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Research Article

Growth and some physiological characteristics of alfalfa (*Medicago sativa* L.) in response to lead stress and *Glomus intraradices* symbiosis

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Abstract

Lead is a nonessential element that has a negative effect on plant growth and development. Plant symbiosis with arbuscular mycorrhizal fungi (AMF) in soils contaminated with heavy metals can affect growth of plants, nutrition and tolerance against heavy metals. In this study, the effect arbuscular mycorrhizal fungi *Glomus intraradices* on the growth, photosynthetic pigments, protein content, proline, phenol and activities of antioxidant enzymes in alfalfa plants under Pb toxicity were examined. The experiment was performed by using two treatments (mycorrhizal and non-mycorrhizal) and five lead concentrations (0, 60, 120, 180 and 240 µm $Pb(NO_3)_2$). Results showed that Pb stress decreased plant growth, photosynthetic pigments and protein content, however, AMF improved them. Amount of proline, phenol and antioxidant enzymes activity increased with lead increasing. The amounts in roots of AMF plants in comparison with non-AMF plants increased. Leaves of AMF plants had more superoxide dismutase activity than non-AMF plants. However proline content, activity of catalase and guaiacol proxidase in leaves of mycorrhizal plants, were lower than those of non-mycorrhizal plants. In addition, mycorrhizal colonization significantly decreased with Pb exposure. The results, suggested that *Glomus intraradices* can decrease Pb toxicity in alfalfa plants.

Keyword: Alfalfa, Antioxidant enzymes, Heavy metals, Lead, Mycorrhiza

Introduction

The contamination of environment by heavy metals (HMs) is of important ecological concern due to its effect on human health through the food chain and its high environmental sustainability (Piechalak *et al.*, 2002). Lead (Pb^{2+}) is one of the most prominent heavy metals in the soil that does not degrade but keeps accumulating. It has numerous direct and indirect effects on plant growth and metabolism (Qureshi *et al.*, 2005). Elevated Pb can be a result of several activities such as mining, electroplating, smelting and the production of leaded gasoline (Eichler *et al.*, 2015). Lead like water, soil and air is naturally present in plants. The absorption of lead from the soil through the roots is an important source in plants. Another important issue is the presence of lead in the atmosphere, which can be another important source of lead in plants by depositing on the leaves of the plants. It has also been reported that the amount of lead in the plants that grow in the air with 1.51 µg/ m^3 Pb are more than plants that grow in the same type of soil but clean air (Torkashvand, 2018). A high amount of this metal impairs plant growth, root elongation, seed germination, chlorophyll production, transpiration, lamellar organization in the chloroplast, and cell division (Sharma and Dubey, 2005). Pb like another HMs induce generation of reactive oxygen species (ROS), which unbalances cellular redox, inactivates enzymes and causes lipid peroxidation (Singh *et al.*, 2010). Plants have some ways to reduce HM toxicity and improve their ability to survive in HM contaminated soil. One way is to scavenge ROS by increasing the activities of antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), guaiacol peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX), to protect plant cells from disruption (Li *et al.*, 2015). Another way is to establish symbiotic association in roots with arbuscular mycorrhizal fungi (AMF) to improve host HM resistance (Joner and Leyval, 2001).

Arbuscular mycorrhizal fungi are one of the most prominent soil microorganisms and main mutual symbiotic organisms of most terrestrial plants. They provide benefits to the host plants (Smith and Read, 1997) such as protection against pathogen and herbivory, alleviation of water stress, enhanced tolerance of salinity, low pH and heavy metals, and biofortification of grain with micronutrients (Ryan and Graham, 2018). AMF also increase plant uptake of relatively immobile nutrients, particularly phosphorus (P), Zinc (Zn) and copper (Cu) (Ryan and Angus, 2003) and consequently they enhance root and shoot biomass and improve plant growth (Vogel-Mikus *et al.*, 2005). In HM polluted soils, AMF can significantly increase the resistance of the host plant to HM by improving plant nutrient acquisition and by affecting the fate of the metals in both soil and plants (Wang *et al.*, 2012). The

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AM symbiosis of *Thlaspi praecox* results in increased phosphate (P) uptake and reduced zinc (Zn) and cadmium (Cd) uptake compared with non-mycorrhizal plants, indicating the major importance of even low amount of symbiosis under HMs stress (Hildebrandt *et al*., 2007). Root AM colonization of plants under heavy metal stress results in expression of specific genes that causes enhance resistance of plants to stress (Rivera-Becerril *et al*., 2005), e.g., metal transporter genes, which are expressed at different levels, and AM symbiosis can regulate the transcription of some genes (Lanfranco *et al*., 2002). In addition to the effect on the uptake of elements, AMF also can affect the translocation from root to shoot by forming extensive hyphal networks. The external mycelium is able to produce glycoprotein (glomalin) with heavy metal binding properties, which prevent the transfer of metals to the aerial parts (Citterio *et al*., 2005). AM fungi also have been shown to enhance antioxidant levels in plants to protect their cells from oxidative stress by scavenging ROS (Gopi *et al*., 2007). According to the sources, colonization of mycorrhizal fungi can protect the host plant from toxic metals. Occurrence of this condition is associated whit improvement of phosphorus uptake that resulted in thinning toxic metals in plants (Lokhandwala *et al*., 2017; Wu, 2017). This study was conducted due to the problem of pollution caused by lead and the dangers of this pollution for plants and humans and also importance of alfalfa as a forage plant that is a major food for livestock. The aim of this study was to investigate the effect of lead on growth and some physiological parameters in symbiosis with mycorrhizal fungi.

