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Mycoremediation of Cd and Pb from Contaminated Soil using Arbuscular Mycorrhiza *Glomus leptotichum*

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Abstract: The objective of study was to investigate the possibility of utilization of arbucular mycorrhizae (AM) to clean up the soil contaminated with two heavy metals, Cd and Pb using sunflower plants (*Helianthus annuus*) as a test plants. AM fungi used was *Glomus leptotichum*. Study was carried out under controlled conditions and sterilized soil. There was significant increase in dry weight of the root system of the AM inoculated plants compared to the negative control . The results showed variable effect of heavy metals on the dry weight of shoot system. Root and shoot dry weight, in response to increasing concentrations of heavy metals applied to the soil, had a significant adverse or negative effect of Cd and non significant effect at the concentration 50 and 100 ppm of Pb on plants inoculated with AM fungi. The AM showed high efficiency for extraction of these heavy metals from the soil at low concentrations and reduction of this efficiency at high concentrations. Compared to shoot rate of accumulation was almost five times lower than the roots. The rate of Cd accumulation in the seeds of the plant was within the toxic levels and recorded 12.9 ppm. in the plants inoculated with *G. leptotichum*. This indicates the efficiency of this AM species to remove this metal from the soil, i.e. within the toxic levels and probably present a risk to human health.

***Keywords*:** Vesicular mycorrhizae, Heavy metals, Glomus *leptotichum*, Cd, Pb

Environmental systems are polluted with heavy metals due to industrial and electronic waste, including cadmium (Cd) and lead (Pb), which cause harm to public health. These heavy elements cannot be converted by plants into a non-toxic form inside their cells and they bind with soil particles and ions to form insoluble complexes and sediments. It may be part of the composition of silicates in the soil (Cobbett and Goldsbrough 2000). The process of biological extraction is used including maycorrhizal fungi that encourage the extraction of cadmium, lead and other elements through the colonization at the root of the various families including the sunflower (Chandrashekara et al 1995, Joner and Leyval 1997, Hossein 2010, Gaur and Adholeya 2004). There are other microorganisms which play a role in these systems including nitrogen-fixing bacteria, fungi, yeasts and others. The arbuscular mycorrhiza fungi (AM) is a type of the soil microorganisms that create a symbiotic relationship with most of the plants to ensure that physically remain connected directly between the soil and plant roots. The sunflower plants incurred for the collection and accumulation of heavy metals (Davies et al 2001). Hence the aim of this research was to study the role of *Glomus leptotichum* in the absorption and accumulation of heavy metals in different parts of the plant and its role in cleaning up the environment biologically.

MATERİAL AND METHODS

Bio-fertilizer starter culture: The stock inoculum containing *G. leptotichum* spores with dry sand soil was used in the study. To ensure the presence of fungal spores, the method of wet sieving was used. The activation process was conducted using a sterile sand soil by autoclave for three hours and 121^{0}C and pressure of 15 Ib. in^{-1} and dried in an electric oven at 60^{0}C until drying. The efficiency of sterilization tested by taking sample of each batch and cultured in nutrient broth and incubated 30 and 37^{0}C. Sterile soil was distributed in plastic pots, 1kg soil each. Fifty grams of AM inoculum (starter fungal fertilizer) added by pad method 3 cm depth and covered with a similar amount of soil in pots of each treatment (Mosse and Hepper 1975). The field capacity of pots containing sterile soil and a nutrient solution of stocks containing the necessary nutrients were measured (Davies et al 2002).

Onions bulb as the host plant was sterilized by 95% ethanol and 2% mercuric chloride and washed several times with sterile distilled water to remove disinfectant traces. This bulb was transplanted in the pots contained 1 kg sterile sand soil with three bulbs for each and added 200 m nutrient solution to soil , before two days of planting. Two weeks after planting, another 200 ml of the nutrient solution was added to each pot (Davies et al 2001). After four months the shoot removed, and the soil with chopped roots was air dried in trays and then keept in sterile plastic bags in a cool and dry place until use as a stock culture. The roots of the onions were stained using acid fuchsin dye (Kormanik et al 1980).

The percentage of infected roots with mycorrhizal fungi were accounted (Davies et al 2002).

$$\text{Percentage of colonization} = \frac{\text{Total colonized with AM - Total non-colonized}}{\text{Total non-colonized}} \times 100$$

Sand soil was dried and passed through 2 mm diameter sieve and washed with water to remove most of the organic matter and fertilizer (Davies and Linderman 1991). The soil was sterilized by autoclave for 3 hours and 121 ^{0}C and pressure of 15 Ib. in^{-1}. The amount of phosphate, total nitrogen and heavy metals in the soil were estimated (Table 1).

