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Lead phytotoxicity on some plant growth parameters and proline accumulation in mycorrhizal tomato (*Lycopersicon esculentum* L.)

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Abstract

Interaction between mycorrhizal fungi and plants under heavy metal polluted condition can alleviate stress condition for plants. This may be due to the better nutrition in mycorrhizal plants than non-mycorrhizal ones. In this study we assayed the chlorophyll content, leaf area, proline concentration, phosphate and lead contents, mycorrhizal dependency and efficiency of photosystem II (PS II) of plants. Tomato plants were inoculated either with *Glomus intraradices* or *Glomus mosseae*. Control plants were left un-inoculated as non-mycorrhizal treatments. Four levels of Pb^{2+}, including 0, 50, 100 and 150 mg L^{-1} were used as $Pb(NO_3)_2$. Increasing Pb^{2+} concentration decreased leaf area, efficiency of photosystem II, chlorophyll content, root colonization rate, shoot and root phosphate content significantly. Also, increasing lead increased root lead content and root to shoot ratio of Pb^{2+} concentration. Raising the lead concentration has no significant effect on shoot lead content. Mycorrhizal dependency increased with increasing the lead concentration. Generally, 150 mg L^{-1} of lead treated mycorrhizal plants had better growth in comparison to non-mycorrhizal plants. It can be concluded that mycorrhizal inoculation increases tomato plant tolerance to lead which may be related to either modified prolin metabolism (specially in *Glomus intraradices* inoculated plants), better phosphorus nutrition or lead sequestration in mycorrhizal roots.

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Introduction

Heavy metals are considered as important abiotic factors limiting plant growth and yield in many areas on earth. Heavy metals such as lead, mercury, arsenic, copper, zinc and cadmium get noticed as potent environmental pollutants. Lead is an important pollutants in the agricultural soils that originates from various sources like long-term application of urban sewage sludge and agrochemical usage (Khan, 2006). Heavy metals toxicity affects major metabolic activities such as respiration and photosynthesis. The severity of symptoms depends on the lead concentration, exposure time and the stage of plant growth. Heavy metals cause proline accumulation in plants as it occurs with other stresses such as salinity, temperature, drought and pathogen invasions (*Verbruggen and Hermans, 2008)*. It seems that proline can increase the plant tolerance through osmoregulation, inhibition of macromolecules denaturation and the protection of protein synthesis (Kuznetsov and Shevyakova, 1997; Ruiz-lozano, 2003). However only a small proportion of toxic metals achieves the chloroplasts, photosynthetic processes are the main domain which affected by heavy metals toxic effect (Sarvari, 2005). In polluted areas, microbial activities in the plant rhizosphere can alter the bioavailability of metals to plants. One of the most important microorganisms that usually are known as modulatig the toxic effect of metals to plant, are AM (arbuscular mycorrhiza) fungi (Biro and Takacs, 2007). There are two possible mechanisms for mycorrhizal plants to tolerate metal toxicity. Mycorrhizal fungi in roots possibly play a role as a barrier against metal transportation from roots to the shoot. This is attributed to metal adsorption on the hyphal walls by several chemical groups like hydroxyl, carboxyl, phosphoryl and phenolic groups or metal complexation with organic acids such as citric and oxalic acids in the rhizosphere (Joner *et al.*, 2001). Another possible tolerance mechanism is the dilution effect. Dillution effect be accomplished by increased plant growth because of better nutrition of mycorrhizal plants in comparision to non-mycorrhizal ones. But all types of fungi are not effective in protecting the host plants equally (Huang and Tao, 2004).

Our hypothesis was that mycorrhizal inoculation can modulate the toxic effects of lead in tomato plants. In the present work to test our hypothesis, we assayed effect of three concentrations of lead on some plant growth and photosynthetic parameters and proline in inoculated plants with two AM fungal species in comparision to non-inoculated plants.