Material and methods

Alfalfa (*Medicago sativa* L.) seeds were surface disinfected in 1% sodium hypochlorite solution for 10 mins. and then transferred to petri dishes for germination. Seeds were germinated in the dark and 5-day-old seedlings were placed in pots containing washed and sterilized sand. Mycorrhizal treatment consisted of 20% of the pot volume AMF-inoculum. AMF-inoculum used in the experiments consisted of *Glomus intraradices* was that purchased from Turan Biotic Company, Semnan, Iran. Non-mycorrhizal treatments received mycorrhiza free inoculum. This experiment was performed in Tehran Kharazmi University and glasshouse conditions with high/low temperatures were 25°C/18°C respectively, and 16/8 (day/night) photoperiod. Because high phosphate of soil causes low colonization by AMF, plants were nourished twice a week by modified Hoagland's nutrient solution with half of phosphate concentration. After 45 days of sowing, we made sure that plants consisted of mycorrhiza, the plants were treated with different concentrations of lead. Lead was supplied as $Pb(NO_3)_2$ at five concentrations (0, 60, 120, 180 and 240 µm) and added to Hogland solution. Then, the plants were irrigated twice a week with Hoagland solution containing Pb and plants were harvested after 75 days of growth.

Mycorrhizal colonization: Root samples were washed and stained with cotton blue in lacto-phenol and fungal colonization was assessed according to Trouvelot *et al*. (1986).

Growth parameter: After harvesting, leaves, roots and stems fresh weight was measured. Dry weights were measured after drying in an oven at 70°C for 2 days (Evans and Hughes, 1962).

Total protein: 0.1 gr leaf and root samples were homogenized in 2 mL of 0.1 mol L^{-1} phosphate buffer (pH 6.8). The homogenate was centrifuged at 15000 × g for 20 mins. at 4°C, and the supernatant was used for total protein assessment following the technique described by Bradford (1976).

Photosynthetic pigments: Chlorophyll a, chlorophyll b, total chlorophylls and carotenoid were determined by using the method described by Lichenthaler (1987). 0.2 gr fresh leaves were homogenized in 10 ml of 80% acetone. The absorbance of acetone extract of the leaves was recorded at 470, 647 and 663 nm using a spectrophotometer.

Pigment content was calculated as follows:

Chlorophyll *a*: 12.25 A663 − 2.79 A647

Chlorophyll *b*: 21.5 A647 − 5.1 A663

Carotenoids: (1000 A470 − 1.82 Chlorophyll *a* − 85.02 Chlorophyll *b*) /198

Proline content: To measure proline contents in plant leaves and roots, 0.3 gr fresh parts were homogenized in 10 ml of 3% sulfo salicylic acid. The suspension was centrifuged for 10 mins. at 10000 g. Then, 2 ml of extract were mixed with 2 ml of ninhydrin reagent and 2 ml of pure acetic acid, and the final solution was boiled for 1 hrs. Then the samples were transferred to $0^{o}C$ water for 20 minutes and 4 ml of toluene was added to each sample. Finally, the absorbance of samples was measured at 520 nm (Bates *et al.*, 1973).

Total phenol content: To measure phenol contents in plant roots, 0.1 gr of fresh roots was homogenized in 5 ml acidic methanol (methanol 99.5% and HCl 1%, ratio 99 at 1). Then the extract was placed for 24 hours at $4^{\circ}C$ in darkness. Then, extract centrifuged at 4000 g for 10 mins. and 2 ml of the supernatant was added to 2 ml eter. Finally, the absorbance of samples was measured at 280 nm (Dai *et al*., 2006).

Enzyme extraction and assay: For enzyme extraction 0.25 gr leaf and root samples were homogenized in 2 mL phosphate buffer (0.1 mol L^{-1}; pH 6.8) and centrifuged at 15000 g for 20 mins. at $4^{\circ}C$, and the supernatant was used for superoxide dismutase (SOD), guaiacol proxidase (GPX) and catalase (CAT) assays.

SOD activity: SOD activity was assayed by monitoring its inhibition of the photochemical reduction of metyl tiazol tetrazolium (MTT) at 560 nm. Each 3 mL reaction mixture contained 50 mmol L^{-1} phosphate buffer (pH 7.3), 13 mmol L^{-1} methionine, 75 mmol L^{-1}

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MTT, 0.1 mmol L^{-1} EDTA, 4 mmol L^{-1} riboflavin. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of MTT reduction, and SOD specific activity was expressed as units mg^{-1} protein (Giannopolitis and Ries, 1977).

SOD activity was assayed by monitoring the inhibition of photochemical reaction of MTT, as described by Giannopolitis and Ries (1977). The amount of enzyme required to cause 50% inhibition of the reduction of MTT at 560 nm was defined as 1 unit of SOD activity.

GPX activity: To estimate the activity of guaiacol proxidase enzyme, 3 ml reaction mixture was prepared. It contained 2750 µl Phosphate buffer (25 mM, pH 6.8), 100 µl guaiacol (25 mM), 100 µl hydrogen peroxide (40 mM) and 50 µl enzyme extract. The guaiacol peroxidase activity was measured at 470 nm. Enzyme activity was calculated based on the changes in absorbance at 470 nm per mins. per mg protein (Dazy *et al*., 2008).