Heavy metal solution: This was prepared as suggested by Chandrashekara et al (1995). Implemented a full factorial sectors of randomization experiment (RCBD) using two types of heavy metals in four concentrations (Table 2). The plastic pot was filled with 3 kg sterile sand soil, chemical fertilizers were added (Davies et al 2002). The 15g of mycorrhizal fertilizer was added to each pot by pad technique (Mosse and Hepper 1975). Five sterile seeds of sun flower were sown in pot after the addition of nutrient solution and irrigated with 200 ml of sterile water and Cd and Pb were added, according to the concentrations listed in the experimental design. Plants were harvested after four months and then cut off the shoot at soil surface level and dried in an electric oven at 60 ^{0}C for 48 hours. The roots were washed well and dried by using the same method.

Table 1. Available phosphate, total nitrogen and heavy metals in the soil

pH	7.58
Available phosphate	0.9 gm Kg^{-1}
Total nitrogen	1.7 gm Kg^{-1}
Cd	Nil
Pb	6 ppm

Analysis of the soil after the harvest: Soil N and P content was estimated for all treatments (Bremner and Mulvaney1982, Watanable and Olsen 1965). The total heavy metals (Cd and Pb) were analyzed by flame atomic absorption photometer (Kumpulainen and Paakki 1987).

Plant analysis after harvest: Shoot, root and seed samples were analyzed to estimate total nitrogen, total phosphorous, cadmium and lead in all treatments as per procedure suggested by McKeague (1978).

Impact of heavy elements in the dry weight: Fresh shoot, root and seed samples washed with water were dried separately in an electric oven 60^{0}C till constant weight to calculate dry weight. The mycorrhizal conducting dependency, which is a plant dependent on AM when the value of mycorrhizal dependency higher than 50% (Davies et al 2001).

RESULTS AND DISCUSSION

The onion was used as host plant for activating mycorrhizal fungi through infected root. The pad technique was used to add myco-fertilizer, which helps to inoculate the onion bulb near *Glomus leptotichum* propgules which thereby increases the chance of colonization on roots with AM (Owusn,-Bennoah and Mosse 1979). *Glomus leptotichum* succeeded in infecting the roots of onions plant and when stained with acid fuchsin dye, infection rate was 70-80% when propagule structures was examined under optical microscope.

Cadmium treatment: The results in treatment 20 ppm of Cd indicate that *G. leptotichum* showed higher efficiency in extracting Cd from the soil and that this differential impact perhaps because of genetic differences for AM fungi which led to the difference in efficiency of AM infection in the soil contaminated with heavy metals and toxic effects (Chandrashekara et al 1995). AM have an additional role in increasing the accumulation of heavy metals in the plant and this is consistent with the findings of Joner and Leyval (1997)

Table 2. Treatments and heavy metals concentration and mycorrhizal fungi used in this study

Treatment	Fungal treatment	Heavy metal
1	Negative control (- AM)	-H.M.
2	Positive control (+ AM)	-H.M.
3	+ AM	Cd concentration of 0.15 is equivalent to 1 mg kg $^{-1}$ of CdSO4.8H2O
4	+ AM	Cd concentration of 1.5 is equivalent to 10 mg kg $^{-1}$ of CdSO4.8H2O
5	+ AM	Cd concentration of 3.0 is equivalent to 20 g kg $^{-1}$ of CdSO4.8H2O
6	+ AM	Cd concentration of 6.0 is equivalent to 40g kg $^{-1}$ of CdSO4.8H2O
7	+ AM	Pb concentration of 7 equivalent to 12.5 mg kg $^{-1}$ of (CH3COO)Pb.3H2O
8	+ AM	Pb concentration of 14 equivalent to 25 g kg $^{-1}$ of (CH3COO)Pb.3H2O
9	+ AM	Pb concentration of 28, equivalent to 50 g kg $^{-1}$ of (CH3COO)Pb.3H2O
10	+ AM	Pb concentration of 56 equivalent to 100 mg kg $^{-1}$ of (CH3COO)Pb.3H2O

who observed that extraradical hyphae for AM fungi is responsible for the transfer of Cd from soil to plant. This also increased the amount of phosphorus at concentrations 1 and 10 ppm of Cd in roots inoculated with *G. leptotichum*, which indicates not affected by taking phosphorus added Cd.

Low phosphorus content in the roots was 0.57 g kg^{-1}, and about 0.7 g kg^{-1} in the positive control which treated with *G. leptotichum*. This indicates the effectivity of AM to increase the availability of phosphorus.The total phosphorus content in the shoot increased from 1.7 to 4.5 g kg^{-1} at AM fertilization with *G. leptotichum* but different concentrations of Cd resulted in decrease to 2.5 g kg^{-1}, although it remained higher than the phosphorus content in the negative control, which indicates the effectiveness of AM. Rivera-Becerril et al (2002) observed no significant difference between mycorrhizal plants with phosphorus content when treatment with Cd.

