Glomalin production by two Glomeral fungi in symbiosis with corn plant under different Pb levels

Shaabani Zenoozagh Vahideh, Aliasgharzad Nasser, Oustan Shahin

Abstract: Glomalin is a specific glycoprotein which produced by the fungi belonging to the order Glomerales in phylum Glomeromycota.. Given the widespread symbiotic relation of these fungi with a large number of plants, considerable amount of glomalin is entered to the soil ecosystems, annually. Heavy metals can influence the both symbionts physiology and hence glomalin production by the fungi. In this study, the effects of Pb levels were investigated on mycorrhizal establishment in maize plants inoculated by either *Glomus mosseae* or *G.intraradices* as well as glomalin production by the system. Lead levels of 0, 500 and 1000 mg Pb. kg^{-1} soil (as $Pb(NO_3)_2$) were applied to the pots before plant culture. Non-mycorrhizal treatment was left un inoculated as control. Eeasily extractable glomalin (EEG) and total glomalin (TG) were determined by the Bradford method after extraction from soil. With increasing Pb concentration in soil, shoot and root fresh and dry weights, P concentration in shoot and root, root colonization, EEG and TG decreased and proline concentration in leaves, Pb concentration in shoot and root increased. The results showed that, glomalin levels in fungal treatments significantly increased compared to the Non-mycorrhizal control. The result also showed a positive correlation between the measured glomalin and the percent of root colonization.

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Introduction

Global climate change in recent years and the importance of soil quality, resulted in a change of attitude towards the soil carbon management, so that it has shifted management strategies to increase in soil carbon reserves (Merino et al 2004).

Soil organic carbon (SOC) stocks plays an important role in soil ecosystem dynamics and comprises a significant portion of the carbon resources in the world (Hail-Mariam et al 2007). Cycle of these reserves between soil and atmosphere can have a significant influence on atmospheric composition and temperature of the gas atmosphere (Bai et al, 2009).

Arbuscular mycorrhizal fungi of the phylum Glumeral form symbioses with about 70 percent of plants. Gloumeral fungi constitute a major portion of soil biomass (Olsson et al, 2010).

Glomalin is a reddish brown glycoprotein that produced by arbuscular mycorrhizal fungi. Glomalin can stabilize a significant amount of carbon in global scale. The available evidence suggests that it sometimes hiding place for a third of the world's stored soil carbon. So it plays a key role in storing soil organic carbon (Treseder and Turner, 2007). In addition to raising aggregate stability, Glomalin reduces the availability of heavy metals via fixing them, thus reducing the risk of toxicity and the availability of other soil microorganisms and plants (Gonzalez-Chavez et al, 2004). Abundant production, hardness and inseparability of this molecule in the soil (Rillig et al 2001), show the importance of the above results. During their life cycle, plants are usually exposed to a wide variety of environmental stresses including heavy metal stresses. Lead has a relatively higher mobility than other heavy metals in soil and plant, so it is easily absorbed by plants and show their toxic effects. Vogel et al (2005) have observed that as concentrations of zinc, lead and cadmium increases, the colonization of AM fungi reduces. Considering the effects of lead heavy metals on mycorrhizal symbiosis, it is assumed that the production of Glomalin is affected differently. It is thought that as levels of lead increase in the soil, the amount of symbiosis of Glomeral fungi is reduced and organic carbon flow from photosynthesis to root is minimized and Glomalin secretions will be decreased.

Materials and Methods

A loamy sand soil was sampled from depth of 0-20 of Khalat Pooshan research station, Tabriz University. Soil pH was 7.81, organic carbon, 0.221%, available Phosphore 4/4 mg kg^{-1} soil and available Potassium was 182/6 mg kg^{-1} soil. Soil samples were sterilized in an autoclave at 121° C for 2 hours. To prepare inoculum, fungal species of *Glomus mosseae* (Gm) and *G.intraradices* (Gi) were inoculated with sterile soil and corn and were kept in greenhouse conditions for four months. At the end of this period, the shoots of corn plants was cut from the soil surface and the contents of the pot, including hyphae, spores and mycorrhizal roots were used as a inoculum in the original experiment. Fungal colonization percentage of roots were determined in inoculums (Aliasgharzadeh et al, 2001).

