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Evaluation of the effects of mycorrhizal inoculation on lead (Pb) uptake and growth of alfalfa in Pb-contaminated soil

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ABSTRACT- Establishment of arbuscular mycorrhizal (AM) symbiosis in plant roots can affect plant physiological and morphological characteristics and may induce tolerance to heavy metals in plants grown in polluted soils; therefore, it can play an important role in phytoremediation. In the present study, to investigate the effect of mycorrhiza on alfalfa growth and lead (Plumbum, Pb) uptake, a factorial experiment was designed with two factors: (1) plants non-inoculated (NM) or inoculated with *Rhizophagus intraradices* (Ri) or *Funneliformis mosseae* (Fm) and (2) soil non-contaminated (Pb_0) and contaminated with 200 (Pb_1), 400 (Pb_2) and 600 (Pb_3) mg kg^{-1} Pb^{2+}. All plants were evenly inoculated with *Sinorhizobium meliloti*. Results showed that at high levels of Pb^{2+}, both fungi compared to the NM controls enhanced root nodulation and phosphorus nutrition. The dry weight of shoots, leaf area and chlorophyll index significantly increased in mycorrhizal-inoculated plants compared to the NM plants. Mycorrhizal dependency increased significantly ($p < 0.05$) by four- and three-folds in Ri and Fm plants, respectively, at Pb_3 level in comparison with the non-polluted condition. Moreover, Pb translocation from root to the shoot significantly ($p < 0.05$) declined at Pb_3 level in Ri plants by 1.75-folds as compared with NM plants. Comparison of the results obtained by principal component analysis demonstrated that *R. intraradices* symbiosis was more efficient for host plant protection against the phytotoxic effect of Pb. These results highlight the assisting role of AM fungi in protecting plants from metal toxicity and in plant establishment in Pb polluted soils.

INTRODUCTION

Heavy metal (HM) contaminated soil, as an important environmental problem, is becoming increasingly widespread. Mining activities, the smelting industry, addition of sewage sludge, fertilizers and pesticides to soils are common sources of anthropogenic contamination of lead (Pb^{2+}) in the polluted soils (Kachenko and Singh, 2006; Lermen et al., 2015). In contrast to heavy metals like zinc, copper, and nickel, lead is not necessary for plant nutrition and development and has no biological function. Passive diffusion and low-affinity metal transporters govern the entering of non-essential HMs like Pb to the plant root (Göhre et al., 2006). High concentration of HMs can interfere with metabolic activities of plant cells and may cause toxicity symptoms, especially growth retardation because of the disordered vital processes like photosynthesis (Göhre et al., 2006; Sharma et al., 2009). Since heavy metals cannot be easily degraded in soils, utilization of biological approaches like the soil microbiota and the host plants interactions can be important alternative approaches for the treatment of soils polluted with HMs (Ashraf et al., 2017).

Microbial activity in the rhizosphere can modulate the availability and toxicity of metals to plants (Bíró and Takács, 2007). Arbuscular mycorrhizal (AM) fungi are prominent as potent soil biota to modulate several biotic and abiotic stresses in plants which have adverse effects on plant growth and yield. More than 80% of vascular plants growing under natural conditions have mycorrhizae (Smith et al., 2010). Several lines of

evidence indicate that the establishment of AM symbiosis in the plant roots affects its growth, nutrition, and tolerance to HMs in the contaminated areas. Therefore, mycorrhizae would have a significant role in phytostabilization by sequestration of HMs and helping plants to survive in polluted soils (Christie et al., 2004; Gu et al., 2017; Firmin et al., 2015). In fact, the mycorrhizal association may increase the efficiency of phytoremediation as an emerging plant-based in situ remediation technology (Chaudhry et al., 1998). It is stated that adsorption of HMs to polyphosphate compounds existing in fungal cell walls can protect plants against HMs toxicity (Wong et al., 2007). Components of AM fungi cell walls including free amino, hydroxyl and carboxyl, and chitin can bind to toxic elements and sequester them (Kapoor and Viraraghavan, 1995). Furthermore, proteins such as glomalin (as a hyphal cell wall component) produced by AM fungi can sequester metals (Gonzalez-Chavez et al., 2004; Driver et al., 2005). In such a case, the fungus can restrict metal transport to the plant shoot. On the other hand, it may be the result of a dilution effect since mycorrhizal fungi can promote the plant growth by improving its nutritional state compared to non-mycorrhizal ones. Establishment of the AM associations may increase (Joner and Leyval, 2001; Chen et al., 2005; Jiang et al., 2016) or decrease (Andrade et al., 2004; Lermen et al., 2015; Hristozkova et al., 2016) the heavy metal uptake by host plants, which seems to be highly dependent on the available metal concentrations in the soil. AM symbioses have not the same behavior in different types of metals, host plant species, fungal species involved as well as different concentration of metals in the soil (Al-Garni, 2006). However, it seems that AM fungi can decrease phytotoxicity caused by toxic trace elements (Khan, 2006; Bíró and Takács, 2007).

Biological N_2 fixation during the symbiotic relation of alfalfa and *S. meliloti* has utmost importance regarding crop production, soil fertility, and the global N cycle balance. Legumes could take advantage of additional nitrogen source through the establishment of N_2 fixing bacteria in their roots (Hack et al., 2019; Chen et al., 2017). Hence, alfalfa-bacterium system responses to phytotoxic effects of metals are critical to explore (Bandyopadhyay et al., 2015). Souza et al. (2013) reported a severe decline of root nodulation in a Pb polluted soil irrespective of AM fungi treatment. However, stimulated root nodule formation and improved N and P uptake in mycorrhizal legume species were reported by Lin et al. (2007).

Alfalfa (*Medicago sativa* L.) is considered as a fast-growing perennial ryegrass and important food-chain crop that is not normally consumed directly by humans. Therefore, it has good potential to be used for the phytoremediation of polluted soils (Miller et al., 1995). Investigating the role of mycorrhizae in plant heavy metal uptake especially in plants used as forage is a particular interest to minimize the risks of heavy metal pollution in soil (Chen et al., 2007). The objective of this study was the investigation of the contribution of AM fungi to uptake and translocation of Pb in alfalfa plants and plant growth under Pb stress in the presence of associated rhizobial symbiont. In this way, we used u

nivariate and multivariate analyses to study the response of mycorrhizal plants to each fungal species at the particular levels of soil Pb contamination.