The accumulation of cadmium in the soil and its association is non-motile in the alkaline soil may be a change in the level in various concentrations due to the AM action (Fig. 1), working to extract ingredients from the soil and transported to the plant. The reason for the survival of large amounts of cadmium in the soil due to the impact of the toxic activity and effectiveness of AM in disrupting mechanical bio-extraction. The results indicate that the transaction *G. leptotichum* showed higher efficiency in extracting Cd from the soil in the low concentrations rather than in high concentrations (Chandrashekara et al 1995). The findings show significant differences in cadmium transactions which increased in the positive control. The accumulation of this metal in the shoot in spite of this part of the plant is not a nutritional importance due to the lack of nutrients and large number of fibers with a lack of content protein but that refers to the transmission element from the soil to the roots and shoot because of the presence of genetic tankers like AtNramp3 which transport cadmium and collects in the food vacuoles in the shoot as well as TgMIP1 tankers that transport cadmium to the leaf vacuoles and tankers TcZNT1 that transport cadmium to the shoot also, but the amount is less than the accumulated in the roots (Hall and Williams 2003). The amount of cadmium accumulated in the roots with shoot, indicate that the roots contain about 5 times more amount than the shoot and these results are consistent with Rivera-Becerril et al (2002). The studies of mycorrhiza type *G. intrakadisces* with *Pisum satium* indicated that cadmium accumulates in the roots 20-50 times more than the shoot, and existence of several plant species stand in front of the transfer of cadmium to shoot and this behavior is one of several strategies to accumulate pollutants. Mycorrhizal fungi play an additional role in increasing the accumulation of heavy metals in the plant. The mycorrhizal extraradical hyphae is responsible for the transfer of cadmium from soil (Zhitong et al 2012). The variation in the accumulation of cadmium in the seed was maximum 7.5 ppm/ seed weight treated with *G. leptotichum*, which showed high efficiency in the extraction of cadmium and then moving to the seeds, and exceeds the limit, which ranges between 0.05-0.2 ppm, and up to toxic ranging limit 5-30 ppm, when compared with the amounts of cadmium accumulated in the roots and seeds with the shoot parts. The cadmium did not arrive to the seeds (Fig. 1). It was explained by the existence of a strategy to link the heavy metals with the cell wall of roots and shoots, which reduces the concentrations of these elements in the seeds. There are other mechanical plants tolerant to the toxicity of heavy metals depends on detoxification and the secretion of substances to cells vacuoles by metallothioneins molecules and phytochelatins (Cobbett and Goldsbrough 2000, Cobbett 2000).

The dry weight of roots and shoot of the sunflower has dropped significantly when comparing fungi and concentration of heavy metal which refers to the inhibitory effect of cadmium on the shoot (Table 3). The result showed that treatment of AM with high toxicity of cadmium, colonization decreased and led to a significant inhibition of the growth of shoot compared to its influence on root, which was evident in the growth during the study period where plant's dependence on AM and accompanied by atrophy of the shoot of high toxicity at high concentrations of cadmium. High concentration of heavy metals in soil have an adverse effect on AM fungi and microbial processes due to thier toxicity for living organisims (Sergio et al 2012). Davies et al (2001) also observed that this has negative relationship with heavy metals. But the treatment plants of different concentrations of cadmium display slight impact of the *G. leptotichum* indicating significant changes in vital operations and photosynthesis because of heavy metals contamination.

The percentage of positive control with the dependency of the plant on AM fungi, was 109.4%, while the plants which treated with cadmium in the treatment with AM dependency on AM the percentage dropped to 33.5% and reached the highest level cadmium concentration of 40 ppm (Table 3).

Lead treatment: *G. leptotichum* was highly effective in the availability of shoot phosphorus and increased efficiency in the concentration of 12.5 ppm. Very ambiguous, only author can tell what he want to say. This had reached to maximum effect 100 ppm concentration. The figure 2 shows that AM fungi have highly efficient in taking the lead from the soil where they were drawn tainted completely in the first concentration 12.5 ppm, in addition to what is present in 6 ppm and continued efficiency even at the highest concentration of 100 ppm. The amount of lead in the roots increased with increasing concentrations and with a significant effect for the type *G. leptotichum*, despite the fact that there is a genetic sole which limit transmission of lead, a known channels (GNGC) (Fig. 2). These channels transporting lead through the plasma membrane into the cell and is working to accumulate inside the plant cells, where the results of the current study, agreed with the findings. The viability of accumulative high in the tissues of plant roots to lead, reaching the highest accumulative amount of lead 212.5 µg/gm dry weight and this amount is within the limits allowed globally. There was non significant increase in the shoot between the positive control and negative control which refers to the positive impact of fungi AM. Gaur and Adholeya (2004) observed that *G. mossese* has weak susceptibility to compile lead in the vegetative part. The increase the surface absorption by extended hyphea in addition to the root hairs form a barrier against the transfer of heavy metals into the plant shoot. At the genetic level, the responsibility for the transfer of lead into the cells through the plasma membrane is GNGC channels that play a key role in the accumulation of lead in the plant process (Gaur and Adholeya 2004). There is a high percentage of lead up to 85 ppm accumulated in the sun flower seeds (Fig. 2). This is within the acceptable limits determined by international standards 30-300 ppm which is not considered harmful to public health when ingested directly by human food or animal feed (Medina et al 2003).

