

Agronomic Properties of Mycorrhizal Alfalfa as Affected by Pb and Zn using Rhizobox System and Assessing its Ability for Phytoremediation Purposes

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Abstract – The objective of present paper is assessing the responses of alfalfa to lead and zinc when it is contributed with mycorrhizal symbiosis in a precise condition, using rhizobox system. Soil with low levels of Zn and Pb and soil with high amounts of Zn and Pb have been considered as media for mycorrhizal alfalfa. Mycorrhizal symbiosis resulted in 1.39 fold and 1.32 fold increases in the dry weight of roots and shoots, respectively. Contrary to zinc, in all treatments lead concentration in roots was more than shoots. It seems that it is because of more mobility of zinc in comparison to lead. Pollution and fungi inoculation and their interaction significantly affected Pb concentration in roots. Zn and Pb contamination and fungi inoculation and their interaction had a significant effect on Zn concentration in roots adjacency to rhizospheric soil and increased it to $104.87 \mu\text{g g}^{-1}$, although Zn concentration in other treatments of these roots were 51.12 , 30.87 and $29.37 \mu\text{g g}^{-1}$ in polluted without inoculation, low amounts of metals, non- inoculated and inoculated, respectively. Results showed that alfalfa can be an eligible plant for accumulating Pb in their roots while their shoots will be edible for animals. In highly contaminated soils, alfalfa cannot be an important accumulator for zinc, in addition zinc can transfer to shoots and in toxic concentrations can enter animals and the human food chain.

Keywords – Alfalfa, Mycorrhizal Symbiosis, Rhizobox, Pb, Zn.

I. INTRODUCTION

Heavy metals can enter the soils via different pathways and these metals are one of the most important sources of soil pollution [7], [5],[10]. These pollutants can enter into the food chain and imperil human health. Also soil heavy metal contaminations can greatly reduce crop biomass, soil microorganism population and consequently reducing the soil quality for agricultural or horticultural uses.

Contemporary attempts has been made to remove or reduce the soil pollution using different biological, physical and chemical methods [3]. Phytoremediation is one of these methods in which some plants such as maize, alfalfa or clover may be employed to uptake a substantial amount of heavy metals from soil. Direct and indirect roles of rhizosphere microorganisms has a positive impact on phytoremediation methods in increasing soil fertility and producing more plant biomass. Mycoremediation is an

innovative biotechnology that uses living fungus for in situ and ex situ cleanup and management of contaminated sites [27].

The process typically begins with field collection of fungi from a local area and continues with steps of culturing, screening, and preconditioning of native species to remediate the specific contaminants, if necessary, such as petroleum hydrocarbons, biological pathogens, organophosphates and metals, at increased efficiency under particular environmental regimes[20],[25]. In a mycorrhizal association, a symbiotic relationship forms between a fungal species and the roots of a host plant. Plants with a mycorrhizal symbiont are often better able to tolerate environmental stress as well. These special phytoremediate plants in addition to their microbial contribution in heavy metal polluted areas, can produce an appropriate biomass for fuel or energy and purges the polluted soils. Endomycorrhizae fungi are one of the soil microorganisms which have the highest potential for reducing and removing heavy metals contaminations from soils and increasing the soil fertility [27]. In earlier researches on the mycorrhiza fungi, it's been often reported the positive effects of this symbiosis relationships on mineral nutrition of plants but recently new researches have shown that also the non- nutritional effects of this symbiosis such as its ability to expulsion of toxic ions, control the pathogens extension, effect on photosynthesis and soil- plant relations, increase the plant resistance to stresses derived from heavy metal contaminations [1]. Mycorrhizoremediation technology has implications in reducing a plant's exposure to potentially toxic elements. Some reports showed that external mycelium of AMF is the main place for heavy-metal localization [11],[21]. However, other researchers have indicated the role of chitinous cell walls in selective absorbing and hence removing toxic and nontoxic elements [28] or the roles of extracellular lycoprotein, glomalin[29],[30] and intracellular precipitation [12] , [13]. Alfalfa can be the host in microbial symbiosis with mycorrhiza fungi and uptake heavy metals from polluted soil [18].