MATERIALS AND METHODS

Soil Preparation and Mycorrhizal Fungi Propagation

The study was conducted on a sandy soil that was collected from Khalat-Pooshan Research Station at the depth of 0-20 cm with the following characteristics: 7.4 mg kg^{-1} available P (Olsen and Sommers, 1982), 182.6 mg kg^{-1} K (Knudsen et al., 1982), 0.1% of organic carbon (Nelsons and Sommers, 1982) with pH 7.79 (Mclean, 1982) and field capacity 12.5% (Cassel and Nielsen, 1986). The soil sample was passed through a 4mm sieve and sterilized by autoclaving at 121°C (1.2 bar). Each pot was filled with 1.5 kg soil and Pb levels of 0, 200, 400 and 600 mg kg^{-1} soil (Pb_0, Pb_1, Pb_2 and Pb_3, respectively) were added to the soil using Pb $(NO_3)_2$ solution. Lead nitrate was filter sterilized through 0.45µm acetate cellulose filter paper and mixed with the soil by uniform spraying. The pots were left for four weeks at 20 ± 5 °C and field capacity moisture to reach the relative equilibrium of Pb with the solid phase. Also, nitrate concentrations in all pots equalized using sodium nitrate. After incubation, soil available Pb was measured using DTPA method (Lindsay and Norwell, 1978). DTPA extractable concentrations of Pb were 156.6, 311.72, and 492.73 at Pb_1, Pb_2 and Pb_3, respectively. In control treatment (Pb_0), Pb was not detected.

Plant Culture, Treatments, and Analyses

Healthy and uniform alfalfa seeds (*Medicago sativa* L.) were sterilized with ethanol (96%) for 30 seconds followed by sodium hypochlorite (1%) for 15 minutes and then were washed carefully with sterile distilled water. Surface sterilized seeds were placed in 1% water-agar at 20 °C for seed germination. Pre-germinated alfalfa seeds were inoculated with bacterial suspension of *Sinorhizobium meliloti* (one ml of bacterial suspension per seed with a population of 10^9 cells per ml of suspension) and sown in pots (twenty seeds per pot) (Bandyopadhyay et al., 2015). This bacterial strain was provided by soil fertility lab of University of Zanjan. Also, 50 g of AM inoculum (*Rhizophagus intraradices* (Ri) or *Funneliformis mosseae* (Fm)) was mixed with upper third of soil in pots (c.a. 11 spores per gram of each inoculant) and sterile substrate used for inoculum preparation was added in case of non-inoculated (NM) treatments. AM fungi species were provided by Soil Biology lab of University of Tabriz. AM fungi were multiplied by *Zea maize* as the host plant in a sterilized sandy soil for four months.

After 20 days of growth, seedlings were reduced to ten per pot. The pots were kept in a growth room with 14/10 hours of day/night period, 60% relative humidity and 10,000 lux intensity of light at 23 ± 5 °C, for three

months. In this period, irrigation of plants was performed with distilled water, up to 80% of field capacity by daily weighing of the pots.

Chlorophyll index (SPAD values) was measured by SPAD chlorophyll meter (Minolta, Osaka, Japan) prior to harvest. Shoots were cut from the crown and their fresh weights were measured. The leaf areas were obtained with a leaf area meter (LI-3100, LI-COR, Lincoln, NE, USA). After removing the roots from the soil, they were washed with distilled water. To determine nodulation rate of alfalfa, the nodules were separated from the roots and their dry weights were determined. To assess the percentage of root colonization the gridline intersect method was used (Giovannetti and Mosse, 1980) after staining 50 pieces of 1 cm long fine root samples at each pot with trypan blue method (Phillips and Hayman, 1970). Shoots, roots and nodules were dried at 70 °C to constant weight. Dry weights were determined and 1 g samples of dried materials were dry-ashed in a muffle furnace. Afterwards, 1:3 ratios of nitric acid and hydrochloric acid were used to extract ash. Vanadate –Molybdate method (Chapman and Pratt, 1961) was applied to determine phosphorus concentration. Pb was measured in acid digests using atomic absorption spectrometer (Shimadzu, Japan) (Waling et al., 1989).

Experimental Design and Statistical Analysis

A completely randomized factorial design was applied with two factors: (1) plants non-inoculated (NM) or inoculated with *Rhizophagus intraradices* (Ri) or *Funneliformis mosseae* (Fm) and (2) soil non-contaminated (Pb_0) and contaminated with 200 (Pb_1), 400 (Pb_2) and 600 (Pb_3) mg kg^{-1} Pb^{2+}. Overall, the study was carried out with 12 treatments in four replicates and a total of 48 pots. Data analyses were performed using two-way analysis of variance (ANOVA) through SPSS software (Version 23). Duncan Multiple Range Test was used to detect significant differences among treatments. Significance was accepted at probability of p <0.05.

Principal Component Analysis (PCA)

PCA is a linear transformation method widely used for dimensionality reduction, visualization, and exploration of multivariate data. This method is an unsupervised pattern recognition method which could be applied for visualizing data trends in a dimensional space and outlier detection. PCA decomposes the data matrix with m rows (samples) and n columns (variables) into the product of a score and a loading matrix. The scores are the positions of the samples in the space of the principal components (PCs), which gives information about the similarity of samples. The data set consisted of 48 samples, including 16 non-inoculated samples, 16 Ri-inoculated and 16 Fm-inoculated. The variables included P and Pb contents and concentrations, fresh and dry weights of root, dry weight of nodules and root colonization percentage for root, and concentrations and contents of P and Pb, fresh and dry weights of shoot, leaf area and chlorophyll index for shoot. Mean-centering was applied as a pretreatment technique and root and shoot results were processed separately by PCA. The PCA model was in-house programmed using MATLAB (the MathWorks, Version 6.0, Natick, MA).