Pb levels (from the source of $Pb(NO_3)_2$) consisting of three levels of 0, 500 and 1000 mg Pb kg^{-1} soil (respectively Pb_0, Pb_1 and Pb_2) were created in sterile soil (Ippolito et al, 2010).Thus, about 54 kg of sterilized soil was divided into three parts with the exception of lead-free treatment (control), in each treatment approximately 18 kg of sterilized soil was scattered on sterile plastic plates, and the needed lead nitrate salt was solved in the necessary water to reach the moisture of 0.8 FC and was sprayed into the soil, and while being sprayed, it was regularly blended with sterile shovel, and was kept in the same situation to reach balance between solid and liquid phase for 15 days (with moisture of 0.8 FC). For the assimilation of nitrate, taking into account the highest lead treatments, equal concentrations of nitrate were established in all treatments using calcium nitrate ($Ca(NO_3)_2.4H_2O$).

After two weeks, 70 g fungal inoculum was placed as a thin layer at a depth of 5 cm of soil. Seeds of maize (Zea mays L.) cultivars (single cross 704) as disinfected, were planted as four seeds in each pot. During the culture period, the measured soil moisture was adjusted to 80% of field capacity with distilled water. At the end of the vegetative growth phase (two days before harvesting plants) leaf proline concentration was measured. Proline measurement was done with Irigoyen et al method (1992). For this purpose, 0.5 g of fresh leaves with 5 ml of 95% ethanol was crushed in a Chinese mortar. The supernatant was removed and its sediment were washed again with 5 ml of 70% ethanol and the upper phase was added to the supernatant. The resulting solution was centrifuged for 10 min at 3500 RPM. To determine the concentration of proline, one ml of the above alcoholic extract was diluted with 5 ml of distilled water and 2.5 ml of ninhydrine reagent was added. Then, 2.5 mL of glacial acetic acid was added to it and the obtained mix was put in boiling water bath for 45 minutes after stirring. After taking the samples from bolied water bath and cooling them, 5 ml of Toluene was added to any of the samples and was shaken well so that proline complex and ninhydrine complex enter Toluene phase. Standards of proline (0 to 0.1 µmol mL^{-1}) were prepared. Finally, the absorbance of standard solutions and samples were measured at a wavelength of 515 nm and with a spectrophotometer (Hack DR/2000) (Irigoyen et al, 1992) and the calibration curve was prepared using standard solutions. Finally Proline was calculated in micromoles per gram fresh weight of leaf.

After the end of three-month culture period, shoot and root of plants were taken separately from each pot and fresh weights of shoot and root were separately measured. Parts of tiny roots were stabilized in 50% ethanol after being washed with water, and then were stained through Kormanic and McGraw method (1982). Percentage of root colonization was determined with gridline intersect method (GIM) (Norrif et al, 1992), so that stained roots spread into a Petri dish and was observed under binocular. Counting the roots intersect and organs of mycorrhizal roots with grid lines within Petri dish, the percentage of root colonization was determined. Lead concentrations were measured in shoot and root by dry ashing method with hydrochloric acid 1N and reading by atomic absorption (Shimdzu, AA-6300), and P concentrations of shoot and root with Vanado nitro-molybdate method (Cottenie, 1980) using spectrophotometer (Hack DR/2000). Easily extractable glomalin (EEG) and total glomalin (TG) were extracted from soil. For extracting EEG, one gram of soil (passed through a 2 mm sieve) was put into a centrifuge tube (autoclavable) and 8 ml of 20 mM sodium citrate solution was added and vortexed for 30 seconds. Other stages were similar to EEG extraction. Then it was autoclaved for 60 minutes at 121°C. Then it was centrifuged with 5000 RPM for 15 minute. The supernatant was removed into a clean tube. For Extraction of TG, 8 ml of 50 mM sodium citrate solution, was added on the same soil sample (from the previous step) and vortexed for 30 seconds. Other steps were Similar to EEG (Wright and Upadhyaya, 1996). The glomalin concentration in extract was measured with Bradford method (Bradford, 1976) and bovine serum albumin standards were measured at 595 nm. The experiment was factorial in a completely randomized design with three replications. Factors were as follows: Pb levels (0, 500 and 1000 mg kg^{-1} soil respectively, Pb_0, Pb_1 and Pb_2) and three fungal levels (fungi species G.mosseae and G. intraradices and non-mycorrhizal control). For statistical analysis of data, MSTATC software and for the charts Excel software were used.