In assessing the risks related to the contaminated soils by heavy metals, bioavailability which is associated with the metal chemical forms in the rhizosphere is more important than the total concentration of metals. Roots and their penetrations stimulate soil microorganisms, these

activities cause the biological, physical and chemical changes in soil rhizosphere properties in comparison with bulk soil [4]. Some factors such as pH changes resulting from roots activities, metals banding with root penetrations, microbial activities resulting from root growth and metal uptake by roots affect the changes of metal chemical forms and consequently their bioavailability in the rhizosphere [8], [16]. It seems necessary to have precise information about rhizosphere medium for increasing the knowledge about plant nutrition and soil pollution [22],[23]. The objective of present study is to assess the influence of mycorrhizal symbiosis on Zn and Pb uptake by alfalfa and assessing the responses of mycorrhizal symbiosis in a precise condition, using rhizobox system.

II. MATERIALS AND METHODS

Soil selection and preparation: In this study the soils were selected from Zanzan province (Iran), on the basis of reports from previous studies indicating the levels of heavy metal contaminations in the selected area. Table 1 depicts the properties of selected soils. The selected soils were air dried and were passed through a 2 mm sieve. This

research was a factorial on the basis of completely randomized design with 4 replications,. Total and DTPA extractable concentration of zinc and lead were measured in soil samples[14] and two soils with different levels of pollution were selected. Soil 1 was tagged as low polluted with metals and soil 2 was called high polluted with higher concentrations of heavy metals. Other than these pollution treatments, two other treatments were applied to these soils: inoculation with arbuscular mycorrhiza fungi (M1) and without inoculation with arbuscular mycorrhiza fungi (M0). This experiment was conducted in a greenhouse with 25°C temperatures during the days and 15°C temperature during the nights. For preparation the soils for greenhouse cropping, 10 kg of each soil was weighted and put in the plastic bags, then 5 ml urea box⁻¹ and 5ml box⁻¹ potassium DI hydrogen phosphate were added to each plastic bag. In the next step, 9 kg of high polluted soil (HH) and 9 kg of low polluted soil (LH) were weighed. Highly polluted soils were treated with 2 g urea in 1000 ml water and 1.2 g potassium DI hydrogen phosphate in 1000 ml water. Low polluted soils were treated with 2 urea and 1.2 g potassium DI hydrogen phosphate in 1000 ml water . Then soils in each plastic bag were mixed precisely to achieve the homogeneous soils.

Table 1: Physical and chemical properties of selected soils

The region of sampling	Soil 1 (LH)	Soil 2 (HH)
	Zanzan- Dandi, areas around calcinations crop	Zanzan- Dandi, areas around Batele dam
Landuse type	Non- irrigated wheat farms	alfalfa farms
Geographical coordination	36°40'12"N, 48°20'53"E	36°33'47"N, 47°37'16"E
Depth (cm)	0- 30	0- 30
Clay (%)	33	25
Silt (%)	33	37
Sand	34	38
Texture	Loam clay	Loam
pH	7.7	7.7
Saturation percentage (%)	35	48
ECe (dSm ⁻¹)	1.14	1.02
Organic carbon (%)	0.61	1.23
Calcium carbonate (%)	18	17.6
available potassium (mg kg ⁻¹)	347	256
available phosphorus (mg kg ⁻¹)	8.8	4.2
available zinc (mg kg ⁻¹)	8.66	89.6
available lead (mg kg ⁻¹)	3.12	9.9
total lead (mg kg ⁻¹)	34	91.6
total zinc (mg kg ⁻¹)	126	258.1