Material and methods

Plant culture and arbuscular mycorrhizal fungi inoculation

Healthy and uniform *Lycopersicon esculentum* L. seeds were surface-sterilized with 1% sodium hypochlorite and then they were sown in plastic basins containing autoclaved perlite (autoclaved at 121°C for 60 min) and AM fungi *Glomus mosseae* (Gm) or *Glomus intraradices* (Gi). The inocula were mixed (50%v/v) with perlite (full volume). Non-mycorrhizal (NM) treatment received the same amount of autoclaved inoculum. After 25 days the inoculated and control plantlets were taken gently to the polyethylene pots containing 3 liters acid washed and autoclaved sand (1.2–2 mm). The soil based mycorrhizal inoculum (spores, roots and hyphae) of Glomus species including *Glomus intraradices* or *Glomus mosseae* (isolated from The *Angooran Lead* and *Zinc Mine* located in Zanjan province-*Iran*) obtained from Shiraz University were used in this study.

Plant treatments and growth conditions

The pots were kept in growth room with 16h photoperiod, max 60% relative humidity and temperatures of 28 ± 2°C (day) and 20 ± 2°C (night) and 200 µmol/m² s light irradiance. The plants were received Long-Ashton nutrient solution with 64 µM phosphate (Hewitt, 1966) twice weekly and they were watered intermittently as needed with distilled water. Four levels of Pb^{2+}, including 0, 50, 100 and 150 mg L^{-1} (Pb0, Pb1, Pb2 and Pb3, respectively) were used as $Pb(NO_3)_2$ form. Adding the lead treatments started after transplanting. Also, sodium nitrate ($NaNO_3$) was added to the nutrient solution at a rate that would

equalize nitrate concentration in all pots.

PS-II efficiency and Chlorophyll content measurements

Plants were harvested before flowering and nine weeks after potting. Chlorophyll content was measured by chlorophyll-meter (*SPAD-502, Konica Minolta Sensing Inc*, Osaka, Japan) prior to harvest. SPAD readings were taken around the midpoint near to midrib of each leaf sample and averaged. PS-II efficiency measured based on ratio of F_v/F_m (maximal photochemical efficiency of photosystem II) by using portable fluorometer (*OS-30, OPTI-SCIENCES Inc*, Hudson, USA). The leaves were dark-adapted for 30 minutes, then F_o, F_m and F_v/F_m were recorded ($F_v=F_m-F_o$) (F_o: minimal fluorescence yield of dark-adapted state, F_m : maximal fluorescence yield of dark-adapted state). The shoots were cut from the crown. The leaf areas were measured by leaf area meter (*LI -3100, LI-COR Inc.*, Lincoln, USA).

Assessment of mycorrhizal root infection

To determine the percentage of root colonization, about 30 pieces of stained roots with 1 cm length, were mounted on slide and the percentage of fungal organs in the root length was estimated. For each sample at least two slides were prepared and the average of them was calculated (Leung *et al.,* 2007).

Determination of phosphorus, lead and proline

All plant materials were dried at 65°C for three days and their dry weights were measured. In order to create a uniform composition of the samples, dry tissue was ground and they were passed through one mm sieve. Plant samples were dry-ashed in a muffle furnace at 300° C for 3h and at 550° C for 5h. The ash was dissolved in 1:3 ratio of nitric acid and hydrochloric acid. Phosphorus was measured colorimetrically by the *vanadate-molybdate method* (Cottenie, 1980) and lead was measured using atomic absorption (*AA-3600, Shimadzu Corporation*, Kyoto, Japan) (Waling *et al.,* 1989). Proline determination was done colorimetrically using the method described by Bates *et al.* (1973).

Mycorrhizal dependency

The mycorrhizal dependency (M.D) of the plants was calculated according to Gerdemann (1975) as:

$$M.D = \frac{\text{DM of inoculated plant at a particular level of metal}}{\text{DM of non inoculated plant at the same level of metal}} \times 100$$

Experimental design and statistical analysis

The experiment was conducted as factorial completely randomized design with four replications. The factors included lead with four levels (0, 50, 100 and 150 mg L^{-1} Pb^{2+}) and fungus with three levels (two species of fungi and the control without fungus). The d*ata were analyzed* using two-way ANOVA and means were separated by *Duncan's* Multiple Range Test using SPSS software.

Results

Root Colonization

Lead treatment reduced mycorrhizal colonization and the colonization with *Glomus intraradices* was higher than *Glomus mosseae* at Pb1 and Pb2 levels. Fig. 1(B) shows that there is no significant difference between two fungal species at Pb0 and Pb3 levels.