Computation of Plant Performance in the Transfer of Lead from Root to Shoot and Mycorrhizal Growth Dependency (MGD)

To determine plant efficiency in lead translocation from roots to shoots, the following equation was used (Gabos et al., 2009):

TI (%) = TQ (mg / pot) / WPQ (mg / pot) × 100 where, TI= Translocation Index; TQ= element accumulation in the shoots; WPQ= element accumulation in the whole plant. Moreover, root to shoot ratio of Pb concentration was also calculated.

Mycorrhizal growth dependency (MGD) was calculated as (Smith et al., 2003):

$$\mathrm{MGD} = \frac{\text{Plant DW}(+\text{AM}) - \text{Plant DW}(-\text{AM})}{\text{Plant DW}(+\text{AM})} \times 100$$

RESULTS AND DISCUSSION

Mycorrhizal Colonization of Roots

Root colonization significantly decreased by increasing the concentration of Pb in the soil (Fig. 1A, p <0.01). AM treatments significantly affected root colonization rate (Fig. 1A, p <0.01). Root colonization by *R. intrarradices* was significantly higher than that of *F. mosseae* at Pb_0, Pb_1 and Pb_2 levels. There was no significant difference in root colonization percentage between the *R. intraradices* and *F. mosseae* at Pb_3 level (p <0.05). The interactive effect of AM fungi and Pb treatments was not significant on root colonization percentage (Fig. 1A).

Chlorophyll Index and Leaf Area

High levels of Pb significantly decreased chlorophyll index (SPAD values) (Fig. 1D, p <0.001). SPAD values of AM plants were higher than those of NM plants and there was a significant interaction between the levels of Pb and fungal treatments (Fig. 1D, p <0.001). Differences between AM and NM plants at Pb_0 and Pb_1 were not significant while AM plants had significantly higher SPAD values at Pb_2 and Pb_3, compared to NM plants (p <0.05) (Fig. 1D).

Generally, increasing soil Pb concentration decreased leaf area (Fig. 1B, p <0.001). Inoculation of plants with mycorrhizal fungi increased leaf area (Fig. 1B, p <0.001), and the interaction of fungi and Pb levels significantly affected this parameter (Fig. 1B, p <0.01). Ri plants showed greater leaf area by 55.24%, 49.68%, 84.7% and 178.5% at Pb_0, Pb_1, Pb_2 and Pb_3 levels, respectively when compared with NM plants. However, Fm plants had significantly higher leaf area than NM plants only at Pb_3 level (Fig. 1B).

Nodule Dry Weight

Increasing soil Pb concentration had a negative effect on nodule dry weight (Fig. 1C, p <0.01). AM fungi showed a significant effect on nodule dry weight (Fig. 1C, p <0.001); however, the interaction of experimental factors did not affect this parameter significantly (Fig. 1C). Mycorrhizal inoculation significantly increased nodule dry weight at Pb_2 and Pb_3 levels (Fig. 1C, p <0.05). It should be noted that no nodules in the NM plants at Pb_3 level were observed while in AM inoculated treatments alfalfa-rhizobium symbiosis was established.

Plant Biomass and Mycorrhizal Growth Dependency (MGD)

Mycorrhizal inoculation and Pb treatments significantly affected dry and fresh weights of shoot (Table 1, p <0.01) and root (Table 2, p <0.01). Interaction of mycorrhizal fungi and soil Pb concentration also had a significant effect on shoot (Table 1, p <0.05) and root (Table 2, p <0.05) biomass.

Dry and fresh weights of shoot in the Ri plants were significantly higher than NM plants at Pb_0, Pb_2 and Pb_3 levels. However, Fm plants had significantly higher shoot dry weight compared with NM plants only at Pb_3 level (Table 1, p <0.05).

In roots, Ri plants showed significantly higher dry weight than NM plants at Pb_1, Pb_2 and Pb_3 levels while Fm plants had higher dry weight of roots compared with related NM plants at Pb_2 and Pb_3 levels. Moreover, inoculation with both mycorrhizal fungi induced higher root fresh weight at Pb_1, Pb_2 and Pb_3 levels compared with NM plants (Table 2, p <0.05).

Increasing soil Pb concentration increased MGD of both inoculated plants (Table 1). Although all inoculated plants became colonized by AM fungi species, alfalfa plant did not show a positive response to *F. mosseae* at non-contaminated soil (Table 1).

Phosphorus

Increasing soil Pb concentration significantly decreased P concentration of shoots (Table 1, p <0.01) and roots (Table 2, p <0.01). Moreover, mycorrhizal colonization had a significant effect in this respect, and P concentration of inoculated plants in both shoots and roots was higher than that of non-inoculated plants. The interaction of Pb levels and mycorrhizal inoculation on shoot and root P concentrations was not statistically significant (Table 1 and 2).