Rhizobox: In this study we used the rhizobox sytem introduced by [23]. All parts of this system have been made from acrylic or plexi glass for avoiding the external environmental effects on the system. This system constitutes the soil- plant components (139 ×64 ×50 cm, height, width and length, respectively). There is a narrow slit with 1 cm width, in the bottom of this part. Plant roots can move vertically downward through this slit and enter

into the next part. This part of the system is limited by the glass sheet and nylon membrane. Nylon membrane is a porous nylon textile which has pores with 40 µm diameter. This membrane separates root sheet from rhizosphere soil. Roots cannot pass through these pores, but water, nutrients and organic compounds molecules move through these pores, easily. Therefore molecules exchanges between roots and rhizosphere occur without direct contact between

soil and roots. This nylon membrane is tightening by the special tool. There should be about 2mm distance between transparent window and nylon membrane. This distance simulates the real mechanical pressure on the roots from soil media. Soil- plant system and rhizospheric soil is irrigated with wicks (4 mm diameter) made of fiberglass. The other side of these wicks is placed in the 1 liter water tank and the wicks pass through silicon hose. This kind of irrigation causes the homogenous and perennial flux of water (according to a potential gradient caused with evapotranspiration of plants) to the roots. Soil- plant part and rhizospheric soil part were filled and compacted with soils until achieving the bulk density equal to 1.4 cm^{-2} . Then in treatments with mycorrhizal inoculations, 50 cm^3 of arbuscular mycorrhiza, *Glomus intraradices*, *mosseae* *etunicatum*, in the same population equal to 80 spore g^{-1} were added to each rhizobox. The layer of mycorrhiza fungi with 5 mm diameter was put in the depth of 10 mm from soil- plant part's surface. Then this layer was covered with soil. Then 3 grown alfalfa (Hamadani var.) seed planted in soil- plant part of the system. Only 15 homogenous plants kept after 30 days in each box. After 40 days, when the roots appeared in the beneath slit of soil - plant parts, we added this part to the rhizospheric soil part. Plants were harvested after 93 days. Then the aerial parts were cut from torque and the roots also were cut. For determining Zn and Pb in roots apoplasm, roots were dipped in 0.05 M CaCl_2 , 3°C for 5 minutes. Then were washed with distilled water and prepared for chemical analysis.

Plant analysis: For determining zinc and lead concentration in plant samples, the samples were digested using dry digestion method described by [2]. The concentration of elements in extractions was measured by atomic absorption (AAS), Perkin Elmer, 311.

Statistical analysis: Statistical analysis was done with SAS (version 9.1) software [19]. Graphs were drawn using EXCEL 2007.

III. RESULTS AND DISCUSSION

Dry weight of aerial parts and roots of alfalfa: There was a significant effect of interaction between treatments on dry weight of shoots and roots of alfalfa in some cases (Table 2). The highest dry weight of shoots was observed in HHM1 and LHM1 and the least dry weight of roots in soil- plant part and total roots were related to HHM0 and LHM0. In other cases there was not significant difference among treatments. In all the treatments roots weight was observed to be more than shoot weight. It means that fungi inoculation can increase the alfalfa yields (shoots and roots) even in polluted treatments.

Concentration and total absorption of Zn and Pb in aerial parts of alfalfa: Anova analysis (data has not been shown) imparts that levels of metals soil pollution and inoculation with mycorrhiza significantly ($\alpha= 0.01$)

affected the Zn concentration in the aerial parts of alfalfa, but these treatments didn't affect the total absorption of Zn to the aerial parts of the plant. The effects of mycorrhizal inoculation on the total absorption of Pb to alfalfa shoots was significant at the 5 % level of probability while it was not significant on the Pb concentration in alfalfa shoots. Interactions between treatments were significant in some cases. Table 3 shows the effects of pollution and mycorrhiza on concentration and total absorption of Zn and Pb in the aerial parts of alfalfa which has been grown in rhizoboxes. As it is illustrated in Table 3, the mean concentration of zinc in alfalfa shoots grown in HH soil ($46.06 \mu\text{g g D.W}^{-1}$) is significantly more than its concentration in plant grown in LH soil ($34.56 \mu\text{g g D.W}^{-1}$). Mycorrhiza inoculation caused a significant increase in Zn concentration in alfalfa grown in HH in comparison with the plant grown in LH soil. Soil pollution neither fungi inoculation didn't have any significant effect on the total absorption of Zn to alfalfa shoots. Soil pollution showed significant effect on Pb concentration and total Pb absorption. In both cases the amount of Pb was higher in LH treatment. But fungi inoculation only affected total Pb absorption significantly. This absorption was higher in M1 treatment. Table 4, shows the effect of interaction among treatments on concentration and total absorption of Zn and Pb. According to this table mean interaction between soil pollution and Mycorrhizal inoculation on these parameters was significant and caused a significant increase in Zn concentration in HHM0 in comparison with other treatments. The interaction among the treatments increased Pb total absorption in LHM1 more than other treatments.