Leaf area and chlorophyll content

It was observed that increasing the lead concentration decreased the chlorophyll content (Fig. 1C) and leaf area of tomato plants significantly (Fig. 1A). Chlorophyll content was affected by mycorrhizal inoculation at Pb3 level. With increasing lead concentration leaf area was reduced in all levels of lead. The leaf area of Gi inoculated plants was higher than non- mycorrhizal ones at Pb0, Pb1 and Pb3 levels ($p<0.05$).

Mycorrhizal dependency

Increasing the lead concentration increased the M.D ($p<0.05$). Plants showed 183% dependency on the Gi and 143% on the Gm at Pb3 level. Our results showed the effect of fungal species on M.D was dependent on the lead concentration. At Pb_0 the effect of both species was same. At Pb1 and Pb3 the plants inoculated with Gi fungi showed more M.D in

comparison to Gm inoculated plants but at Pb2 M.D was higher in Gm inoculated plants (Fig. 2A).

The efficiency of photosystem II

In the presence of lead specially at Pb3 the F_v/F_m in Gi plants was significantly higher than control plants. Increasing the lead concentration decreased the F_v/F_m in control and Gi plants (Fig. 1D).

Shoot and root proline concentrations

Interaction between lead treatment and mycorrhization on shoot and root proline concentration was not significant ($p<0.05$). Also, lead treatment and fungal inoculation had no significant effect on shoot proline concentration (Table 1). Inoculation with *G. mossseae* and *G. intraradices* induced a significant effect in root proline concentrations. Proline concentrations of Gi and Gm plant roots increased by 1.68 and 1.89 times respectively, in comparison to control plants (Fig. 3).

Shoot and root phosphorus content

Interaction between lead treatment and mycorrhizal inculation was not significant on shoot and root phosphorus content ($p<0.05$). Raising the lead concentration decreased shoot and root phosphorus content and mycorrhizal plants showed more phosphorus content in their shoots and roots than control plants (Table 1).

Table 1. Shoot and root proline concentrations, shoot and root P contents, shoot lead content and root to shoot ratio of lead in tomato plants non-inoculated or inoculated with *Glomus mosseae* or *Glomus intraradices* at different levels of lead.

Treatments	Shoot proline [µM g^{-1} FM]	Root proline [µM g^{-1} FM]	Shoot P content [mg pot^{-1}]	Root P content [mg pot^{-1}]	Shoot Pb content [mg pot^{-1}]	Root to shoot ratio of Pb
NMPb0	0.657 ± 0.105	0.204 ± 0.041	3.55 ± 0.36	0.432 ± 0.006	nd	-
NMPb1	0.467 ± 0.076	0.223 ± 0.022	2.52 ± 0.14	0.336 ± 0.013	0.119 ± 0.119	3.18 ± 0.14
NMPb2	0.583 ± 0.064	0.225 ± 0.004	1.30 ± 0.15	0.156 ± 0.033	0.097 ± 0.097	4.78 ± 0.21
NMPb3	0.453 ± 0.072	0.181 ± 0.064	1.09 ± 0.08	0.103 ± 0.017	0.160 ± 0.16	4.94 ± 0.78
GiPb0	0.593 ± 0.055	0.281 ± 0.018	6.51 ± 1.05	0.602 ± 0.157	nd	-
GiPb1	0.607 ± 0.023	0.332 ± 0.057	4.68 ± 0.75	0.685 ± 0.077	0.160 ± 0.16	5.49 ± 0.79
GiPb2	0.597±0.097	0.492 ± 0.098	2.49 ± 0.18	0.367 ± 0.039	0.137 ± 0.137	9.76 ± 1.06
GiPb3	0.617 ± 0.018	0.304 ± 0.009	2.55 ± 0.42	0.320 ± 0.06	0.227 ± 0.227	9.45 ± 1.26
GmPb0	0.667 ± 0.064	0.343 ± 0.010	5.65 ± 0.56	0.504 ± 0.078	nd	-
GmPb1	0.657 ± 0.018	0.464 ± 0.06	3.5 ± 0.54	0.388 ± 0.047	0.132 ± 0.132	5.47 ± 0.74
GmPb2	0.543 ± 0.107	0.446 ± 0.09	3.10 ± 0.09	0.436 ± 0.069	0.175 ± 0.175	8.38 ± 1.86
GmPb3	0.443 ± 0.073	0.327 ± 0.073	2.11 ± 0.29	0.267 ± 0.034	0.163 ± 0.163	7.32 ± 0.57
Significance						
Fungi	ns	**	**	**	**	**
Pb	ns	**	**	**	**	**
Fungi ×Pb	ns	ns	ns	ns	ns	ns

Data are the means of four replicates ± standard error. ns: non significant. ** Significant at 0.01 level. nd: not detectable. Pb0, Pb1, Pb2 and Pb3, respectively 0, 50, 100 and 150 mg L^{-1}. Gi, Gm and NM respectively *Glomus intraradices*, *Glomus mosseae* and non-mycorrhizal plants. FM is fresh mass.

