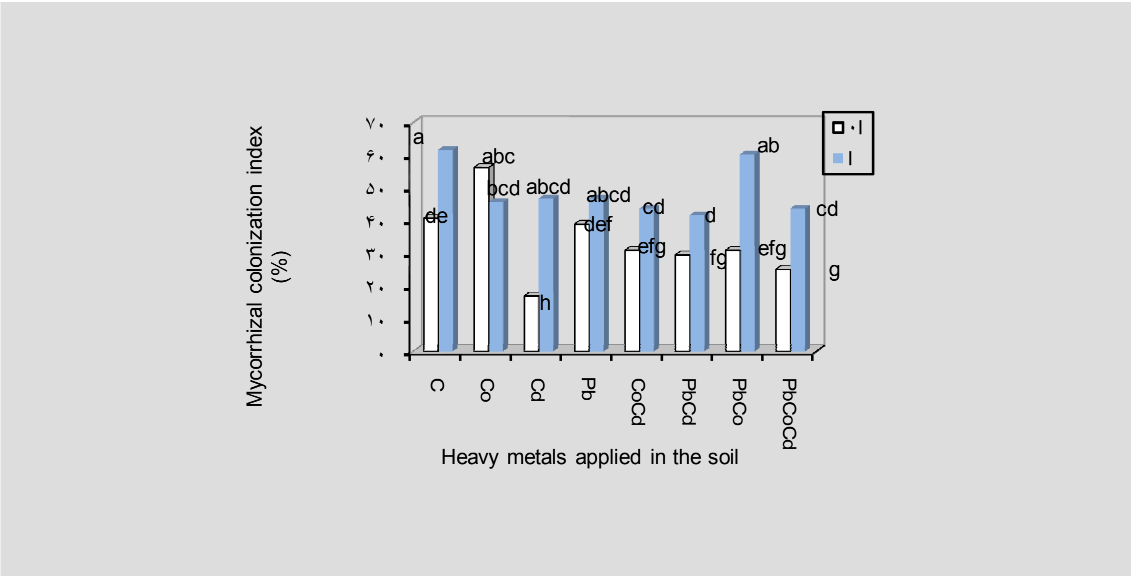

Figure 4. Mean comparisons of interaction effect between inoculation and contaminations based on Duncan's multiple range test (*P<0.01*). I0: non-inoculated plants, I: inoculated plants, C: control. Same letters indicate non-significant differences between interactions.