Concentration and total absorption of Zn and Pb in roots: Anova analysis indicates that differences among treatments in this experiment affected the Pb concentration in roots and Pb total absorption to alfalfa in soil- plant part of the rhizobox system. Effect of soil pollution on Zn concentration and Zn total absorption in roots in soil- plant part was substantially significant. But there was not a significant effect of mycorrhiza inoculation on these parameters. The mean concentration of Pb and Zn in roots related to soil- plant part in soil with high pollution (HH) was $46.75 \mu\text{g g dry weight}^{-1}$ and $244.56 \mu\text{g g dry weight}^{-1}$, respectively and in soil with low pollution (LH) was $10.81 \mu\text{g g dry weight}^{-1}$ and $48.63 \mu\text{g g dry weight}^{-1}$. The mean concentration of Pb and Zn in roots related to soil- plant part of M1 treatment was $21.43 \mu\text{g g dry weight}^{-1}$ and $140 \mu\text{g g dry weight}^{-1}$ respectively and in M0 treatment was $36.12 \mu\text{g g dry weight}^{-1}$ and $153.19 \mu\text{g g dry weight}^{-1}$ respectively (Figs. 1 and 2). These treatments also affected the Pb total absorption. Interaction among pollution and fungi inoculation was significant (Fig. 1). The interaction between treatments has affected Zn and Pb concentration and Zn total absorption significantly. The highest amount of Zn concentration and Zn total absorption was related to HHM0 and HHM1. The highest amount of Pb concentration was related to HHM0, HHM1 and LHM0 and LHM1 respectively.

Table 2: Means of shoots and roots dry weight for each experimental

	Shoots dry weight (g box ⁻¹)	Roots dry weight in soil- plant part (g box ⁻¹)	Roots dry weight in rhizospheric soil part (g box ⁻¹)	Total roots dry weight (g box ⁻¹)	Ratio between shoots to roots dry weight
HHM0	6.97 ^b	16.86 ^{bc}	2.87 ^a	19.73 ^c	0.35 ^a
HHM1	9.14 ^{ab}	21.96 ^{ab}	2.29 ^a	24.26 ^{ab}	0.38 ^a
LHM0	7.77 ^{ab}	14.75 ^c	3.46 ^a	18.22 ^{bc}	0.42 ^a
LHM1	10.29 ^a	24.56 ^a	3.79 ^a	28.36 ^a	0.36 ^a

Table 3: Effect of pollution and mycorrhiza on concentration and total absorption of Zn and Pb in aerial parts of alfalfa

		Zn concentration (µg g ⁻¹)	Total Zn absorption (µg box ⁻¹)	Pb concentration (µg g ⁻¹)	Total Pb absorption (µg box ⁻¹)
Soil Pollution	LH	34.56 ^b	311.02 ^a	10.81 ^a	98.28 ^a
	HH	46.06 ^a	365.73 ^a	8.78 ^b	70.63 ^b
Least Square Difference (LSD)		4.46	62.49	1.45	18.62
Inoculation with Micorhyza	M0	43.50 ^a	318.05 ^a	9.75 ^a	72.15 ^b
	M1	37.12 ^b	358.69 ^a	9.84 ^a	96.76 ^a

Concentration and total absorption of Zn and pb in roots of alfalfa in adjacency to rhizospheric soil:

Anova analysis related to this part of rhizobox showed that the effect of different levels of soil pollution and mycorrhiza inoculation on Zn and Pb in roots adjacency to rhizospheric soil was significant at 1% and 5% level of probability. The interaction between treatments was significant at the 5 % level. The mean concentration of lead in the roots adjacency in rhizospheric soil in soils with high metals pollution (HH) was 15.96 µg g dry weight⁻¹ and in soils with low metals pollution was 7 µg g dry weight⁻¹. This concentration in M1 treatments was 13.81 µg g dry weight⁻¹ and for M0 Treatments was 9.15 µg g dry weight⁻¹ (Fig. 2). The mean concentration of zinc in the roots adjacency to rhizospheric soil in soils with high metals pollution (HH) was 78 µg g dry weight⁻¹ and in soils with low metals pollution (LH) was 30.13 µg g dry weight⁻¹. This concentration in M1 treatments was 67.13 µg g dry weight⁻¹ and for M0 Treatments was 41 µg g dry weight⁻¹ (Fig. 1). The mean amount of Zn total absorption in HH and LH treatments was 180.35 µg g dry weight⁻¹ and 110.33 µg g dry weight⁻¹, respectively. The effect of pollution and Mycorrhizal inoculation on Pb total absorption was not significant. compares the interactions between treatments. According to this table zinc and lead concentration have been affected by interaction between pollution and fungi inoculation as the highest concentration of Zn and Pb was related to HHM1. Fig. 3

shows that the lead concentration in roots was more than shoots. This result agrees with [24] studies on canola and sunflower for absorption and accumulation of lead in its organs. Also [26] studied the accumulation of lead in native plants in contaminated soils by heavy metal in Florida and they observed that the Pb concentration in roots was more than shoots. They concluded that this difference is because of low mobility and the fixation of Pb in roots. [17] reported that plant's ability for metals accumulating depends on pollutant properties and plant species. Also this difference in metal absorption by plants is dependent on soil- metal band and plant- metal interactions in roots, which is related to the kind of metal. There are a lot of studies about translocation and distribution of metals in plants [15],[6], [9] showed that the Pb concentration in monocotyledon plants' roots is more than their aerial parts significantly. Lead precipitated as insoluble phosphates, carbonates and hydroxides in soils. Then increasing its solubility in soil is the main factor to increase the metal absorption from the soil. That different properties of metals cause different behaviors in plants. Zn, Ni and Cd are more mobile than Pb and Cu in soils. In addition Zn is a nutritional element for plants but there is no known biological role for Pb in plants, then the absorption of Zn and its translocation in plants is done easier and with less resistance of plant in comparison to Pb.

Table 4: Compares the interactions among treatments in roots of alfalfa in soil- plant part.

	Zn concentration (µg g ⁻¹)	Total Zn absorption (µg box ⁻¹)	Pb concentration (µg g ⁻¹)	Total Pb absorption (µg box ⁻¹)
HHM0	261.37 ^a	4411.49 ^a	58.25 ^a	985.31 ^a
HHM1	227.75 ^a	5109.39 ^a	35.25 ^b	814.42 ^a
LHM0	45.00 ^b	672.70 ^b	14.00 ^c	212.12 ^b
LHM1	52.25 ^b	1292.95 ^b	7.62 ^c	190.51 ^b

In each column means with the same letter doesn't have significant difference in 5% level. LH= soil 1 with lower level of metals pollution. HH= soil2 with higher level of metals pollution. M0= non- inoculated with mycorrhiza. M1= inoculated with mycorrhiza.