Shoot and root lead content

Interaction between lead treatment and mycorrhizal inculation was not significant on shoot lead content (Table 1), but it had a significant effect on root lead content (Fig. 2B). Gi treated plants showed more lead content in their root in comparison to Gm and control plants. Specially there was a considerable difference at Pb3 level.

Root to shoot ratio of lead concentration

Interaction between lead treatment and mycorrhizal inculation was not significant (Table 1). Increasing the concentration of lead increased the ratio. There was no significant difference between levels Pb2 and Pb3 in this respect. On the other hand the ratio was higher in mycorrhizal plants significantly in comparison to non-mycorrhizal plants (Fig. 4).

Discussion

Pb toxicity can affect the plant growth, transpiration, chlorophyll biosynthesis and cell division unfavorably. Decrease in photosynthetic rate in plants exposed to Pb can be results from inhibition in chlorophyll, plastoquinon and carotenoids synthesis, damged chloroplast ultrastructure and changed pigment composition, hampered electron transport, inhibited activities of Calvin cycle enzymes, inhibition in PSII activity and decreased carboxylation efficiency of RUBISCO. Also, lead can alter thylakoid membrane lipids including change in total amount and degree of saturation. Pb^{2+} ions stimulate dehydrogenation of fatty acids (Mishra and Dubey, 2005; Sharma and Dubey, 2005). The decrease in chlorophyll content may be due to impairment in essential nutrient uptake such as Mg^{2+} and Fe^{2+} (required for the synthesis of chlorophylls) by plants (Haneef *et al.,* 2013). High concentration of heavy metals like Pb^{2+} can alter cation balance in plants due to competition of metals in uptake or translocation processes. Drazkiewicz (1994) showed that chlorophyll degradation increased in lead treated plants due to increased chlorophylase activity. Many studies have shown that PSII is the main target of heavy metal stress. However, the exact mechanism is not clear yet (Sarvari, 2005). Based on the results of some experiments performed with isolated chloroplasts or PSII particles exposed to Cu, Pb, Zn or Hg, the inhibition was related to the dissociation of the oxygen-evolving complex proteins and to the displacement of the essential cofactors for water splitting like Ca^{2+},Cl^{-}, and Mn^{2+} by these heavy metals (Sarvari, 2005). Rashid and Popovic (1990) showed that lead inhibits water oxidizing complex function through the competing for binding near the Ca^{2+} and Cl^{-} binding sites in this complex. Our results indicated that inoculation with AM fungi enhanced efficiency of the PS II in tomato plants under metal stress conditions. Mycorrhiza can support a higher chlorophyll concentration by improving Mg^{2+} and Fe^{2+} uptake, subsequently lead to a higher production of photosyntates and biomass (Haneef *et al.,* 2013). Higher chlorophyll content, leaf area, better PS II function, more biomass in mycorrhizal plants specially at Pb2 and Pb3 levels are evidence for these findings. Similar results have been reported for photosynthetic enhancement in AM symbiosis of eucalyptus plants in the soil contaminated by lead (Arriagada *et al.,* 2005) and increase in chlorophyll and leaf area of beans in the soil contaminated by cadmium and zinc (Saleh-al-Garni, 2006).