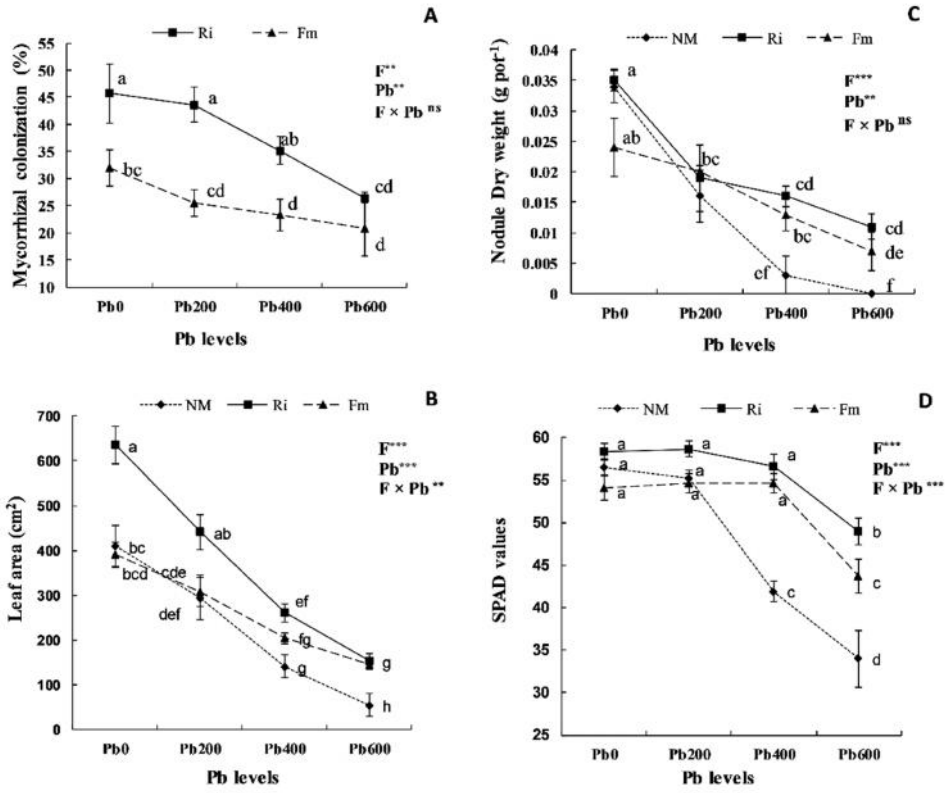

Fig. 1. Effect of mycorrhizal inoculation (F) and soil Pb (Pb) concentrations on root mycorrhizal colonization percentage (A), leaf area (B), nodule dry weight (C), and SPAD values (D). NM, Ri and Fm represent non-mycorrhizal, *R .intraradices* and *F. mosseae* inoculated plants, respectively. Pb0, Pb200, Pb400 and Pb600 represent non-contaminated and contaminated soils with 200, 400, and 600 mg Pb kg^{-1} soil, respectively. The error bars represent the standard error (SE). Treatment effects were tested by two-way ANOVA and are shown with strikes in the figure (ns: non-significant. *, **, ***: Significant at 0.05, 0.01 and 0.001 probability levels). Different letters indicate significant differences according to Duncan's multiple range test ($p < 0.05$).

Table 1. Mycorrhizal growth dependency, shoot fresh and dry weights, P and Pb concentrations and contents in the shoot of alfalfa non-inoculated or inoculated with *R. intraradices* or *F. mosseae* at different levels of lead.

Fungi	Pb Levels	MGD (%)	Pb content (mg pot^{-1})	Pb concentration (mg g^{-1})	P concentration (mg g^{-1})	P content (mg pot^{-1})	Fresh Weight (g pot^{-1})	Dry Weight (g pot^{-1})
	Pb_0	−	−	n.d.	0.929 ± 0.036^{cde}	3.814 ± 0.25^{b}	19.291 ± 1.49^{bc}	4.115 ± 0.29^{b}
NM	Pb_1	−	0.280 ± 0.05^{ab}	0.120 ± 0.002^{ef}	0.783 ± 0.026^{ef}	1.836 ± 0.33^{de}	13.139 ± 1.79^{d}	2.307 ± 0.36^{c}
	Pb_2	−	0.191 ± 0.04^{c}	0.134 ± 0.003^{de}	0.702 ± 0.045^{f}	0.965 ± 0.10^{fg}	7.813 ± 1.31^{e}	1.415 ± 0.24^{de}
	Pb_3	−	0.174 ± 0.05^{c}	0.336 ± 0.011^{a}	0.501 ± 0.061^{g}	0.251 ± 0.06^{h}	2.648 ± 0.80^{f}	0.517 ± 0.13^{f}
	Pb_0	12.02 ± 2.8	−	n.d.	1.322 ± 0.044^{a}	6.524 ± 0.53^{a}	26.311 ± 1.88^{a}	4.910 ± 0.25^{a}
Ri	Pb_1	26.59 ± 2.56	0.285 ± 0.02^{ab}	0.101 ± 0.004^{f}	0.989 ± 0.022^{c}	2.783 ± 0.17^{c}	16.044 ± 0.59^{cd}	2.820 ± 0.19^{c}
	Pb_2	47.30 ± 3.1	0.329 ± 0.02^{a}	0.148 ± 0.022^{d}	0.964 ± 0.081^{c}	2.178 ± 0.14^{cd}	11.239 ± 0.70^{d}	2.257 ± 0.13^{c}
	Pb_3	60.28 ± 4.5	0.234 ± 0.01^{bc}	0.175 ± 0.012^{c}	0.850 ± 0.111^{cdef}	1.146 ± 0.18^{fg}	6.786 ± 0.47^{e}	1.340 ± 0.07^{de}
	Pb_0	-22.72 ± 2.05	−	n.d.	1.035 ± 0.023^{cd}	3.704 ± 0.14^{b}	16.652 ± 0.96^{bc}	3.265 ± 0.13^{bc}
Fm	Pb_1	12.35 ± 2.48	0.252 ± 0.04^{abc}	0.107 ± 0.003^{f}	0.946 ± 0.080^{cd}	2.239 ± 0.08^{cd}	11.632 ± 0.87^{d}	2.365 ± 0.06^{c}
	Pb_2	20.36 ± 3.29	0.227 ± 0.024^{bc}	0.148 ± 0.010^{d}	0.841 ± 0.033^{cdef}	1.293 ± 0.12^{ef}	7.431 ± 0.42^{e}	1.527 ± 0.09^{d}
	Pb_3	47.57 ± 3.9	0.188 ± 0.007^{c}	0.200 ± 0.013^{b}	$0.799 \pm 0..040^{def}$	0.755 ± 0.10^{g}	5.281 ± 0.39^{ef}	1.005 ± 0.07^{e}
Significance								
Fungi			**	**	**	**	**	**
Pb			**	**	**	**	**	**
Pb×Fungi			ns	**	ns	**	*	*

Table 2. Root fresh and dry weights, P and Pb concentrations and contents in roots of alfalfa non-inoculated or inoculated with *R. intraradices* or *F. mosseae* at different levels of lead.