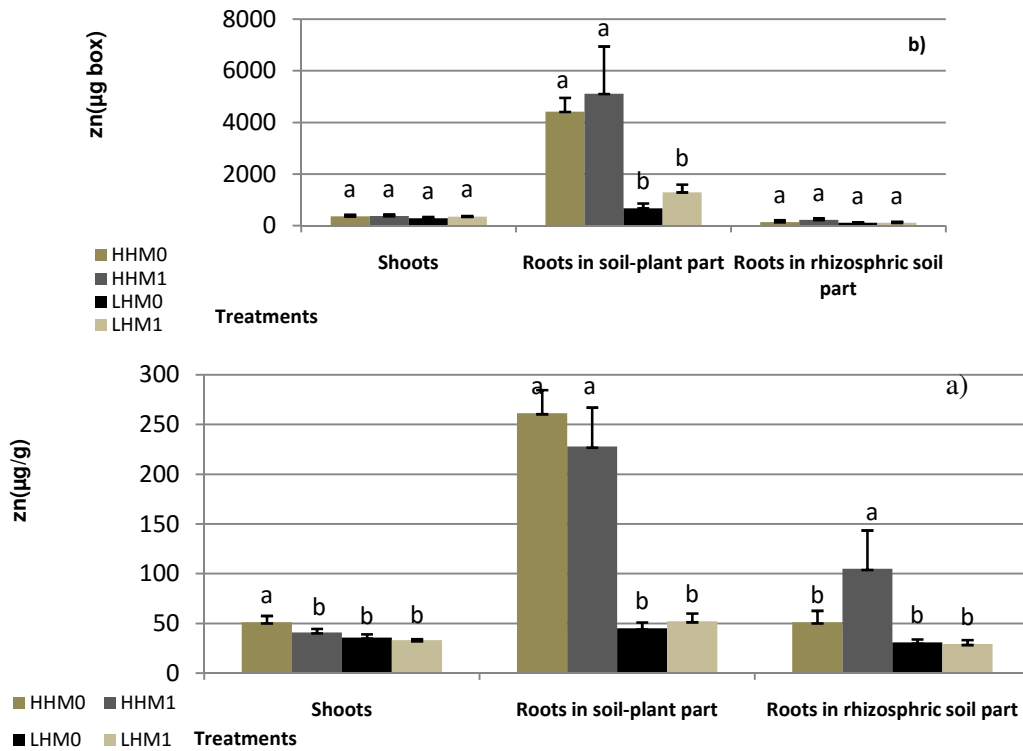


Fig.1. Zn concentration a) and Zn total absorption b) in different parts of plant
LH= soil 1 with lower level of metals pollution. HH= soil2 with higher level of metals pollution.
M0= non- inoculated with mycorrhiza. M1= inoculated with mycorrhiza

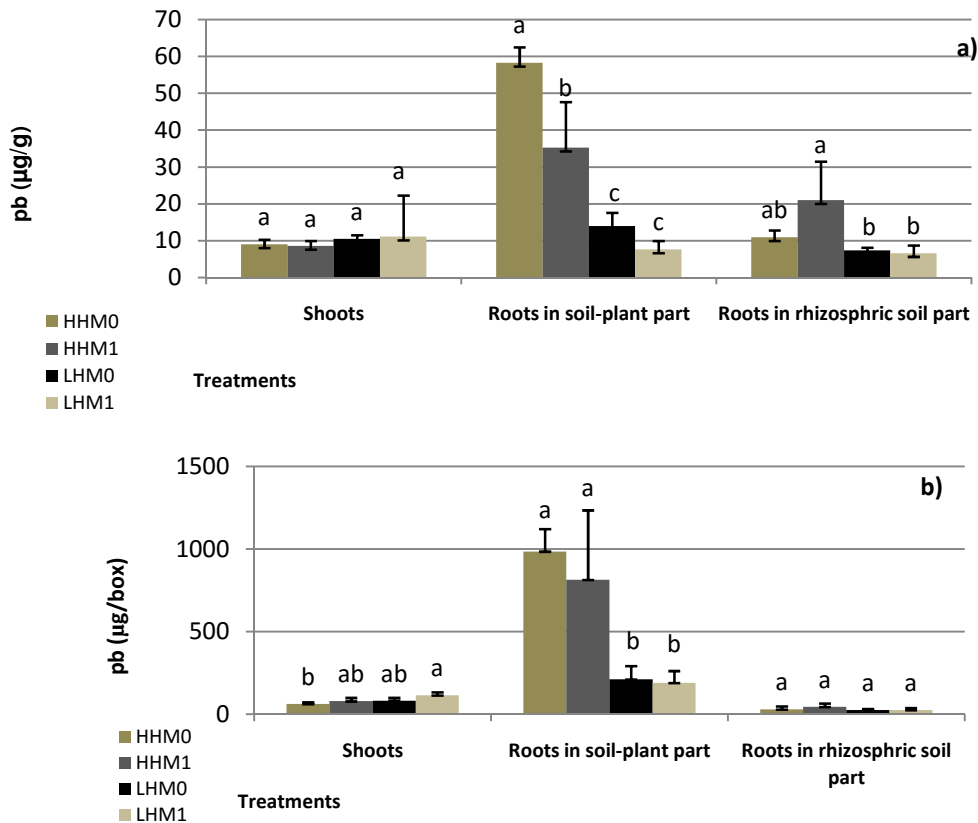


Fig.2. Pb concentration a) and Pb total absorption b) in different parts of plant
LH= soil 1 with lower level of metals pollution. HH= soil2 with higher level of metals pollution.
M0= non- inoculated with mycorrhiza. M1= inoculated with mycorrhiza