Results and Discussion

Proline concentration in leaves

The results of data analysis showed that the main effect of Pb levels and main effect of fungal species on proline concentration in leaves was significant, but the interaction of Pb and fungal species was insignificant. Results of proline density measurement in leaves indicated that with the increase in Pb levels, proline concentration increasing with increases of Pb levels. Based on Duncan test the lowest leaf proline was observed in unleaded control and the highest level was observed in Pb_2 level. This increase in the levels of Pb_1 and Pb_2, was 29.14 and 78.70 percent compared to unleaded control respectively (Fig. 1-a). According to Kuznetsov and Shevyakova (1997), an increase of proline in stressed plant is a defense

mechanism. Proline enhances stress resistance in plants through several mechanisms such as swiping hydroxyl radical, osmotic adjustment, protection and preservation of denaturation of enzymes and protein synthesis. Our results are consistent with the work of these scientists. Contamination by heavy metals such as lead, has adverse effects on growth and metabolism of plant leaves. In the present study, it was seen that corn, like other plants increased free proline to defend against the stress resulting from Pb, which conforms to Ibarra et al (1988). Proline concentration in mycorrhizal plants was approximately less than non- mycorrhizal plants (Fig. 1-b). Proline concentration in mycorrhizal plants was about half of non- mycorrhizal plants. ($P < 0.05$). These findings are consistent with the results of Abdel Latef (2011). Proline reduction in the presence of mycorrhizal fungi shows decreased stress level in plants. Rodriguez et al (2004) found that obligate symbiotic with fungi, enables the plant, quickly adapted to stress, thereby causing a rapid activation of the biochemical reaction of plants to reduce the effects of stress and proline amount is reduced.

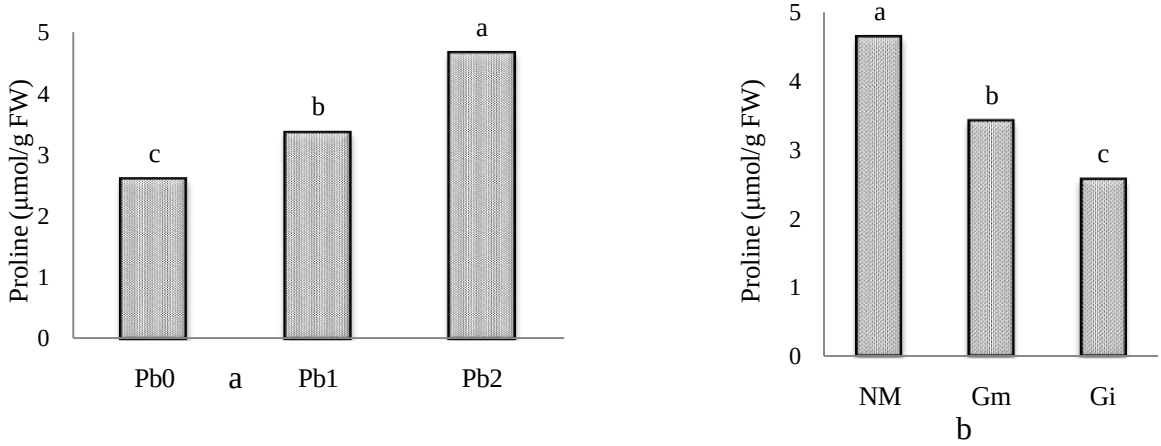

Fig. 1 - Main effects of (a)- levels of Pb added to the soil (mg kg^{-1}soil) and (b)- fungal species on leaf proline concentration.