Fungi	Pb Levels	Pb content (mg pot^{-1})	Pb concentration (mg g^{-1})	P concentration (mg g^{-1})	P content (mg pot^{-1})	Fresh Weight (g pot^{-1})	Dry Weight (g pot^{-1})
	Pb_0	−	nd	0.795 ± 0.04^{abc}	1.93 ± 0.28^{ab}	17.16 ± 1.08^{a}	2.48 ± 0.23^{ab}
NM	Pb_1	0.814 ± 0.19^{c}	0.524 ± 0.07^{fg}	0.582 ± 0.11^{bcd}	0.890 ± 0.17^{bc}	7.60 ± 0.44^{cd}	1.53 ± 0.07^{bcd}
	Pb_2	0.823 ± 0.11^{c}	0.907 ± 0.09^{cd}	0.440 ± 0.10^{cd}	0.443 ± 0.15^{d}	6.26 ± 0.45^{d}	0.92 ± 0.15^{e}
	Pb_3	0.822 ± 0.15^{c}	1.827 ± 0.19^{bc}	0.302 ± 0.07^{d}	0.159 ± 0.08^{e}	3.73 ± 1.02^{e}	0.47 ± 0.13^{f}
	Pb_0	−	nd	1.027 ± 0.06^{a}	2.709 ± 0.22^{a}	16.80 ± 1.89^{a}	2.67 ± 0.09^{a}
Ri	Pb_1	1.26 ± 0.33^{c}	0.490 ± 0.09^{ef}	0.733 ± 0.05^{abc}	1.77 ± 0.29^{ab}	15.29 ± 2.40^{a}	2.46 ± 0.29^{a}
	Pb_2	2.17 ± 0.59^{ab}	0.942 ± 0.17^{de}	0.752 ± 0.1^{abc}	$1.69 \pm 0.32b^{ab}$	14.32 ± 1.67^{a}	2.21 ± 0.23^{abc}
	Pb_3	2.29 ± 0.27^{a}	2.064 ± 0.23^{a}	0.725 ± 0.17^{abc}	0.788 ± 0.24^{cd}	8.31 ± 0.44^{bcd}	1.14 ± 0.18^{de}
	Pb_0	−	nd	0.871 ± 0.08^{ab}	1.88 ± 0.21^{ab}	14.35 ± 2.06^{a}	2.18 ± 0.28^{abc}
Fm	Pb_1	0.894 ± 0.10^{c}	0.438 ± 0.05^{fg}	0.558 ± 0.05^{bcd}	1.12 ± 0.10^{bc}	12.39 ± 1.17^{ab}	2.05 ± 0.22^{abc}
	Pb_2	1.408 ± 0.24^{bc}	1.018 ± 0.22^{cd}	0.657 ± 0.12^{bc}	0.912 ± 0.13^{de}	10.64 ± 0.69^{abc}	1.43 ± 0.10^{cd}
	Pb_3	1.238 ± 0.15^{c}	1.396 ± 0.25^{bc}	0.681 ± 0.07^{abc}	0.635 ± 0.10^{cd}	6.97 ± 0.37^{cd}	0.92 ± 0.09^{e}
Significance							
Fungi		**	**	**	**	**	**
Pb		**	**	**	**	**	**
Pb×Fungi		*	*	ns	*	*	*

Data are presented as means ± standard error (SE). Values labeled with different letters are significantly different (alpha = 0.05) according to the Duncan test. Pb_0, Pb_1, Pb_2 and Pb_3 are 0, 200, 400 and 600 mg Kg^{-1} of Pb, respectively. NM, Ri and Fm are non-mycorrhizal, *R.intraradices* and *F. mosseae* inoculated plants. n.d. is not detected. − is not tested.

Mycorrhizal inoculation and Pb treatments had significant effects on P content of both shoot (Table 1, p <0.01) and root (Table 2, p <0.01). Also, the interactive effect of fungi and Pb treatments significantly affected P content in shoot (Table 1, p <0.01) and root (Table 2, p <0.05). There was a statistically significant difference between the amount of P in the shoot of Ri plants and NM plants at all levels of Pb in soil (Table 1, p<0.05). Plants inoculated with both fungal species had more P content in shoot than NM plants at Pb_3 level (Table 1, p<0.05). The P content of Ri plant root was higher than NM plants at Pb_2 and Pb_3 levels. Moreover, Fm plants showed significantly higher P content of root at Pb_3 level compared to NM plants (Table 2, p<0.05).

Lead

Mycorrhizal inoculation and Pb treatments significantly affected Pb concentration of shoot (Table 1, p <0.01) and root (Table 2, p <0.01). Interaction of mycorrhizal fungi and soil Pb concentration also had a significant effect on shoot (Table 1, p <0.01) and root (Table 2, p <0.05) Pb concentration.

Colonization with both fungal species decreased Pb concentration of shoot at Pb_3 level compared with NM plants (Table 1, p <0.05). Moreover, inoculation with *R. intraradices* significantly increased root Pb concentration at Pb_3, while it did not show a significant effect on root Pb concentration at Pb_1 and Pb_2 levels (Table 2, p <0.05).

Increasing Pb concentration in soil significantly decreased the Pb content of shoot (Table 1, p <0.01). Plants inoculated with *R. intraradices* showed higher Pb content compared with NM and Fm plants (Table 1, p <0.01). The interaction of Pb levels and mycorrhizal inoculation on shoot Pb content was not statistically significant (Table 1).

In roots, interaction of Pb levels and mycorrhizal inoculation on Pb content was significant (Table 2, p <0.05). Alfalfa inoculation with *R. intraradices* increased the Pb content of roots compared with NM plants at Pb_2 and Pb_3 levels by 2.6 and 2.8 times, respectively (p <0.05). In addition, the differences between root Pb content of NM and Fm plants were not statistically significant at all Pb levels (Table 2).