V. CONCLUSION

Mycorrhiza fungi had a significant effect on dry weight of alfalfa roots and aerial parts, in soil- plant part of rhizobox system and total weight of roots. The mean dry weight of roots and shoots in inoculated treatment was more than the treatment without inoculation. But pollution treatment didn't have any significant effect on dry weights of alfalfa organs. Fungi inoculation had significant effect on alfalfa yield and more yield caused more absorption of metals by alfalfa. Pollution treatment had a significant effect on Zn and Pb concentrations in alfalfa shoots and didn't have any significant effect on the total absorption of zinc. Zn concentration in HH soil was more than LH soil. Lead concentration and total absorption in LH treatment was more than HH treatment. Contrary to zinc, Pb concentration in roots was more than shoots. It seems that it is because of the difference in mobility of Zn in comparison to Pb. Pollution and fungi inoculation and their interaction significantly affected Pb concentration in roots of soil- plant part of rhizobox. Pollution and fungi inoculation and their interaction had a significant effect on Zn concentration in roots adjacency to rhizospheric soil. The results of present paper impart that alfalfa can be an effective plant for accumulating Pb in their roots and their shoots will be edible for animals because the lead concentration in aerial parts is less than critical values. It seems that in high polluted soils alfalfa cannot be an important accumulator for zinc, in addition zinc transfer to shoot and in toxic concentrations can enter animals and the human food chain.