CAT activity: To estimate the activity of catalase enzyme, 3 ml reaction mixture was prepared. It contained 2800 µl Phosphate buffer (50 mM, pH 7), 100 µl Hydrogen peroxide (15 mM) and 100 µl enzyme extract. The absorbance of the prepared extraction were measured at 240 nm. Enzyme activity was calculated based on the changes in absorbance at 240 nm per mins. (Chance and Maehly, 1955).

Statistical analysis: All results were expressed as the means ± SD of four independent replicates. Analysis of variance (ANOVA) was used to test all experimental data. Significant differences were detected using Duncan's multiple range test ($P<0.001$) by means of statistical product and service solutions SPSS software.

Results

Mycorrhizal colonisation: The presence of arbuscules and vesicles in the roots of AMF plants was observed in figure 1a. Also, figure 1b showed the percentage of root colonization by AMF decreased with increasing Pb concentrations.

Growth parameters: Table 1 showed the growth responses of alfalfa plants grown under different Pb concentrations in the presence and the absence of AMF. Results indicated that lead significantly reduced fresh and dry weight of the leaves and stems but root fresh weight was not affected by the lead. AM fungi inoculations significantly increased the fresh and dry weights of the leaves, roots and stems of the alfalfa plants in presence of AMF compared to the observed non-AMF plants ($P<0.001$).

Protein contents: Significant changes were observed in total protein content of leaves and roots alfalfa plants by different concentrations of Pb (Figure 2). Mycorrhizal plants showed higher protein content compared to the non-AM plants, although this difference was not statistically significant.

Photosynthetic pigments: Photosynthetic pigments (chlorophyll a, chlorophyll b and total chlorophylls) significantly decreased with lead treatment in both AMF and non-AMF plants (Table 2). AMF plants showed significantly higher pigments contents than that observed in non-AMF plants.

Carotenoid content: Also, table 2 showed a significant decrease in carotenoids content with increasing Pb concentrations in both AMF plants and non-AMF plants ($P<0.001$). Mycorrhizal plants also showed higher carotenoids content compared to the non-AM plants, although this difference was not statistically significant.

Proline content: Our results revealed that amount of proline in the leaves of AMF plants and non-AMF plants increased significantly by increase in Pb concentration (table 2). Amount of proline in the root of AMF plants in comparison with non-AMF plant increased significantly ($P<0.001$).

Total phenol content: Our results indicated that the total phenol content of roots increased significantly by lead concentrations (Table 2). Amount of phenol in the root of AMF plants in comparison with non-AMF plant increased, but mycorrhiza had no significant effect on content of phenol content in roots.

Enzymes activities: SOD activity in the roots and leaves of AMF plants and non-AMF plants increased by increasing Pb $(NO_3)_2$ concentration, however, this change in roots was not significant. Activity of SOD in both of roots and leaves of AMF plants increased significantly compared with the non-AMF plants (Figure 3).

GPX and CAT activities for both leaves and roots, significantly increased under Pb treatments in AMF and the non-AMF plants. Activities of those enzymes in the leaves of non-AMF plants in comparison with AMF plants increased, but this was no significant (Figure 4 and 5).

Discussion

Mycorrhizal colonization: In this present study, mycorrhizal colonization significantly decreased with Pb exposure. The results suggest that contamination with high Pb concentration could inhibit the development of AMF in soil, as Chen *et al*. (2005), indicated the inhibition of mycorrhizal spore germination and extra radical hyphal growth under a stressful environment. Reduction in mycorrhizal colonization by heavy metals has been extensively studied (Andrade *et al*., 2004; Liao *et al*., 2003). The toxicity of heavy metals limits the extension of intracellular hyphae and high concentrations of HM effect on the extracellular hyphae. On the other hand, the expansion of symbiotic depends on the conditions of the host plant and unfavorable conditions of the host, colonization is also effective (Pawlowska and Charvat, 2004).

Growth parameters: Pb stress decreased fresh and dry weight of the roots, leaves and stems significantly. Plant growth retardation from Pb exposure may be attributed to disruptions in the processes of nutrient metabolism and photosynthesis (Pourrut *et al*., 2011;

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Table 1. Effect of $Pb(NO_3)_2$ on growth characteristics of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants

$Pb(NO_3)_2$ (µM)	AMF	Leave fresh weight	Root fresh weight	Stem fresh weight
		(g)		
0	M	0.0726 ± 0.003 [a]	0.088 ± 0.0055 [a]	0.081 ± 0.005 [a]
	NM	0.0594 ± 0.003 [bc]	0.083 ± 0.0058 [a]	0.069 ± 0.0041 [abc]
60	M	0.064 ± 0.002 [ab]	0.084 ± 0.0044 [a]	0.079 ± 0.0068 [ab]
	NM	0.0506 ± 0.001 [de]	0.075 ± 0.0035 [a]	0.062 ± 0.0038 [c]
120	M	0.06 ± 0.002 [bc]	0.078 ± 0.0037 [a]	0.070 ± 0.0056 [ab]
	NM	0.046 ± 0.002 [ef]	0.051 ± 0.0049 [b]	0.046 ± 0.0034 [d]
180	M	0.0603 ± 0.001 [bc]	0.076 ± 0.0031 [a]	0.068 ± 0.0045 [abc]
	NM	0.0409 ± 0.002 [e]	0.043 ± 0.0044 [b]	0.041 ± 0.0022 [d]
240	M	0.0554 ± 0.002 [cd]	0.074 ± 0.0030 [a]	0.065 ± 0.0023 [bc]
	NM	0.0383 ± 0.004 [e]	0.038 ± 0.0049 [b]	0.036 ± 0.0026 [d]

Values are means ± SE. Values with similar letters are no significantly different

Continue of table 1.