Lead Translocation from Root to Shoot

As Pb concentration increased in soil, translocation index significantly reduced (Fig. 2A, p <0.001). The effect of mycorrhizal inoculation was also significant on this factor (p <0.01). Translocation of Pb from root to shoot significantly declined at Pb_3 level in *R. intraradices* inoculated plants by 1.75-fold as compared with non-mycorrhizal plants (Fig. 2A, p < 0.05). However, the interaction of Pb treatment and mycorrhizal inoculation was not significant in this respect.

Mycorrhizal inoculation (Fig. 2B, p <0.05), Pb treatments (Fig. 2B, p <0.01) and their interaction (Fig. 2B, p <0.05) had a significant effect on the root to shoot ratio of Pb. Increasing the Pb concentration in soil increased the root to shoot ratio of Pb in Ri plants considerably. Furthermore, there was no statistically significant difference between Fm and NM plants at all Pb levels (Fig. 2B).

Principal Component Analysis (PCA)

PCA was applied on the data to provide an overview about the relation between the samples by focusing on the effect of mycorrizal fungi and different levels of Pb concentration. The first step was the PCA applied on Ri inoculated by the non-inoculated samples. By using the first three principal components on the shoot data, the total explained variation is equal to 99.54% (PC1 95.29%, PC2 2.94%, PC3 1.31%) indicating that the amount of information which would be lost taking into account the first three PCs is not considerable. The scores for the first three PCs according to four levels of Pb are plotted as a scatter diagram in Fig. 3. It is clear that two distinct clusters are formed in all levels of Pb, corresponding to the samples that were inoculated with Ri and the non-inoculated samples. Moreover, it seems that the effect of Ri is more significant in the presence of higher levels of Pb.

The second step was the PCA applied on Fm inoculated by the non-inoculated samples. By using the first three principal components on the shoot data, the total explained variation is equal to 99.52% (PC1 94.23%, PC2 4.01%, PC3 1.28). In this case, the overall results show that the effect of Fm in high levels of Pb is more significant than the lower levels (Fig. 4). Similar results were also found for roots of inoculated plants. Comparison of the results obtained by PCA demonstrates that Ri symbiosis is more efficient for host plant protection against phytotoxic effect of Pb.

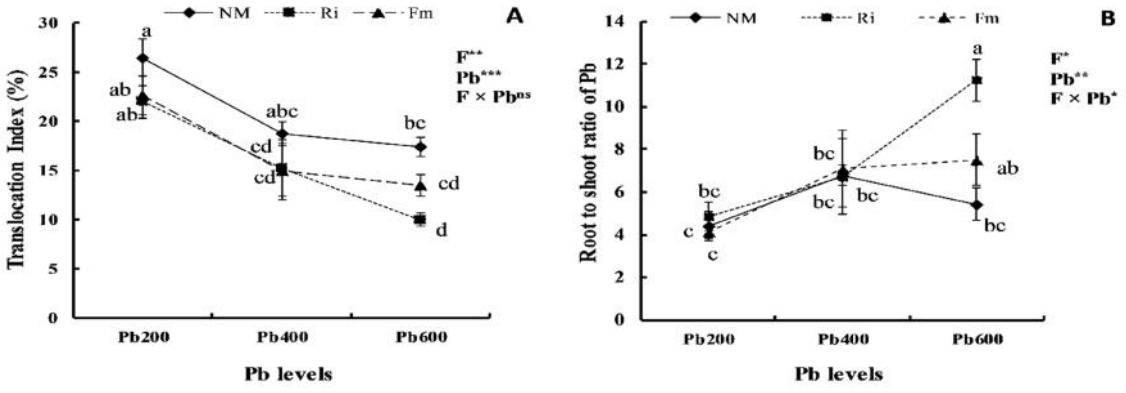

Fig. 2. Effect of mycorrhizal inoculation and soil Pb concentrations on translocation index (A) and root to shoot ratio of Pb concentration (B). The description of symbols is the same as Fig. 1.

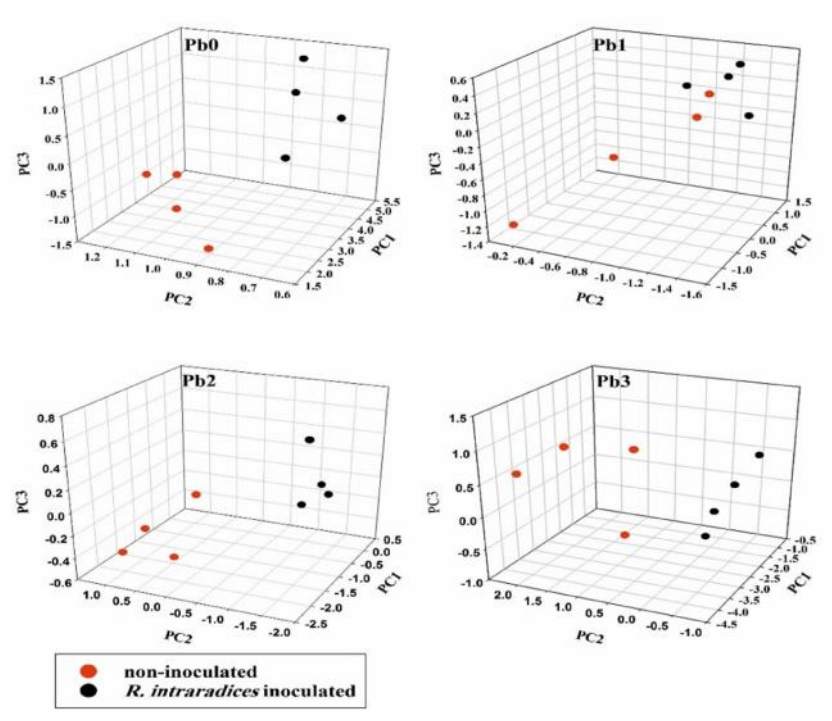

Fig. 3. PCA score plot of non-inoculated samples and inoculated samples with *R. intaradices* derived from plant parameters (plant biomass, chlorophyll content, leaf area, Pb and P concentration and content)