REFERENCES

- [1] R.M.Auge, X.Duan, R.C. Ebel , A.J.W.Stodola , Nonhydraulic signaling of soil drying in mycorrhizal maize. *Planta* 193,2001,pp. 74-82.
- [2] A.Amani, Methods of plant analysis Vol.1. Bulletin No. 982. Soil and Water Research Institute, Tehran, Iran,1996.
- [3] Bio-Wise, Contaminated Land Remediation: A Review of Biological Technology, London.DTI, 2003.
- [4] G.D.Bowen, A.D. Rovira, The rhizosphere,1991, pp. 641-669. In Y. Waisel et al. (ed.) *Plant roots: The hidden half*. Marcel Dekker. Inc, New York.
- [5] G.Bridge, Contested terrain: mining and the environment, *Annu. Rev. Environ & Resour.* 29,2004,pp. 205-259.
- [6] H.M.Chen, *Environment Agrology*. Science Press China. Beijing, 2005.
- [7] V.Dembitsky, Natural occurrence of arseno compounds in plants, lichens, fungi, algal species and microorganisms. *Plant Sci* 165, 2003,pp. 1177-1192.
- [8] W.H.O.Ernst, Bioavailability of heavy metals and decontamination of soils by plants. *Appl Geochem* 11, 1996, pp. 163-167.
- [9] J.Fang , B. Wen ,X. Shan ,J. Lin, G. Owens, Is an adjusted rhizosphere based method valid for field assessment of metal phytoavailability application to non contaminated soils. *Environ Pollut* 150, 2007,pp. 209-217.
- [10] W.L.Fitz,W.W.Wenzel, Arsenic transformations in the soil-rhizosphere-plant system: Fundamentals and potential application to phytoremediation. *J Biotechnol* 99, 2002,pp. 259-278.
- [11] M. Kaldorf,A.J. Kuhn, Schroder ,W.H., Hildebrandt, U., Bothe, H. Selective element deposits in maize colonized by a heavy metal tolerance conferring arbuscular mycorrhizal fungus. *J Plant Physiol* 154, 1999,pp.195-206.
- [12] A.G.Khan, Mycorrhizas and phytoremediation. In: Willey N, editor. *Method in biotechnology- phytoremediation: methods and reviews*. Humana Press, Totowa, USA. 2005.
- [13] A.G.Khan., Mycorrhizoremediation - An enhanced form of phytoremediation. *Journal of Zhejiang University Science B* 7, 2006,pp. 503-514.
- [14] W.L.Lindsay, W.A. Norvell , Development of DTPA tests for zinc, iron, manganese and copper. *Soil Sci Soc Am J* 42, 1978,pp.421-428.
- [15] N.E.Longnecker, A. Robson , Distribution and transport of zinc in plant; Robson A.b.(ed.) *zinc in soils and plants*. Kluwer Academic Pub., Dordrecht, the Netherlands, 1993,pp.79-91.
- [16] H.Marschner, V.Romheld, Root-induced changes in the availability of micronutrients in the rhizosphere. In Y. Waisel et al. (ed.). *Plant roots-The hidden half*. Second ed. Marcel Dekker Inc, New York, USA. 1996.
- [17] R.Naidu., D. Oliver, S. McConnell, Heavy metal phytotoxicity in soils . In proceeding of the fifth national workshop on the assessment of site contamination, 2003,pp. 235-241.
- [18] M.Rezvani, M.R.Ardakani. Interaction between alfalfa (*Medicago sativa* L.) mycorrhizal roots traits and heavy metals (Cd, Co and Pb). International Symposium "Root Research and Applications Root RAP, 2-4 September 2009, Boku, Vienna, Austria.
- [19] SAS Institute Inc. *SAS/STAT user's guide*. Version 6, 4th ed. Cary North Carolina: Statistical Analysis Institute Inc. 1988.
- [20] S.A.Thomas., P. Becker, M.R.Pinza, J.Q. Word, Mycoremediation: A Method for Test-to Pilot-Scale Application. PNL- SA- 30881, in Proceedings, Battelle In Situ and On-Site Bioremediation, Fifth International Symposium, April 19-22, 1999, San Diego, California.
- [21] K.Turnau , Heavy metal content and localization in mycorrhizal *Euphorbia cyparissias* from zinc wastes in southern Poland. *Acta Soc Bot Pol* 67, 1998,pp.105-113.
- [22] W.W.Wenzel ,Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. *Plant Soil* 321,2009,pp. 385-408.
- [23] W.W.Wenzel, G. Wieshammer, W.J. Fitz, M .Puschenreiter, . Novel rhizobox design to assess rhizosphere characteristics at high spatial resolution. *Plant Soil.* 237, 2001,pp. 37-45.
- [24] G.Wieshammer, R.Unterbrunner,T.B. Garcia., M.F.Zivkovic,M. Puschenreiter,W.W. Wenzel , Phytoextraction of Cd and Zn from agricultural soils by *Salix spp.* and intercropping of *Salix caprea* and *Arabidopsis halleri*. *Plant Soil* .298, 2007,pp.255-264.
- [25] Y. Xiaoe,Y. Feng, He, Z., P.J Stoffella, Molecular mechanisms of heavy metal hyperaccumulation and Phytoremediation. *Journal of Trace Elements in Medicine and Biology* .18, 2005, pp. 339-353.
- [26] J. J. X. Yoon,Q. Cao, U.Zho, U. , Ma, L.Q..Accumulation of pb.cu.and Zn in native plants growing on a contaminated florida site. *Sci Total Environ.* 360, 2006,pp.456-644.
- [27] F.Zafarian, M. Rezvani, M.R.Ardakani, F.Rejali , M. Miransari , Impact of Mycorrhizae Formation on the Phosphorous and Heavy Metal Uptake of Alfalfa (*Medicago sativa* L.). *Commun Soil Sci Plant Anal* 44, 2013.,pp.1340-1352.
- [28] J.L.Zhou, Zinc biosorption by *Rhizopus arrhizus* and other fungi. *Appl Microbiol Biotechnol* .51, 1999,pp.686- 693.
- [29] S.F. Wright, and A . Upadhyaya, A survey of soil aggregate stability and lomalin, a glycoprotein produced by hyphae of mycorrhizal fungi. *Plant Soil* .198,1998, pp. 97-107
- [30] M.Ae.N.Rajkumar, & H.Freitas, Endophytic Bacteria and Their Potential to Enhance Heavy Metal Phytoextraction. *Chemosphere*, Vol.77. No.2,2009, pp. 153- 160, ISSN 0045-6535