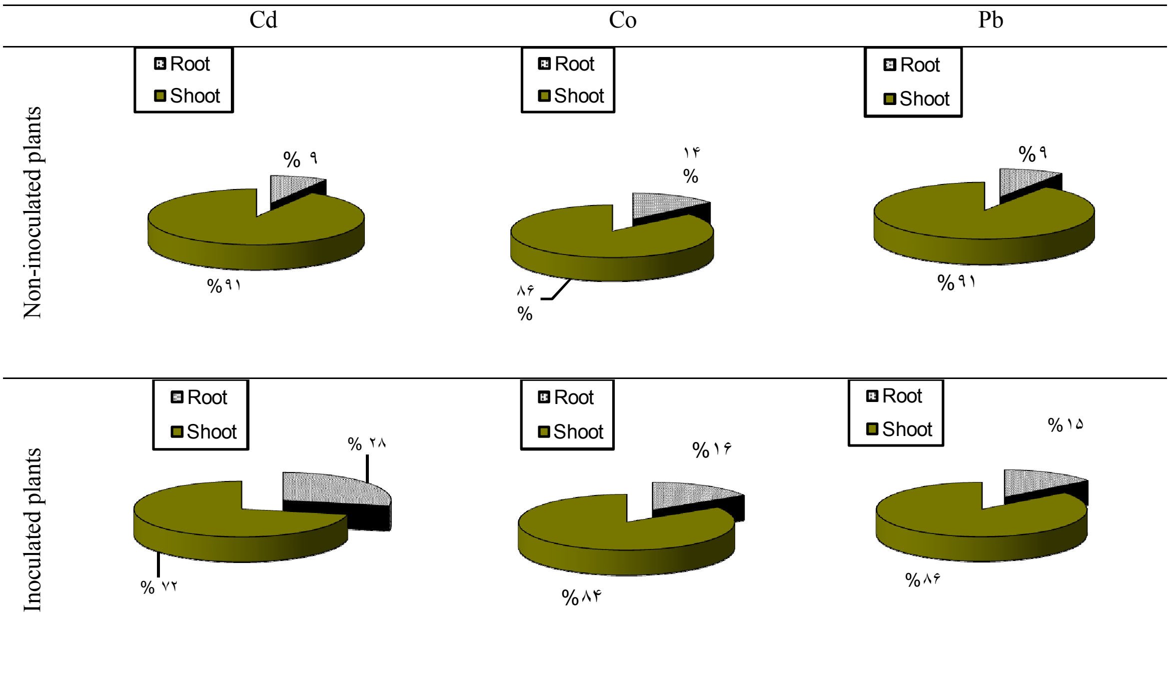

Figure 5. Heavy metals distribution in alfalfa root and shoot.

References

ATSDR (Agency for Toxic Substances and Disease Registry) (2000). ATSDR's toxicological profiles on CD-ROM. Florida: CRC Press.

Anderade, S.A.L., C.A. Abreu, M.F. De Abreu and A.P.D. Silveira (2004). Influence of lead addition on arbuscular mycorrhizae and Rhizobium symbioses under soybean plants. Applied Soil Ecology, 26: 123–13.

Assuncao, A.G.L., H. Schat and G.M. Aarts (2003). *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. New Phytologist, 159: 351-360.

Baker, A.J.M., K. Ewart, G.A.F. Hendry, P.C. Thorpe and P.L. Walker (1990). The evolutionary basis of cadmium tolerance in higher plants. 4th International Conference on Environmental Contamination, Barcelona. Edinburgh: CEP Consultants Ltd.

Bartolome-Esteban, H. and N.C. Schenck (1994). Spore germination and hyphal growth of arbuscular mycorrhizal fungi in relation to soil aluminium saturation. Mycologia, 86: 217– 226.

Brooks, R.R. (2002). Phytochemistry of hyperaccumulators. In R.R. Brooks (ed.), Plants that Hyperaccumulate Heavy Metals. New York: CAB International.

Chen, B.D., H.Q. Tao, P. Christie and M.H. Wong (2003). The role of arbuscular mycorrhiza in zinc uptake by red clover growing in a calcareous soil spiked with various quantities of zinc. Chemosphere, 50: 839-846.

Citterio, S., N. Prato, P. Fumagalli, R. Aina, N. Massa, A. Santagostino, S. Sgorbati and G. Berta (2005). The arbuscular mycorrhizal fungus *Glomus mosseae* induces growth and metal accumulation changes in *Cannabis sativa* L. Chemosphere, 59: 21-29.

Das, P., S. Samantaray and G.R. Rout (1997). Studies on cadmium toxicity in plants: a review. Environmental Pollution, 98: 29–36.

Dehn, B. and H. Schupp (1990). Influence of VA mycorrhizae on the uptake and distribution of heavy metals in plants. Agriculture, Ecosystem and Environment, 29: 79-83.

Del Val, C., J.M. Barea and C. Azcon-Aguilar (1999). Assessing the tolerance to heavy metals of arbuscular mycorrhizal fungi isolated from sewage sludge-contaminated soils. Applied Soil Ecology, 11: 261–269.

Diaz, G., C. Azcon-Aguilar and M. Honrubia (1996). Influence of arbuscular mycorrhizae on heavy metal (Zn and Pb) uptake and growth of *Lygeum spartum* and *Anthyllis cytosoides*. Plant and Soil, 180: 241-249.