Shoot and root fresh and dry weight

According to the results, the main effects of Pb level and fungal species on fresh weight of root and shoot and interaction between Pb and fungal species on shoot and root dry weight were significant (Fig. 2). With increasing concentration of Pb in soil, fresh and dry weight decreased. At Levels Pb_1 and Pb_2, compared with control, 23.27 and 35.43% respectively reduction in shoot fresh weight was observed (Fig. 2- a). Photosynthesis of plants in contaminated soil with Pb was reduced, possibily resulting from tribulation in electron transfer, prevention from activity of Kelvin cycle and substitution of Pb for chlorophyll central atom (Sharma and Dubey, 2005). Although in the mycorrhizal plants, reduced shoot dry weight, but in general mycorrhizal plants had higher fresh and dry weight in all levels of Pb than non-mycorrhizal plants ($P < 0.05$). The dry weight of plants inoculated with Gi fungus was higher than the plant inoculated with the fungus Gm (Fig. 2).

High fresh and dry weight of mycorrhizal plants compared with non-mycorrhizal plants, is mainly due to the release of mycelium AM fungus and formation of an additional absorption system as the complementary of plant root system and production of plant growth hormones such as oxin and cytokinin, and this can improve and enhance the growth of mycorrhizal plants. Better feeding of Phosphor as an important factor increases the growth of mycorrhizal plants and generally it seems that mycorrhizal plants have better chance of survival and growth in soils contaminated with heavy metals (Andrade et al, 2004). Wright et al (1996) showed that the dry weight of mycorrhizal clover in a growth period of 80 days was significantly increased compared to non-mycorrhizal control plants

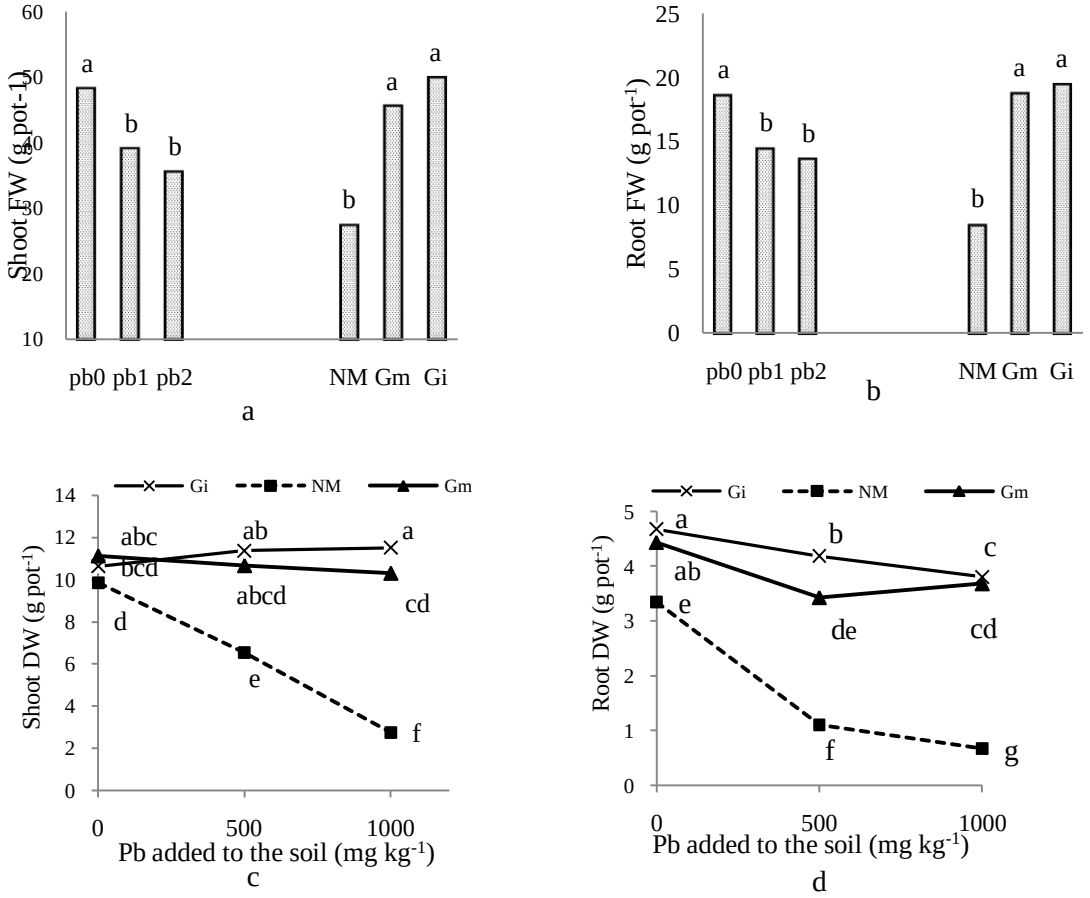

Fig. 2- The main effect of lead and fungal species on (a)– shoot fresh weight (b)- roots fresh weight (c)- interaction between Pb and fungal species on shoot dry weight, and (d)– Root dry weight root.