The root dry weight of the positive control may outweigh the negative control and that may be due to the influence of AM fungi the root to increase the surface area for the absorption of reflecting on the obvious differences in dry weight of shoots by AM fungi. Dry weight also increased in roots inoculated with *G.leptotichum* treatments 12.5 and 25 ppm due to increased dry weight of the root compared with shoot due to the toxic effects of lead. In plants treated with different concentrations of lead the shoot showed an increase in the content of the total nitrogen in the fertilized treatment with *G. leptotichum* especially in 12.5 ppm concentration and then started to decrease to concentration 100 ppm reached 0.93. The results of AM myccorhizal dependency index indicte that the toxicity of lead to sunflower at higher concentration influenced shoot root ratio compared to the negative control. The percentage of the effect of AM may be zero in all treatments suggesting AM failed to infection due to the toxic effect of lead on the AM fungi. But when inoculated with *G.leptotichum* plants in the positive control and was dependent on AM fungi (109.4%), but when added the lead concentration 12.5 ppm and other concentrations to 100 ppm, plant was not supported on AM even with independency ratio of 6.1%.

Table 3. Effect of different concentrations of cadmium in the dry weight of sunflower (*Helianthus annuus*) infected by AM fungi type *G. leptotichum*

Cadmium treatment/ ppm	Shoot (g dry weight)	Root (g dry weight)	Total plant (g dry weight)	Mycorrhizal dependency (%)	Root/shoot ratio (%)
Negative control	3.51	0.42	3.93	---	0.12
Positive control	7.33	0.9	8.23	109.414	0.123
1	4.75	0.5	5.25	33.587	0.105
10	4.35	0.46	4.81	22.391	0.106
20	4.51	0.37	4.88	24.173	0.082
40	2.45	0.34	2.79	-29.007	0.139

Table 4. Effect of different concentrations of lead in the dry weight of the plant sunflower (*Helianthus annuus*) infected with AM fungi type *G. leptotichum*

Cadmium treatment/ ppm	Shoot (g dry weight)	Root (g dry weight)	Total plant (g dry weight)	Mycorrhizal dependency (%)	Root/shoot ratio (%)
Negative control	3.51	0.42	3.93	---	0.12
Positive control	7.33	0.9	8.23	109.414	0.123
12.5	4.4	0.7	5.1	29.77	0.159
25	4.19	0.66	4.85	23.409	0.157
50	3.98	0.62	4.6	17.048	0.155
100	3.14	0.55	3.69	-6.106	0.175

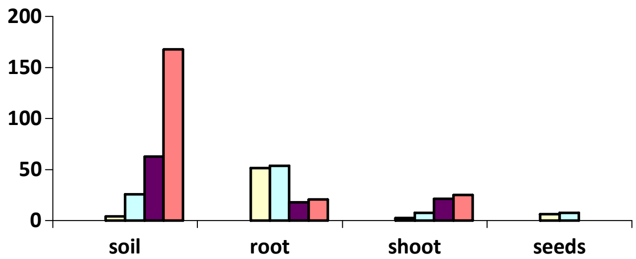

Fig. 1. Concentration of cadmium accumulated rates in the soil, roots, shoot and seeds sunflower plants inoculated with AM fungi

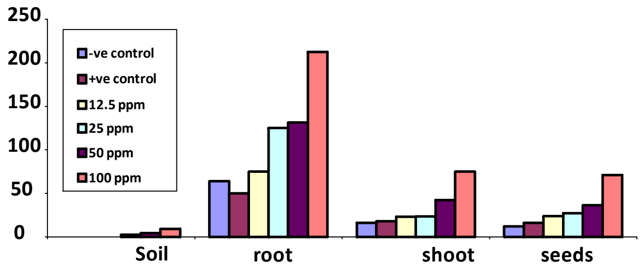

Fig. 2. The concentration of lead accumulated in the soil, the roots, the vegetative parts and sunflower seeds and in plants inoculated with VAM fungi

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