$Pb(NO_3)_2$ (µM)	AMF	Leave dry weight	Root dry weight	Stem dry weight
		(g)		
0	M	0.0097 ± 0.0004 [a]	0.0085 ± .0006 [a]	0.0109 ± 0.0007 [a]
	NM	0.0086 ± 0.0005 [abc]	0.0069 ± 0.0004 [bc]	0.0083 ± 0.0006 [bc]
60	M	0.0091 ± 0.0005 [ab]	0.0080 ± 0.0004 [ab]	0.01 ± 0.0012 [ab]
	NM	0.0072 ± 0.0006 [cd]	0.0058 ± 0.0004 [cd]	0.0071 ± 0.0007 [cd]
120	M	0.0085 ± 0.0003 [abc]	0.0069 ± 0.0004 [bc]	0.0089 ± 0.0005 [bc]
	NM	0.0062 ± 0.0004 [de]	0.0048 ± 0.0004 [de]	0.0056 ± 0.0003 [de]
180	M	0.0083 ± 0.0004 [abc]	0.0063 ± 0.0003 [bc]	0.0086 ± 0.0005 [bc]
	NM	0.0055 ± 0.0004 [e]	0.0043 ± 0.0003 [de]	0.0054 ± 0.0002 [de]
240	M	0.0079 ± 0.0005 [bc]	0.0065 ± 0.0002 [bc]	0.0078 ± 0.0002 [c]
	NM	0.0049 ± 0.0004 [e]	0.0039 ± 0.0004 [e]	0.0049 ± 0.0003 [e]

Values are means ± SE. Values with similar letters are no significantly different

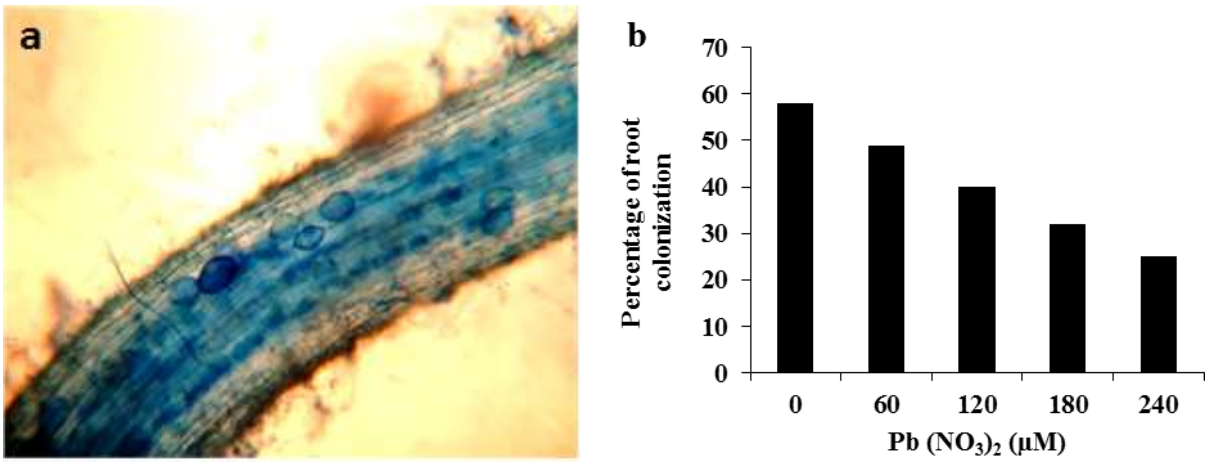

Figure 1. Longitudinal cut of mycorrhizal root and presence of vesicles and arbuscules in the root zone cortex with magnification (×10) (a), Effect of $Pb(NO_3)_2$ on the Percentage of colonization in the mycorrhizal plants (b)

Zanganeh *et al*., 2019).

The present study observed that the fresh and dry weight of roots, leaves and stems of the mycorrhizal plants were higher compared to that observed in non-mycorrhizal plants. Gupta *et al*. (2009) have also reported similar results in *Zea mays* subjected to pb stress. The results are also in agreement with prior studies conducted using other plant species and metals (Andrade *et al*., 2009; Arriagada *et al.*, 2005), which indicate the major contribution of AMF inoculation to plant growth under metal stress conditions. Mycorrhizae could improve plant growth by increasing P uptake(Zhang *et al*., 2010a). Similar to our results, previous studies have also shown that mycorrhiza increased plant fresh and dry weights in plants (Rivera-Becerril *et al*., 2002; Jurkiewicz *et al*., 2004). The higher density of extra radical hyphae in soil, the higher absorption surface, and the more effective mycorrhizal plants can assimilate these low-mobility metal nutrients (Jansa *et al.*, 2013). Enhanced acquisition of P,

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Table 2. Effect of $Pb(NO_3)_2$ on some characteristics of mycorrhizal (M) and non-mycorrhizal (NM) alfalfa plants