Galli, U., H. Schuepp and C. Brunold (1994). Heavy metal binding by mycorrhizal fungi. Physiology of Plant, 92: 364–368.

Gildon, A. and P.B. Tinker (1983). Interaction s of VAM infections and heavy metals in plants. II. The effects of infection on uptake of copper. Transactions of the British Mycological Society, 77: 648-649.

Giovannetti, M. and B. Mosse (1980). Evaluation of techniques for measuring vasicular arbuscular mycorrhizal infection in roots. New Phytologist, 84: 489-500.

Guo, Y., E. George, H. Marschner (1996). Contribution of an arbuscular mycorrhizal fungus to the uptake of cadmium and nickel in bean and maize plants. Plant and Soil, 184: 195-205.

Heggo, A. and J.S. Angle (1990). Effects of vesicular–arbuscular mycorrhizal fungi on heavy metal uptake by soybeans. Soil Biology and Biochemistry, 22: 865–869.

Hetrick, B.A.D., G.W.T. Wilson and D.H. Figge (1994). The influence of mycorrhizal symbiosis and fertilizer amendments on establishment of vegetation in heavy metal mine spoil. Environmental Pollution, 86: 171-179.

Hildebrandt, U., M. Kaldorf and H. Bothe (1999). The zinc violet and its colonisation by arbuscular mycorrhizal fungi. Journal of Plant Physiology, 154: 171-179.

Janouskova, M., D. Pavlikova, T. Macek and M. Vosatka (2005). Infleunce of arbuscular mycorrhizae on the growth and cadmium uptake of tobacco with inserted metallothionein gene. Applied Soil Ecology, 29: 209-214.

Joner, E.J., R. Briones, C. Leyval (2000). Metal-binding capacity of mycorrhizal mycelium. Plant and Soil, 226: 227-234.

Joner, E.J. and C. Leyval (1997). Uptake of ^{109}Cd by roots and hypae of a *Glomus mosseae/Trifolium subterraneum* mycorrhizae from soil amended with high and low concentration of cadmium. New Phytologist, 135: 353-360.

Khan, A.G. (2006). Mycorrhizoremediation-an enhanced form of phytoremediation. Journal of Zhejiang University Science B, 7: 503-514.

Khan, A.G. (2005). Mycorrhizas and phytoremediation. In: Willey N, editor. Method in Biotechnology—Phytoremediation: Methods and Reviews. Totowa, USA: Humana Press.

Khan, A.G., C. Kuek, T.M. Chaudhry, C.S. Khoo and W.J. Hayes (2000). Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. Chemosphere, 41: 197– 207.

Killham, K. and M.K. Firestone (1983). Vesicular arbuscular mycorrhizal mediation of grass response to acidic and heavy metal deposition. Plant and Soil,72: 39-48.

Leyval, C. and E.J. Joner (2001). Bioavailability of heavy metals in the mycorrhizosphere. In: Gobran, R. G., W.W. Wenzel and E. Lombi (Eds.). Trace Metals in the Rhizosphere. Florida: CRC Press.

Leyval, C., K. Turnau and K. Haselwandter (1997). Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza, 7: 139–153.

Li, T.Q., X.E. Yang and X.X. Long (2004). Potential of using *Sedum alfredii* Hance for phytoremediating multi-metal contaminated soils. Journal of Soil Water Conservation, 18: 79-83.

Liao, J.P., X.G. Lin, Z.H. Cao, Y.Q. Shi and M.H. Wong (2003). Interaction between arbuscular mycorrhizae and heavy metals under sand culture experiment. Academic Chemosphere, 50: 847-853.

Loth, F.G. and W. Hofner (1994). Einfluß der VA-Mykorrhiza auf die Schwermetallaufnahme von

Hafer (*Avena sativa* L.) in Abhangigkeit vom Kontaminationsgrad der Böden. Z. Pflanzenernaehr. Bodenk, 158: 339–345.