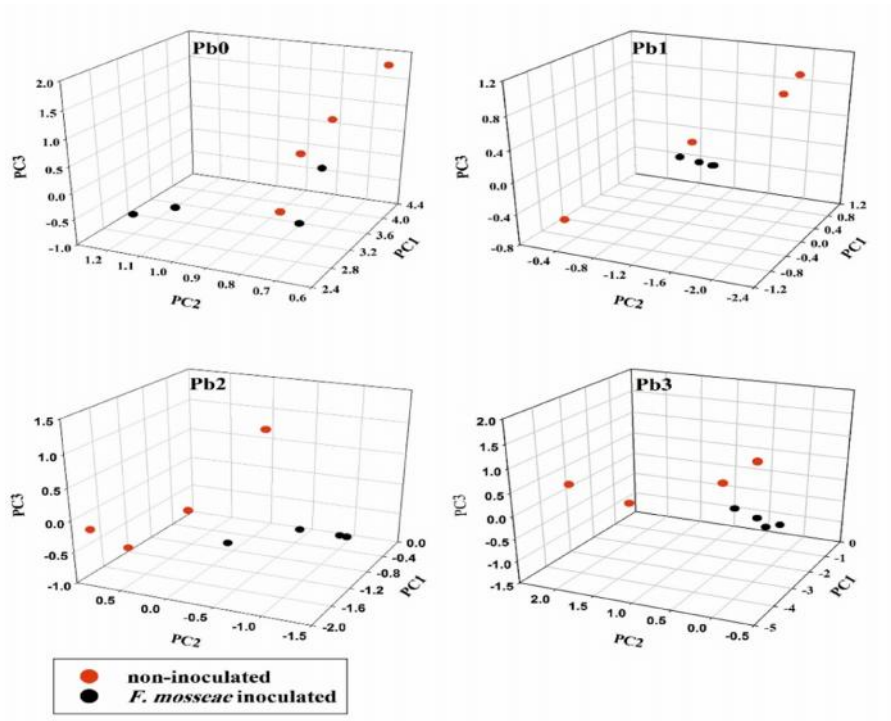

Fig. 4. PCA score plot of non-inoculated samples and inoculated samples with *F. mosseae* derived from plant parameters (plant growth parameters, Pb and P concentration and content)

Phytotoxicity of heavy metals like Pb can be linked to cellular metabolism impairment and disorder in nutrient absorption (Kuper and Kroneck, 2005). It seems that mycorrhizal plants have a greater chance for survival and growth in conditions of heavy metal contaminated soils than non-mycorrhizal plants. Mycorrhizal plants have more contact with the soil bulk due to the widespread net of extraradical hyphae, which can explore a larger volume of the soil that is unavailable for roots in the absence of AM symbiont (Garg and Bhandari, 2016).

Toxicity of heavy metals can inhibit the development of fungal structures including intraradical and extraradical hyphae. Moreover, plant photosynthetic status can determine the fungal colonization rate. It seems that the restriction in carbon supply because of a reduction in chlorophyll content, leaf area, and photosynthesis could negatively affect the establishment of mycorrhizal fungi in roots (Gildon and Tinker, 1983; Pawlowska and Charvat, 2004). In spite of the decreased root colonization percentage, MGD values increased by increasing Pb stress (Table 1). It suggests that mycorrhizal efficiency is partly independent of root cortex colonization by AM fungi.

Enhanced growth of mycorrhizal plants most often is explained in connection with better P nutrition (Andrade et al., 2004). AM fungi are quite effective in P uptake since their hyphae have a greater surface area per unit volume in comparison with plant roots and these fungi can transfer P to the cortical cells of host plant efficiently (Miyasaka and Habte, 2001). On the other hand, lower K_m (higher affinity) for P than plant roots leads to better absorption of P by the fungus, which means that at low concentrations of the ion, the uptake rate of the fungi is greater than that of the roots (Miyasaka and Habte, 2001; Powell, 2018). Other mechanisms, including the decrease in rhizosphere reaction and production of chelating agents by AM fungi, may lead to efficient nutrient uptake by mycorrhizal plants (Miyasaka and Habte, 2001).

Results indicated decreased P concentration in mycorrhizal and non-mycorrhizal plants as soil Pb concentration increased (Table 1 and 2). This might lead to dry weight loss in plants. Mycorrhizal plants showed higher P concentration than non-mycorrhizal plants, which is in agreement with other studies in polluted soils by heavy metals (Sudova and Vosatka, 2007; Diaz et al., 1996; Andrade et al., 2004). Pb induced P deficiency was stated as a mechanism of Pb phytotoxicity (Cheyns et al., 2012) likely due to precipitation of available phosphates as lead phosphates whose solubility is very low (Shotyk and Roux, 2005). The reduction in colonization rate, improper development of extraradical hyphae as well as impairment in the transport of P to the host might be the reasons for the decreased P concentration with increasing soil Pb level in AM plants (Bíró et al., 2012). Despite the greater concentration of P in Fm plants compared to NM plants, the shoot biomass was not significantly different between them at the levels Pb_0, Pb_1 and Pb_2. Conversely, Ri inoculated plants showed higher values for both P concentration and shoot biomass than NM plants. Accordingly, the fungal species had different impacts on host plant facing metal stress.

Mycorrhizal symbiosis induced the root nodule formation, especially at higher levels of Pb. Therefore, improved growth of mycorrhizal plants may also be related to better nitrogen nutrition (Lin et al., 2007). The positive effect of AM symbiosis on aerial part biomass has been reported in contaminated soil with heavy metals (Diaz et al., 1993; Sudova and Vosatka, 2007; Wang et al. 2005; Saleh-Al- garni 2006). The mycorrhization increased root dry weight of mycorrhizal plants, which was similar to the findings of other researchers (Wang et al., 2005; Sudova and Vosatka, 2007). Higher chlorophyll and leaf area at Pb_2 and Pb_3 levels emphasize improved nutrition of mycorrhizal plants (Arriagada et al., 2005; Saleh-al-garni, 2006).