$Pb(NO_3)_2$ (µM)	AMF	Cholorophyll *a* content	Cholorophyll *b* content	Total cholorophyll content	Carotenoid content
		(mg g^{-1} FW)			
0	M	1.29 ± 0.091 [a]	0.82 ± 0.025 [a]	2.11 ±0.15 [a]	0.312 ±0.035 [a]
	NM	1.19 ± 0.132 [a]	0.73 ±0.058 [ab]	1.92 ± 0.21 [ab]	0.331 ± 0.036 [a]
60	M	1.11 ± 0.082 [ab]	0.74 ± 0.026 [ab]	1.85 ± 0.14 [bc]	0.295 ±0.015 [a]
	NM	0.99 ± 0.041 [be]	0.62 ± 0.030 [d]	1.61 ± 0.09 [d]	0.282 ± 0.019 [a]
120	M	1.01 ± 0.049 [b]	0.64 ± 0.047 [bcd]	1.65 ±0.09 [cd]	0.225 ± 0.027 [b]
	NM	0.75 ± 0.032 [ed]	0.45 ± 0.049 [e]	1.25 ± 0.10 [e]	0.221 ±0.024 [b]
180	M	0.99 ± 0.071 [bc]	0.61 ± 0.031 [cd]	1.60 ±0.18 [d]	0.138 ± 0.021 [c]
	NM	0.62 ± 0.035 [d]	0.41 ±0.024 [e]	1.02± 0.05 [ef]	0.115 ± 0.034 [e]
240	M	0.89± 0.062 [bc]	0.59 ± 0.023 [d]	1.48 ± 0.17 [d]	0.107 ± 0.009 [c]
	NM	0.60 ± 0.041[d]	0.038 ± 0.0049 [e]	0.98± 0.08 [f]	0.099 ± 0.008 [e]

Means ± SD of three replicates followed by the same letters are not significantly different according to Duncan's multiple range test at P≤ 0.001

Continue of table 2.

$Pb(NO_3)_2$ (µM)	AMF	Leave proline content	Root proline content	Root total phenol content
		(µg g^{-1} FW)		
0	M	8.26 ± 0.5 [d]	32.5 ± 2.8 [c]	2041 ± 465 [b]
	NM	11.13 ± 0.73 [d]	21.5 ± 1.6 [d]	1959.3 ± 428 [b]
60	M	15.8 ± 1.3 [c]	34.6 ± 2.7 [c]	3566 ± 337 [a]
	NM	19.1 ± 0.8 [c]	30.8 ± 2.1 [c]	3229 ± 232 [ab]
120	M	25.08 ± 1.95 [b]	49.8 ± 0.8 [ab]	4387 ± 397 [a]
	NM	25.83 ± 1.6 [b]	43.2 ± 3 [b]	3486 ± 451.5 [a]
180	M	31.16 ± 2.58 [a]	53.7 ± 3.4 [a]	4392 ± 482 [a]
	NM	34.7 ± 1.7 [b]	49 ± 1.5 [ab]	3903.3 ± 510 [a]
240	M	34.2 ± 1.5 [a]	50.3 ± 3.1 [ab]	4616.3 ± 419.5 [a]
	NM	34.8 ± 1.9 [a]	49.4 ± 1.8 [ab]	4373.5 ± 453 [a]

Means ± SD of three replicates followed by the same letters are not significantly different according to Duncan's multiple range test at P≤ 0.001

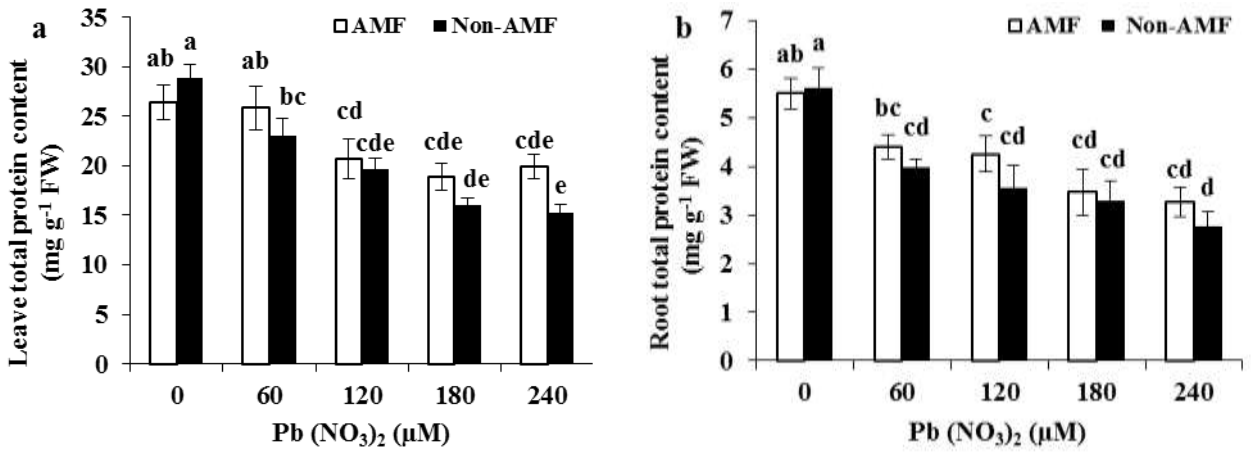

Figure 2. Effect of $Pb(NO_3)_2$ on the total protein content (mg g^{-1} FW) in the leaves (a) and roots (b) of AMF and non-AMF plants. Means ± SD of three replicates followed by the same letters are not significantly different according to Duncan's multiple range test at P≤ 0.001.