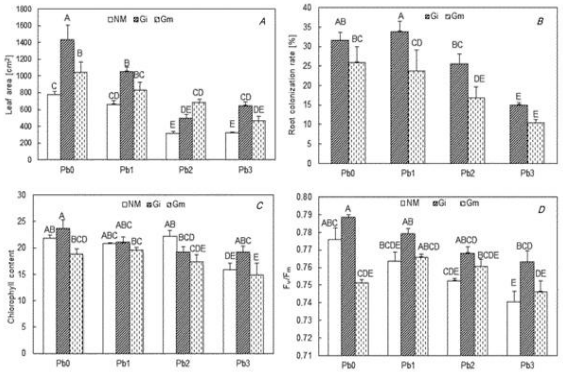

Fig. 1. Effect of mycorrhizal inoculation and Pb concentrations on leaf area (A), root colonization rate (B), chlorophyll content (C) and F_v/F_m **(**maximal photochemical efficiency of photosystem II)(D). The bars represent the standard error of the mean ($n = 4$). Columns with different letters indicate significant difference at $p< 0.05$. Pb0, Pb1, Pb2 and Pb3, respectively 0, 50, 100 and 150 mg L^{-1}. Gi, Gm and NM respectively *Glomus intraradices*, *Glomus mosseae* and non-mycorrhizal plants.

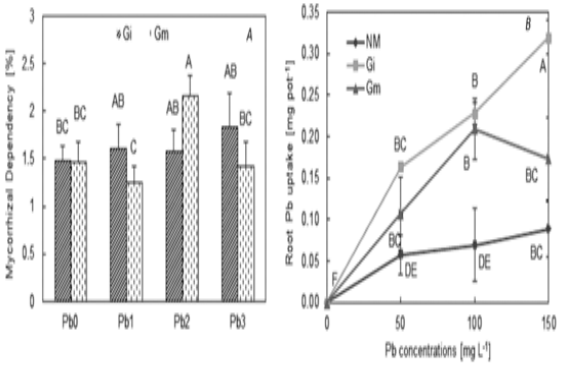

Fig. 2. Effect of mycorrhizal inoculation and Pb concentrations on mycorrhizal dependency (A) and root lead uptake (B). The bars represent the standard error of the mean ($n = 4$). Columns with different letters indicate significant difference at $p< 0.05$. Pb0, Pb1, Pb2 and Pb3, respectively 0, 50, 100 and 150 mg L^{-1}. Gi, Gm and NM respectively *Glomus intraradices*, *Glomus mosseae* and non-mycorrhizal plants.

The proline accumulation has been reported in several plant species exposed to metal stress (Sharma and Dietz, 2006). Some of the proposed roles of proline are to act as an inhibitor of lipid peroxidation, a free radical scavenger also a metal chelator. Also, proline accumulation may play role in osmotic adjustment at the cellular level (Perez-Alfocea *et al.,* 1993) and acts as supply of energy and nitrogen after removing the stress condition (Chandrashekhar and Sandhyarani, 1996). In present study, it was determined that the highest root proline concentration was in Gi plants. It can be concluded that mycorrhizal inoculation caused to increase the proline values to protect plants against lead toxicity. Andrade *et al.* (2009) reported low Zn-induced proline contents in mycorrhizal jack beans with *Glomus etunicatum* at high Zn concentration in soil. Andrade *et al.* (2010) showed higher proline contents in jack bean plant inoculated with *Glomus etunicatum* in Cu^{2+} polluted soil. Ruscitti *et al.* (2011) showed that mycorrhizal inoculation increased pepper plant tolerance to Cr in the soil with modifying proline synthesis. They showed that interaction of mycorrhization and Cr lead to an increase in leaf proline content. Maximum shoot proline accumulation was observed at Pb2 level. Increasing lead concentration to 150 mg Pb declined the proline concentration. John *et al.* (2008) reported maximum shoot proline at 20 mg L^{-1} of Pb in *Lemna polyrrhiza* L. plant. Their results showed that 30 mg L^{-1} of Pb declined the proline to about 25% of the control. It seems that effect of myorrhizal inoculation on proline accumulation pattern depends on the fungal species, plant and heavy metal type and It is necessary to perform more detailed analysis of inoculated plant tissues under stress conditions (Ruscitti, 2011). Root colonization percent shows the success of symbiosis between AM fungi and host plant. Researches have shown that the toxicity of heavy metals has inhibitory effect on development of intraradicle hyphae; moreover, high concentrations of heavy metals affect the development of extraradicle hyphae. Also development of mycorrhizal symbiosis depends on the host plant condition, which means that unfavorable conditions affect the colonization rate (Gildon and Tinker, 1983; Pawlowska and Charvat, 2004).