Easily extractable glomalin (EEG)

study of the interactions between Pb levels and fungal species showed that with increase in the concentration of Pb in soil, concentrations of EEG decreases, so that the highest EEG produced by two species of fungi in control levels. In non-mycorrhizal treatments, with increase in Pb levels from Pb_0 to Pb_1 and Pb_2, 6.21 and 30% reduction in EEG concentration respectively was observed. EEG measured in soil of non-mycorrhizal treatments is related to the initial concentration of glomalin in soil. It was also observed that the increase in soil Pb concentrations significantly decreases glomalin concentration in both species. This reduction was higher in Gm than in Gi. In soil of inoculated with the fungus Gm, with increase in the Pb from Pb_1 to Pb_2 compared to Pb_0, respectively 12.82% and 36.87% and the fungus Gi 34.35 and 46.4% reduction in the concentration of EEG respectively was observed (Fig. 3- a). This finding confirms our first hypothesis on decrease in mycorrhizal fungi growth with the increase in density of heavy metals in soil; however, in other studies the negative effect of heavy metals on growth of mycorrhizal fungi is indicated (Mc Grath et al, 1995). Indeed, the concentration of glomalin measured in different Pb levels indicates the growth of any of mycorrhizal fungi species affected by these levels. Effect of heavy metals on glomalin production is reported to be different. Glomalin production can be negatively related to absorption of heavy metals in plants. In this state, metals may be stabilized on hyphae wall by glomalin, thereby preventing from entry of extra glomalin to soil system. In all levels of Pb, mycorrhizal treatments soil, had higher EEG compared to non- mycorrhizal treatments ($p<0.05$). There were also significant differences between the two fungal species and Gi species had a greater ability to produce glomalin in this experiment (Fig. 3-a). Studies show that species of arbuscular mycorrhizal fungi in soils are the most important factor in the control of glomalin-related

soil protein (GRSP) in soil (Nichols and Wright, 2004).

In a study to describe glomalin produced by hyphae of arbuscular mycorrhizal fungi during active colonization of roots, Wright et al (1996) observed that the amount of protein extracted per unit hyphae weight among species of *Gigaspora gigantea*, *Glomus etunicatum* and *G.intraradices* were different.

The results indicated that a high correlation exists between the EEG concentrations measured by the method of Bradford and the percentage of root colonization, and this relationship is linear and the corrolation coefficient is 0.671. With the increase in percentage of root colonization, EEG is also increased (Fig. 4). Positive correlation between glomalin and percentage of root colonization indicates that the measurement of glomalin produced by AM fungi with Bradford method gives us information about presence and absence and also the population of these fungi in soils (Rosier et al 2008). In the study by Wu et al (2012) it was found that the percentage of root colonization had a significant correlation with EEG and TG. In an experiment conducted by Morton (1990), it was precisely shown through immune-fluorescence that there exists glomalin only in the roots that are colonized by arbuscular mycorrhizal fungi.

Total Glomalin (TG)

The effect of Pb levels and fungal species on the production of TG was significant. In the soil of fungal treatments compared to control without fungus with increase in Pb levels, TG decreased. In soil of non-mycorrhizal treatments with increase in concentrations of Pb in soil from Pb_0 to Pb_1 and Pb_2, 12.53 and 40.43% respectively reduction was observed in TG concentration. This decrease in soil of mycorrhizal treatments inoculated with fungi Gm was 23.68 and 49.71% and the fungus Gi 36.59 and 53.9% respectively. The results showed that inoculation with fungal species Gm and Gi can increase TG production ($p<0.05$) and a significant difference was observed between the two fungal species. In this study, Gi fungi was more efficient than Gm fungi in terms of TG production (Fig. 3-b). It also showed high correlation results between TG and percentage of root colonization. The coefficient of this linear correlation was 0.635. With the increase in percentage of root colonization TG increased and the slope of this increase was higher than the EEG (Fig. 4).