Cu, Zn and Fe by mycorrhizal plants has been extensively studied (Kafkas and Ortas, 2009; Navarro Garcia *et al*., 2011). Allen *et al*. (1982) showed that changes in plant hormone levels occur in mycorrhizal plants. Mycorrhizal plants showed an increase in gibberellin content, which in turn improved plant growth. Increase in the fresh weight of roots in symbiosis with *Glomus intraradices* also increased auxin biosynthesis in corn (Kaldorf and Ludwig-Muller, 2000).

Protein content: In this study, the amount of protein content in both plants mycorrhizal and non-mycorrhizal plants significantly decreased with increase in lead stress. Decreased levels of protein content in heavy metal exposed plants have been previously reported (Garcia *et al*., 2006; Piotrowska *et al*., 2009). Damage

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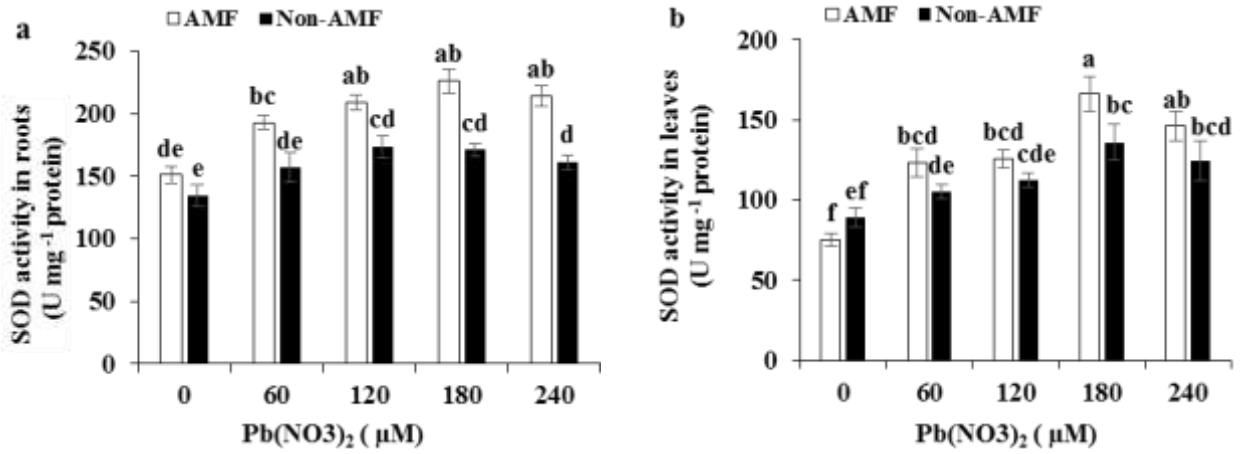

Figure 3. Effect of $Pb(NO_3)_2$ on the activity of SOD in the roots (a) and leaves (b) of AMF and non-AMF alfalfa plants. Means ± SD of three replicates followed by the same letters are not significantly different according to Duncan's multiple range test at P≤ 0.001.

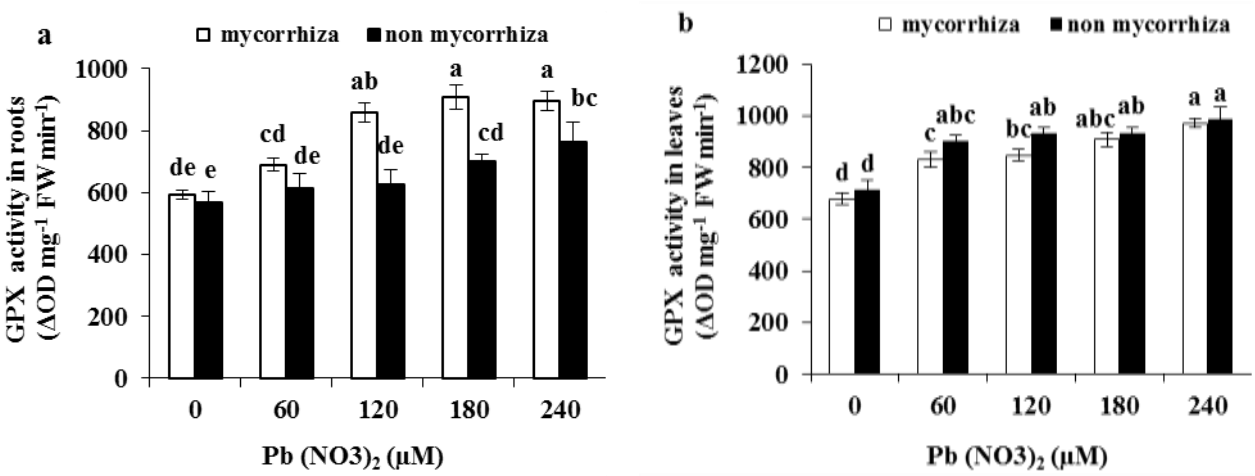

Figure 4. Effect of $Pb(NO_3)_2$ on the activity of GPX in the roots (a) and leaves (b) of AMF and non-AMF alfalfa plants. Means ± SD of three replicates followed by the same letters are not significantly different according to Duncan's multiple range test at P≤ 0.001