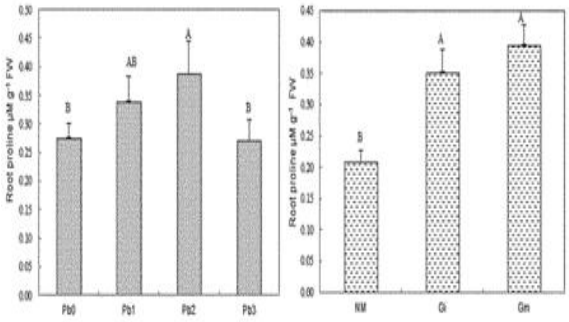

Fig. 3. Effect of mycorrhizal inoculation and Pb concentrations on root proline concentration. The bars represent the standard error of the mean ($n = 4$). Columns with different letters indicate significant difference at $p< 0.05$. Pb0, Pb1, Pb2 and Pb3, respectively 0, 50, 100 and 150 mg L^{-1}. Gi, Gm and NM respectively *Glomus intraradices*, *Glomus mosseae* and non-mycorrhizal plants.

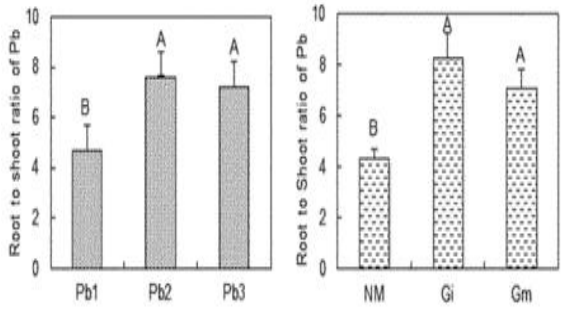

Fig. 4. Effect of mycorrhizal inoculation and Pb concentrations on root to shoot ratio of Pb. The bars represent the standard error of the mean ($n = 4$). Columns with different letters indicate significant difference at $p< 0.05$. Pb0, Pb1, Pb2 and Pb3, respectively 0, 50, 100 and 150 mg L^{-1}. Gi, Gm and NM respectively *Glomus intraradices*, *Glomus mosseae* and non-mycorrhizal plants.

The M.D for biomass production was different between fungal species and it showed dependency on lead treatment. No significant difference of mycorrhizal species was seen at Pb0 level. It can be concluded that root colonization rate was not closely related to biomass production. Both shoot and root P contents decreased by increasing the lead concentration. It seems that with increasing lead concentration, a part of the soluble phosphate were

precipitated as $Pb_3(PO_4)_2$. Also it may be attributed to dry weight loss in plants. These factors can be effective in reducing the phosphorus content at the high levels of lead. Higher content of phosphorus in mycorrhizal plants than non-mycorrhizal plants in terms of pollution by heavy metals has been reported by many researchers (Sudova and Vosatka, 2007; Andrade *et al.,* 2004). One of the reasons that was expected to reduce lead translocation to the shoot by increasing lead concentration is the translocation of lead and other metals occurs through the xylem and it follows transpiration; thus, by reducing plant growth, evapotranspiration rates and transmission in the vessels were reduced (Marschner, 1995). Transpiration rate of plants and root respiration are important factors in the rate of release of ions into the xylem. Reduction in these factors decreased ions release into the vessels (Marschner, 1995). Chen *et al.* (2005) found that AM symbiosis enhances Pb accumulation in root and shoot system. Also AM symbiosis can increase plant growth due to improving P uptake and sequestration of Pb in roots. It seems that due to the heavy metal retention in fungal structures including vesicles, arbuscules and hyphae, the root to shoot ratio of Pb of inoculated plants is lower than NM plants. Also, it seems heavy metal is fixed through the fungi organs by polyphosphate granules (Chen *et al.,* 2005) or is complexed by the compound of fungal wall such as chitin and melanins (Vogel-Mikus *et al.,* 2006). Similar results have been reported by Andrade *et al.* (2004). It has been proposed that plant-fungal symbiosis confers host plants to discover stress more quickly than plant without endophyte and it can lead to the fast activation of plant biochemical response against high stress condition (Ruscitti, 2011). Our data indicated that mycorrhizal plants growth parameters increased compared to the non-mycorrhizal controls. It seems that the most important reasons for the better growth of the mycorrhizal plants are better nutrition, sequestering of lead in mycorrhizal roots and consequently inhibition of metal transport to shoots and modifying proline metabolism in inoculated plants to ensure about effective tolerance response.

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