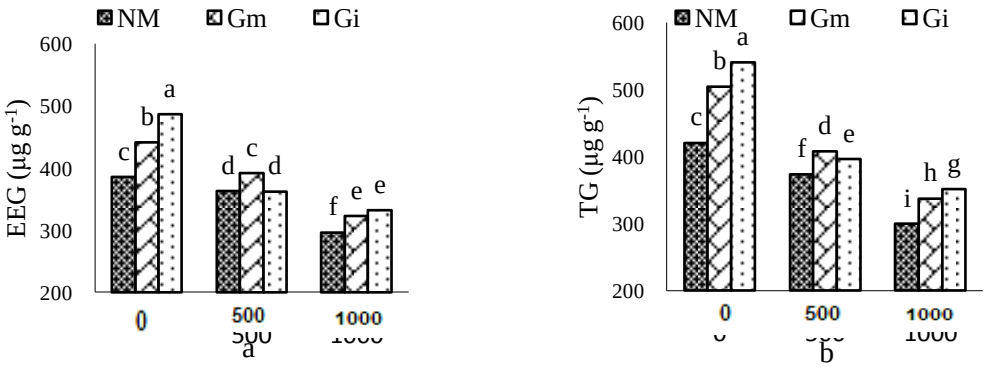

Fig. 3 - Interaction of Pb levels (mg kg^{-1} soil) and the fungal species: (a) – easily extractable glomalin (EEG), (b) - total glomalin (TG).

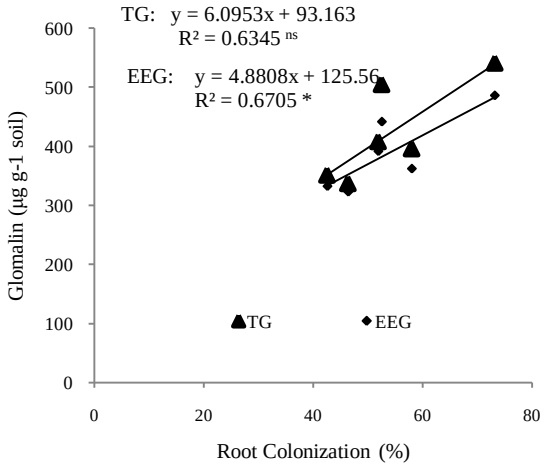

Fig. 4 - Relationship between (EEG) and (TG) and root colonization percentage.

Changes in EEG and TG content per unit of root colonization

With elevated levels of Pb from Pb_0 to Pb_1, EEG and TG concentration decreased per unit of root colonization, but at the level of Pb2 amount of EEG and TG production per unit of root colonization was significantly increased (Fig. 5). Glomalin is a glycoprotein similar to heat shock proteins 60 (hsps60). Researchers believe that glomalin, as a protein produced under stress conditions, may support AM fungi (Rillig and Steinberg 2002). Their study showed for the first time that poor growth conditions may increase production of glomalin. Because AM fungi allocate much of their carbon and nitrogen resources to the production of glomalin, it is thought that the main task of glomalin is to support these fungi.

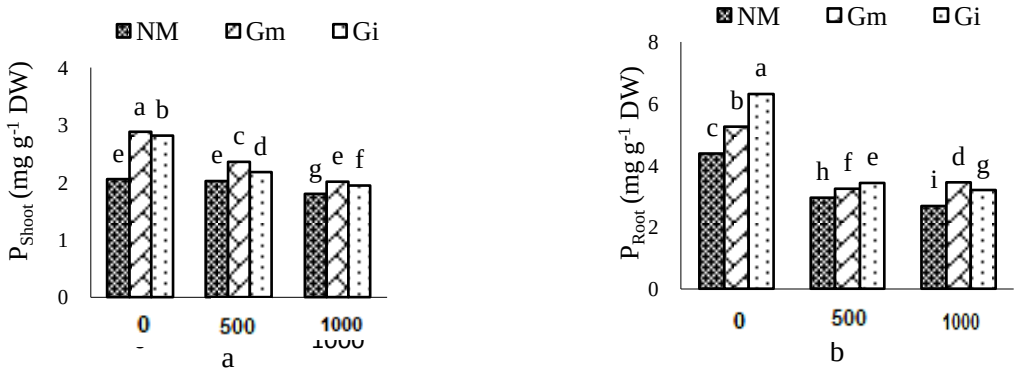

Fig. 5- Change of the (a)- EEG and (b)- TG, per unit of root colonization under influence of Pb levels and fungal species.