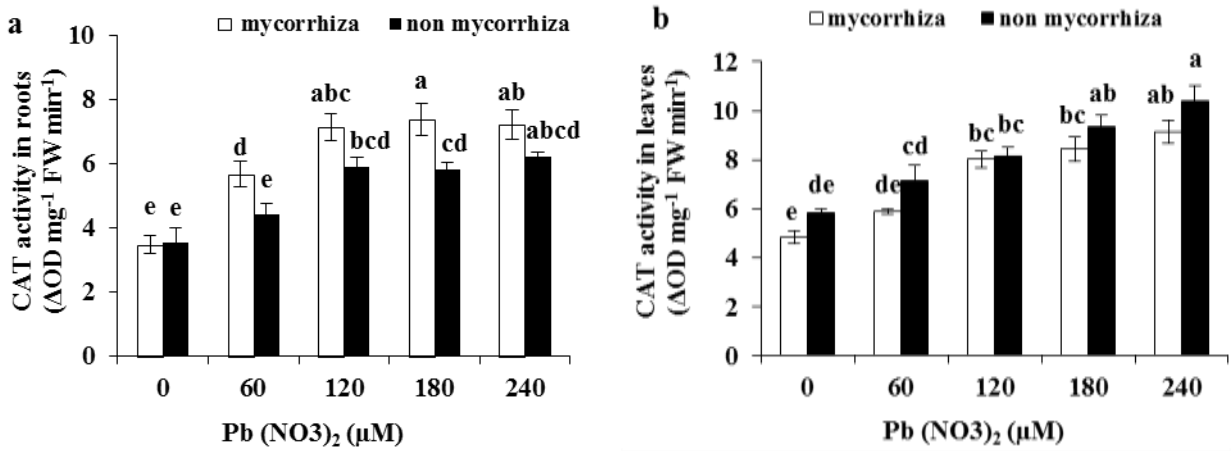

Figure 5. Effect of $Pb(NO_3)_2$ on the activity of CAT in the roots (a) and leaves (b) of AMF and non-AMF alfalfa plants. Means ± SD of three replicates followed by the same letters are not significantly different according to Duncan's multiple range test at P≤ 0.001.

of oxidative stress of ROS, the use of protein for Pb detoxification (Gupta *et al*., 2009), modification in gene expression (Kovalchuk *et al*., 2005) and increased ribonuclease activity (Gopal and Rizvi, 2008) are the principal reasons why protein content is decreased.

The protein levels in mycorrhizal plants were higher

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than the non-mycorrhizal plants in both roots and leaves. Similar results have also been previously reported (Lenin *et al*., 2010; Shinde and Khanna, 2014). Symbiosis with the fungus by host plant contributes to high concentrations of protein storage in the shoots and roots (Subramanian and Charest, 1998). Studies have shown that nitrogen levels increased in mycorrhizal plants, which can be a factor in improving protein content in plants (Cheng and Baumgartner, 2006). AMF plants under heavy metal stress results in expression of several genes responsible for production of proteins (including metallothioneins) that enhance the tolerance of plants to stress (Rivera-Becerril *et al*., 2005). Metallothionein is metal-binding protein produced in many organisms when exposed to high concentrations of heavy metals such as Cu, Pb and Cd.

Photosynthetic pigments: Pigment content in both mycorrhizal and non-mycorrhizal plants decreased significantly with increaseing Pb stress. The results could be partly related to the peroxidation of chloroplast membranes as mediated by Pb, leading to chlorophyll degradation and photosynthesis inhibition (Gajewska *et al*., 2006). It has also been previously reported that carotenoids reduce the effects of Pb exposure (Haider *et al*., 2006). Photosynthesis is adversely affected by Pb, which could be due to metal-induced reductions in the levels of photosynthetic pigments, inhibition of the electron transport system, changes in the fine structure of chloroplasts, and stomatal closure (Li *et al*., 2012). Ahmad *et al*. and Islam *et al*. have described a strong relationship between Pb application and the reduction in whole plant photosynthesis, which is believed to result from stomatal closure (Ahmad *et al*., 2008; Islam *et al*., 2008).

In this study, chlorophyll content was lower in non-mycorrhizal plants compared to mycorrhizal plants under lead stress conditions, indicating the possible mechanism of lead detoxification induced by AMF symbiosis. AMF facilitates P uptake from soil. Therefore, mycorrhizal fungi can increase pigment content by increasing P uptake. Content of chlorophyll in mycorrhizal plants is generally higher than that in non-microbial plants (Song, 2005*; Tohidi Moghadam et al*., 2018). Arbuscular mycorrhizal symbiosis increased the rate of photosynthesis, thus enhanced the rates of photosynthetic storage and export (Auge, 2001). Also AMF reduced translocation Pb to shoot and decrease its toxicity, thus pigments were enhanced in the shoots. These results indicated that the formation of AMF could improve pigment content in leaves. Similar results have been reported by prior-researches (Morte *et al*., 2000; Hongwen Xu and Tong, 2018).

Proline: In this study, the amount of proline was increased significantly by lead stress. Proline has been previously shown to accumulate in plants under heavy metal stress conditions, indicating a protective or a regulatory role (Chen *et al*., 2001; Sharma and Dietz, 2006). Similar results were reported in plants under heavy metal condition (Nalini and Prakash, 2002). Plants under HM stress may also alter changed amino acid contents, especially proline. Accumulation of proline in plants under HM stress was considered to be a traitof adaptation process. One of the proposed roles of proline is to reduce free radicals levels and metal-induced proline accumulation in plant tissues (Abdelmoneim *et al.*, 2014).