Heavy metal effect on rhizobial symbiosis often appears as a reduction in growth, proliferation, survival, and nodulation of rhizobial bacteria in plant rhizosphere (Obbard and Jones, 1993). Casella et al. (1988) have stated that the toxicity of heavy metals causes the loss of *sym* plasmid (containing the genes for symbiosis) in rhizobial bacteria. Researchers have shown that there is a synergistic interaction between nitrogen-fixing bacteria and AM fungi. Nitrogen-fixing bacteria provide nitrogen not only for plants but also for fungi (Saleh-al-garni, 2006; Andrade et al., 2004). Providing phosphate for nitrogen-fixing bacteria by fungi could be another reason for the synergistic relation among the nitrogen-fixing bacteria and mycorrhizal fungi. Toro et al. (1998) have reported that P is an important factor in root nodules formation. On the other hand, nitrogen fixation is a process that needs to spend large amounts of energetic compounds. Hence, plant photosynthetic capacity and existence of sufficient amounts of carbon compounds can determine nodulation rate (Vance and Gantt, 1992). It seems that mycorrhizal plants had a higher root nodulation rate than non-mycorrhizal plants due to their better growth condition. Similar results in soybean plants in the soil contaminated by lead (Andrade et al. 2004) and beans in the cadmium and zinc contaminated soil (Saleh-al-garni, 2006) have been reported.

Results indicated the accumulation of high concentration of Pb in roots of *R. intraradices* inoculated plants at Pb_3 level (Table 2). However, Pb translocation to plant shoots reduced in this treatment, which can be related to various mechanisms which are used by AM fungi under metal stress condition (Meier et al., 2012). Fixation of heavy metals in the fungal organs could occur through polyphosphate granules (Wong et al., 2007; Chen et al., 2005) and biopolymers in cell wall such as chitin and glomalin (Vogel-Mikus et al., 2006; Gonzalez-Chavez et al., 2004).

Increased root to shoot ratio of Pb and decreased translocation index at Pb_3 level in case of *R. intraradices* inoculated plants is more controversial. A significant increase in the root to shoot ratios of Pb suggests metal immobilization by the mycorrhiza. It seems that the protection of photosynthetic structures from the destructive effects of metal toxicity by the accumulation of toxic metals in roots led to higher

tolerance in stressed-plants (Cicatelli et al., 2010). Similar results in some mycorrhizal legumes have been reported (Lin et al., 2007). Also, the possibility of stress alleviation by mycorrhization due to the dilution effect can be considered as another tolerance mechanism.

CONCLUSIONS

In this study, plants showed symptoms of phytotoxic effects of Pb including chlorosis, wilting of old leaves, decrease in plant biomass, dark brown and brittle roots at the higher level of Pb especially in non-mycorrhizal plants. The role of *R. intraradices* in support of alfalfa plants at all levels of Pb was quite clear according to multivariate analysis. Our data indicated a higher translocation index in non-mycorrhizal plants than *R. intraradices* inoculated plants at high Pb level. We found that the growth of *R. intraradices* inoculated plants was higher in comparison to the non-mycorrhizal controls under Pb stress condition. It seems that better P nutrition and sequestering of Pb in mycorrhizal roots and consequently inhibition of metal transport to shoots resulted in better growth of the mycorrhizal plants. The symbiosis of alfalfa with AM fungi can be suggested as an alternative to enhance alfalfa growth for phytoremediation purposes and a practical approach for exploiting soils polluted with heavy metals.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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بررسی اثر مایه‌زنی میکوریزی بر جذب سرب و رشد گیاه یونجه (*Medicago sativa* L.) در خاک آلوده به سرب

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تحلیل مؤلفه اصلی
شاخص انتقال

چکیده- استقرار همزیستی میکوریز آربوسکولار در ریشه می‌تواند ویژگی‌های ریخت‌شناسی و فیزیولوژیکی گیاهان را تحت تاثیر قرار دهد و ممکن است سبب القا مقاومت در گیاهان رشد یافته در خاک‌های آلوده به فلزات سنگین گردد و متعاقباً نقش مهمی در گیاه پالایی ایفا نماید. در مطالعه حاضر به منظور بررسی اثر همزیستی میکوریزی بر رشد گیاه یونجه و جذب سرب، آزمایشی با دو فاکتور شامل (۱) گیاهان بدون مایه‌زنی میکوریزی (NM) و گیاهان مایه‌زنی شده با دو گونه قارچ میکوریزی *Rhizophagus intraradices* (Ri) یا *Funneliformis mosseae* (Fm) و (۲) خاک غیر آلوده (Pb_0) یا آلوده شده با سطوح ۲۰۰ (Pb_1)، ۴۰۰ (Pb_2) و ۶۰۰ (Pb_3) میلی‌گرم سرب بر کیلوگرم خاک طراحی شد. همه گیاهان بطور یکنواخت با باکتری *Sinorhizobium meliloti* مایه‌زنی گردیدند. نتایج نشان داد که در سطوح بالای سرب مایه‌زنی با هر دو گونه قارچی گره‌بندی در ریشه‌ها و تغذیه فسفر را در مقایسه با گیاهان بدون مایه‌زنی افزایش داد. همچنین وزن خشک بخش هوایی، سطح برگ و شاخص کلروفیل گیاهان مایه‌زنی شده با Ri و Fm بطور معنی‌داری در مقایسه با گیاهان NM بیشتر بود. وابستگی میکوریزی در سطح Pb_3 به میزان سه و چهار برابر به ترتیب در گیاهان Ri و Fm در مقایسه با شرایط بدون آلودگی بطور معنی‌دار (p <0.05) افزایش نشان داد. همچنین انتقال سرب از ریشه به بخش هوایی به طور معنی‌داری (p <0.05) به میزان ۱/۷۵ برابر در گیاهان Ri در سطح Pb_3 کاهش یافت. مقایسه نتایج حاصل از تحلیل مؤلفه‌های اصلی نشان داد که همزیستی*R. intraradices* برای حفاظت از گیاه میزبان در برابر اثر سمی سرب مؤثرتر بود. این نتایج نقش مهم قارچ‌های AM در حفاظت از گیاهان در برابر سمیت فلزات و استقرار گیاه در خاک-های آلوده به سرب را نشان می‌دهد.