P concentrations in shoot and root

With the increase in concentration of Pb in soil, P concentration of shoot and root of mycorrhizal and non- mycorrhizal plants declined ($p<0.05$). According to Estrella-Gomez (2009) Lead toxicity in the soil reduces the absorption of minerals such as phosphorus. It seems that with increase in the concentration of lead in soil, part of the absorbed phosphate of the soil will precipitate in form of $Pb_3(PO_4)_2$. In this study, at all levels of lead, phosphorus concentrations in shoot and root of mycorrhizal plants was significantly more than its concentration in non- mycorrhizal plants. The results showed that with the increase in the levels of Pb, Gi mycorrhizal plants have a greater ability to increase P concentration in shoot (Fig. 6). Mycorrhizal Plants through extra-radical hyphae has more contact with soil. The low Km of soil can lead to better absorption of phosphorus, namely in low concentrations of a ion, fungi absorb more rapidly than the roots.

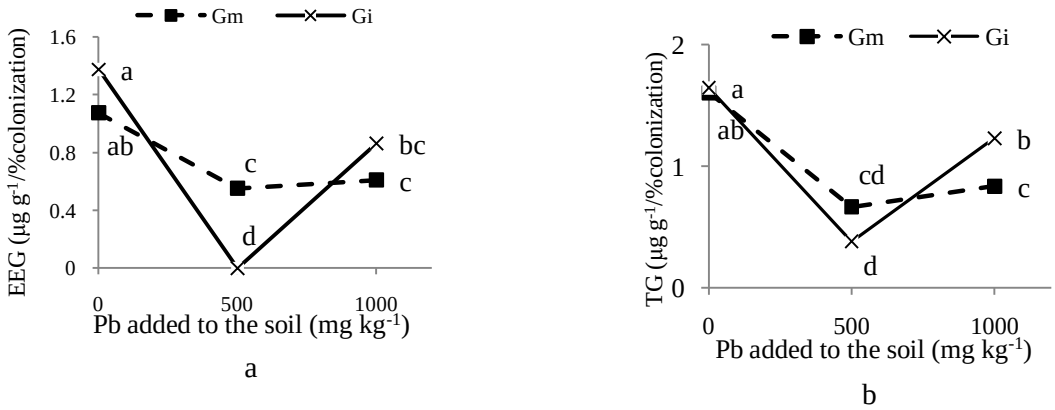

Figure 6 - Interaction of Pb (mg kg^{-1} soil) and fungal species on (a) - phosphore concentration in shoot (b)- root.

Pb concentrations in shoot and roots

Interactions between Pb levels and mycorrhizal fungi on Pb concentration in shoot and root were significant. colonization with two species of Glomeral fungi in both levels of Pb_1 and Pb_2 reduce levels of Pb concentrations in shoot and significant

difference was observed between mycorrhizal and non-mycorrhizal plants and also between the two fungi species. But at Pb_0 level no significant difference was observed between the two species (Fig. 7). Low concentrations of Pb in shoot and roots of mycorrhizal plants compared to non-mycorrhizal plants was because the mycorrhizal plants have plenty of dry matter and lead in it is diluted (dilution effect). Other possible reasons for this relationship, is the maintenance of the heavy metal in fungal structures ranging from vesicles, and hyphae and arbuscule. It is also thought that heavy metal is stabilized by phosphate granules (Chen et al, 2005) or is complexed by fungal wall components such as chitin and melanin (Vogel et al 2005). Another theory in decrease in concentration of heavy metals in mycorrhizal plants is that the glomalin existing on hyphae of mycorrhizal fungi play an important role in immobilizing heavy metals in the space between soil and hyphae, ie before heavy metals enters fungus-plant system (Khan 2006). In this study Gi has a significantly higher efficiency than Gm in reducing the concentration of shoot and roots of corn in all lead levels. Gi fungal colonization percentage was higher than fungus Gm, indicating the success of these fungi in the establishment of symbiosis in lead contamination.