Proline of leaves was found more in non-mycorrhizal plants than mycorrhizal plants. This was due to AM fungi which helps the host plant during heavy metal condition. Mycorrhizal plants synthesize less amount of proline than non-mycorrhizal plants. This clearly indicated that the mycorrhiza helps the plants during lead stress conditions, hence they do not synthesize proline in more concentration, whereas the non-mycorrhizal plants need proline as osmolytes for its survival so that they synthesized more proline than mycorrhizal plants. Zhu *et al*. (2010) reported proline content was lower in the AM maize leaves than that in the non-AM plants. However, Abdel Latef and Chaoxing (2011) found AM tomato plants accumulated less proline than the non-AM plants. Zhu *et al*. (2010) suggested that the change of leaf proline level reflects the degree of injury of mycorrhizal plants by the stress, and if the stress was moderate, there was no need to synthesize more proline for osmotic adjustment protection.

Roots mycorrhizal plants have more proline in comparison with non-AMF plant. This was due to AM fungi which helps the host plant during stress condition. Proline as a nitrogen-containing compound may be greater in mycorrhizal plants as mycorrhizal fungi have the ability to increase nitrogen uptake under stress conditions (Azcon and Tobar, 1998). Porcel and Ruiz-Lozano (2004) found that in comparison with non-mycorrhizal plants one species of *Glomus* increased of proline content in roots and decreases of proline content of leaves in drought stress.

Phenol: In this study, the amount of phenol in both plants mycorrhizal and non-mycorrhizal was increased significantly as there was increase in lead stress. Increasing the amount of phenol in of plants under heavy metal stress has been reported (Michalak, 2006). Phenolic antioxidants inhibit lipid peroxidation by trapping the alkoxyl lipid radical, which depends upon the structure of the molecules and the number and position of the hydroxyl group in the molecules (Senanayake *et al.*, 2013). Phenolic compounds stabilize the membranes by reducing the membrane fluidity and prevent the diffusion of free radicals and limit the peroxidative reaction (Verstraeten *et al*., 2003). In this study, the amount of phenol in the roots of AMF plants in comparison with non-AMF plants increased. Similar results were reported (Lima *et al*., 2015; Pedone-Bonfim *et al*., 2013) that showed it is produced by mycorrhiza. Also Wu *et al*. (2010) revealed contents of secondary metabolites in roots are increased by mycorrhization that is positively associated with resistance to replant disease.

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Enzymes activities: Pb has a high oxidative potential that can produce reactive oxygen species (ROS). ROS cause oxidative damages in plants. In response to oxidative stress, plants have developed defense systems to scavenge the ROS. SOD, CAT and GPX can protect the cells from oxidative injury under Pb-induced oxidative stress (Singh *et al*., 2010; Gupta *et al*., 2009)

In this study, increasing concentrations of Pb caused a significant increase in total SOD, CAT and GPX activities in the leaves and roots of both AMF and non-AMF alfalfa plants. Similar results were reported (Sharma and Dubey, 2005; Chaoui and Ferjani, 2005). The present research showed in the roots, activity of SOD, CAT and GPX in response to lead in AMF plants were higher than in non-AMF plants, may be due to the accumulation of ROS in the roots (Fester and Hause, 2005) that these activities helped to reduce oxidative damage to biomolecules (Becana *et al.*, 2000). Increasing of enzymes activities in roots of mycorrhizal plants than non-mycorrhizal has been observed previously (Labidi *et al*., 2011). SOD activity in leaves of mycorrhizal plants was higher than the non-mycorrhizal plants. Maya and Matsubara (2013) observed activity of SOD of AMF-plants was greater than non AMF plants. However, zero or negative effects of AM symbiosis on some enzymatic activities were observed (Zhu *et al*., 2010; Abdel Latef and Chaoxing, 2011). The aforementioned results suggest that AM plants possess higher antioxidant enzyme activities, but the response of theindividual enzymes varies with respect to the AM fungal and the host plant species (Zhu *et al*., 2017).

Enhance of antioxidant enzyme activity in leaves of mycorrhizal plants, may suggest a better grow under heavy metal stress (Zhang *et al*., 2010b). Yu *et al*. (2009) suggested that mycorrhizal plants had reduced generation of ROS in aerial parts and therefore less antioxidative enzymes were synthesized. However, AMF-plants had lower activities of CAT and GPX in leaves than in non-AMF plants. Increasing of enzymes activities in leaves of non-mycorrhizal plants compared to mycorrhizal plants under heavy metal stress has been reported (Rahmati and Khara, 2011). This finding supports results from numerous studies reporting that AMF often protect plants against high accumulation of toxic element in leaves, as it was reports for Pb (Malvoca *et al*., 2003).

Conclusion

The results of this study showed that alfalfa inoculation with *Glomus intraradices* improves the plant growth in the presence of lead. The AM symbiosis improved plant photosynthetic pigments, total protein and content of phenol under lead stress. Also, *Glomus intraradices* enhanced antioxidant enzyme activities. Therefore, AMF could be an efficient and beneficial for decreasing toxic effects of lead in the studied plant. It is recommended to use for symbiotic with *Glomus intraradices* in soils of HM-polluted for resistance to stress in plants.

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