Marschner, H. (1995). Mineral Nutrition of Higher Plants. San Diego. CA: Press.

Mosse, B. (1973). Advances in the study of vesicular-arbuscular mycorrhiza. *Annual Review of Phytopathology,* 11: 171-196.

Naidu, R., R.S. Kookkuma, D.P. Oliver, S. Rogers and M.J. McLaughlin (1996). Contaminants and the Soil Environment in the Australasia-Pacific Region. Dordecht: Kluwer Academic Publishing.

NandaKumar, P.B.A., V. Dushenkov, H. Motto and I. Raskin (1995). Phytoextraction: the use of plants to remove heavy metals from soils. Environmental Science and Technology, 29: 1232–1238.

Norgberg, G. (1994). Assessment of risks in occupational cobalt exposures. Science of the Total Environment, 150: 207-210.

Phillips, J. M. and D.S. Haymann (1970). Improved procedures for clearing roots and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society, 55: 158-160.

Rezvani, M., M.R. Ardakani, F. Rejali and G. Noormohammadi (2007). Use of mycorrhizal plants in clean up of contaminated soils to heavy metals. Ph.D. Dissertation in Agroecology. Islamic Azad University, Science and Research Branch. Tehran. Iran.

Rivera-Becerril, F., C. Calantzis, K. Turnau, J.P. Caussane, A.A. Belimov, S. Gianinazzi, R. J. Strasser and V. Gianinazzi-Pearson (2002). Cadmium accumulation and buffering of cadmium-induced stress by arbuscular mycorrhiza in three *Pisum sativum* L. genotypes. Journal of Experimental Botany, 53: 1177–1185.

Smith, S.E. and D.J. Read (1997). Mycorrhizal Symbiosis. second ed. London: Academic Press.

Sun, O., X.R. Wang, S.M. Ding and X.F. Yuan (2005). Effects of Interaction Between Cadmium and Plumbum on Phytochelatins and Glutathione Production in Wheat (*Triticum aestivum* L.). Journal of Integrative Plant Biology, 47: 435-442.

Turnau, K., I. Kottke and F. Oberwinkle (1993). Element localization in mycorrhizal roots of *Pteridium aquilinum* (L.) Kuhn collected from experimental plots treated with cadmium dust. New Phytologist, 123: 313–324.

Vivas, A., A. Vörös, B. Biro, J.M. Barea, J.M. Ruiz-Lozano and R. Azco´n (2003). Beneficial effects of indigenous Cd-tolerant and Cd-sensitive *Glomus mosseae* associated with a Cd-adapted strain of *Brevibacillus* sp. in improving plant tolerance to Cd contamination. Applied Soil Ecology, 24: 177–186.

Vogel-Mikus, K., P. Pongrac, P. Kump, M. Necemer and M. Regvar (2005). Colonization of a Zn, Cd and Pb hyperaccumulator *Thlaspi praecox* Wulf. with indigenous arbuscular mycorrhizal fungal mixture induces in heavy metal and nutrient uptake. Environmental Pollution, 133: 233-242.

Weissenhorn I. and C. Leyval (1995). Root colonization of maize by a Cd-sensitive and a Cd-tolerant *Glomus mosseae* and cadmium uptake in sand culture. Plant and Soil, 175: 233-238.

Weissenhorn, I., C. Leyval and J. Berthelin (1993). Cd-tolerant arbuscular mycorrhizal (AM) fungi from heavy metal polluted soils. Plant and Soil, 157: 247– 256.

Weissenhorn, I., C. Leyval, G. Belgy and J. Berthelin (1995). Arbuscular mycorrhizal contribution to heavy metal uptake by maize (*Zea mays* L.) in pot culture with contaminated soil. Mycorrhizae, 5: 245-251.

Xiong L.M. (1993). Vesicular-arbuscular mycorrhizae decrease cadmium uptake by plant. Journal of Plant Resources and Environment, 2: 58-60.