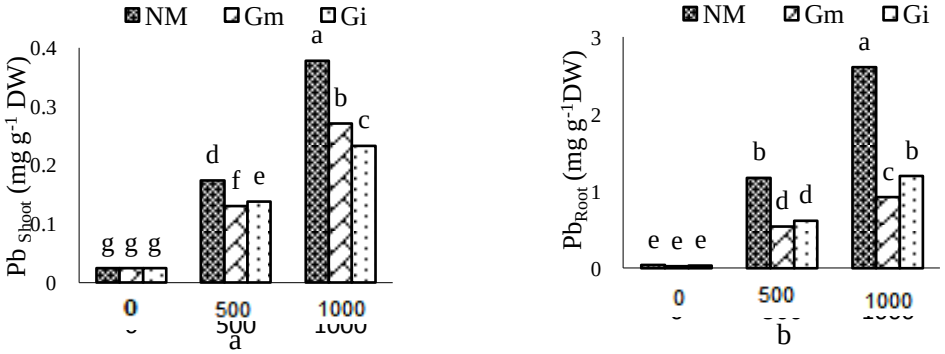

Figure 7 - Interaction between Pb levels (mg kg^{-1} soil) and fungal species on the Pb concentration (a)- shoot (b)- root.

Root colonization percentage

According to the results, the interaction of Pb and fungal species on root colonization percentage was significant. Comparison of The average interactions indicated that increase in Pb concentration in soil decreased root colonization ($p < 0.05$) (Fig. 8). Reduced colonization may be a mechanism to limit the absorption of heavy metals (Oudeh et al, 2002). Some researchers express reduced root colonization as resulting from toxic effects of heavy metals on the organs of arbuscular mycorrhizal fungi. (yasrebi et al 1994). In this study, at all Pb levels, Gi fungus had the highest percentage of colonization, showing the efficiency of these fungi in soil contaminated with lead.

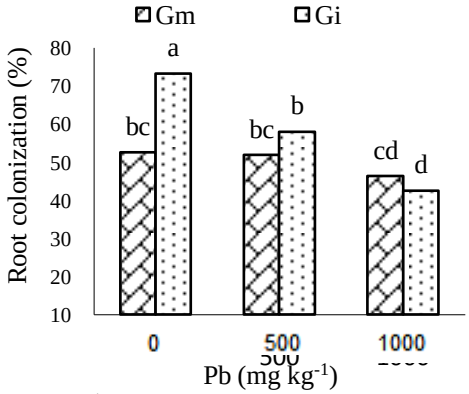

Figure 8- Interaction between Pb levels (mg kg^{-1} soil) and fungal species on the percent of colonization of maize roots.

Conclusion

The results of these experiments showed that Pb concentrations has a negative effect on glomalin production in both fungal species Glomus mosseae and G.intraradices, of course its effect were higher on the fungus G. mosseae. It appears that production of glomalin by mycorrhizal fungi, can be a strategy for

survival in soil microorganisms containing heavy metals. Glomalin sometimes accounted for a third of the total soil organic carbon. So it plays a key role in storing soil organic carbon. Although this combination makes up a significant global reserve of nitrogen and carbon, the environmental pollution impact on production has not been fully analyzed. such research can clarify the ecophysiological tasks of glomalin in glomeral fungi and lead to predictions of glmalin reserves in the ecosystems under global change. Considering the correlation between the concentration of glomalin and the presence of mycorrhizal fungi in the investigation, the need for further studies on the use of mycorrhizal fungi in soil Glvmalyn as an indicator of mycorrhizal fungi is felt. The results proved that the method of Bradford protein measurement is relatively efficient. employing this method in comparison studies in sterilized and greenhouse situation is efficient but is not recommended for soils containing high organic substances. In this study, the use of both types of G.intraradices G.mosseae compared with non-mycorrhizal control, measured in terms of influence on indices was positive, although G.intraradices in terms of glomalin production increase, growth characteristics and reduction of proline concentration has a better efficiency, However, due to the different impact of lead levels on the growth of two species of fungi, it is suggested that in future studies of other species are especially fungal strains resistant to heavy metals can be used are used.

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