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Research Article

The Effects of Inoculation with Three *Glomus* Species on Growth and Pb Uptake by Hemp (*Cannabis Sativa*) in a Pb-Contaminated Soil

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Abstract: Increasing levels of hazardous heavy metals, which enter soil and water ecosystems have had a detrimental effect on global living organisms and needs special attention. The effect of inoculation with three *Glomus* mycorrhizal fungi (*G.mosseae*, *G.etunicatum* and *G.constrictum*) on hemp growth and some biological properties was evaluated in a soil contaminated with different levels of lead (0, 600, 1200 and 1800 $mg \cdot kg^{-1}$ Pb soil) in the form of $Pb(NO_3)_2$. The results show that shoot and root dry weights decreased with increasing Pb levels in soil. But Pb concentration in roots and shoots of hemp increased significantly with increasing Pb levels in soil. However the growth of mycorrhizal inoculated hemps was significantly higher than that of non-inoculated hemp. Mycorrhizal inoculation increased the concentration of Pb in the shoot and root of hemp. In this study, both translocation factor (TF) and enrichment factor (EF) were < 1 and decreased with increasing Pb concentration in soil. The TF was higher in non-inoculated hemp than inoculated hemps. It shows the inhibition effect of mycorrhiza in translocation of Pb from root to shoot. The EF of all inoculated hemps (0.18) was significantly greater than non-inoculated hemp (0.17), showing increased uptake of Pb due to inoculation with mycorrhiza. Simple linear regressions show that the diethylenetriaminepentaacetic acid (DTPA) extractable Pb is more suitable index for evaluating the toxicity effect of soil Pb than the total Pb content.

Keywords: Pb pollution, phytoremediation, mycorrhiza, hemp

1. Introduction

Lead (Pb) compounds, originate mainly from weathering, industries, storage batteries, vehicle fumes, metal-plating factories, mining and smelting of Pb ores, Pb-containing pigments, fertilizers, pesticides, sewage sludges, explosives, additives and gasoline [1-2], all of which are regarded as a principal source of environmental contamination [3]. Contaminated soils contain Pb in the range of 400-800 $mg \cdot kg^{-1}$ soil, although in industrialised areas, this level may reach 1000 $mg \cdot kg^{-1}$ soil [4-5].

There are various methods for cleaning of environment from heavy metals. The application of biological methods, including plant bioremediation and use of microorganisms, has been proven to be more effective and less expensive [6-7].

Arbuscular mycorrhizal (AM) fungi are developing symbiosis with the most of the plants and are occurring in almost all soils [8]. Numerous research works have indicated that arbuscular mycorrhizal (AM) can benefit their host

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plant nutrition and enhance plant tolerance of heavy metal pollution [9-11] by several mechanisms, including extra-and intracellular precipitation, sequestration and biosorption to the cell wall [12].

While some researchers reported that the inoculation with mycorrhizal fungus resulted to an increased uptake of the heavy metals by plants and its transfer from roots to shoots tissues [13-14], others reporters indicated that it led to reduction in the uptake of heavy elements by plants [15] or stabilization of heavy metals within roots [16-18].

Although, AM fungi usually can enhance plant resistance to heavy metals [19-22], but selection of autochthonous AM species from heavy metal contaminated areas that are adapted to this conditions, can be more beneficial [23-24].

At this study the effect of inoculation with three *Glomus* species, isolated from mine soils, has been investigated to study its impacts on the metal tolerance and accumulation abilities of hemp. Previous studies showed that Hemp, *Cannabis sativa*, is a fast growing, metal tolerant, and high biomass producing plant that has evolved mechanisms enabling it to cope with high heavy metal contaminated soils [25-26]. The possibilities of growing in different climates and using its biomass in non-food industries can make hemp a proper crop to recover the heavy metal contaminated soils' productivity, and, although slowly, restore them at the same time.

The objectives of this study were to investigate the influences of different levels of Pb and *Glomus* species on some chemical and biological characteristics of an artificially polluted soil and Pb phytoextraction by *C. sativa*.

2. Materials and methods

An agricultural soil (*Fluventic Haploxerept*) was sampled from 0 to 30 cm depth of a farm located at Joraghan, Hamadan, Iran. The soil was brought to the laboratory, hand-picked to remove stones, air dried, passed through a 2 mm sieve and mixed thoroughly. A sub-sample of the soil was taken and analysed for selected physicochemical properties (Table 1).

Table 1. Physical and chemical properties of the soil used in this experiment

Properties value	value
Texture	Sandy loam
Clay	18 (%)
Sand	56 (%)
Silt	26 (%)
pH	7.7
$CaCO_3$ Equivalent	8 (%)
CEC	8 ($Cmolc \cdot kg^{-1}$)
Organic carbon	0.9 (%)
EC	1.2 ($dS \cdot m^{-1}$)
P (Olsen)	3.2 ($mg \cdot kg^{-1}$)
Pb (total)	70.0 ($mg \cdot kg^{-1}$)

Pb was added to the soil as $Pb(NO_3)_2$ solution for polluting of control (sampling soil had 70 $mg \cdot kg^{-1}$ Pb before Pb adding), 600, 1200 and 1800 $mg \cdot kg^{-1}$ Pb soil. Urea was supplied to equilibrate the N level in $Pb(NO_3)_2$ treated and untreated soils.

After mixing, the soils were placed in plastic cups and incubated at 25-28°C for 90 days. After rapid Pb reaction and its equilibrium in soil [27], each soil was sterilized separately with hot steam by a steam sterilization machine.

Three *Glomus* species including *G.mosseae* (*Funneliformis mosseae*), *G.etunicatum* (*Claroideoglomus etunicatum*)

and *G.constrictum* (*Septoglomus constrictum*) were isolated from Zanjan Zn-Pb mine (Zanjan Province, west of Iran), Dasht-e-Tabriz (East-Azarbayjan Province, northwest of Iran) and Ahangaran Zn-Pb mine (Hamedan Province, South west of Iran), respectively.

Fungi spores were surface sterilized by washing with 10% calcium hypochlorite. The spores were then propagated in sterilized sandy soil by establishing trap cultures using maize as host plant. These pots were sustained for about four months and during this time were irrigated three times a week, once with sterilized Hoagland solutions (with half strength of phosphorus) and the two other times with sterilized distilled water.

The AM inoculums comprised of a mixture of rhizospheric soil from trap cultures containing spores (350 spore·g^{-1} inoculum), hyphae and mycorrhizal root fragments, as much as 70 g per 4 kg·pot, were layered at 5 cm below the hemp seeds. The hemp seeds previously were surface sterilized by soaking in 1% sodium hypochlorite (NaOCl) for 5min and subsequently rinsed thoroughly with sterilized water. Also, for the non-inoculated treatment, a 70 g sterilized inoculum was added. Three replications were applied to each treatment with a total of 48 pots in the greenhouse, College of Agriculture, Bu-Ali Sina University.

At the beginning, each pot was planted with 8 seeds of hemp and then reduced to 4 plants in each pot one week after sowing. The greenhouse temperature during the growing period was 21 to 32°C. During this period, in order to maintain soil moisture at field capacity, tap water was added twice a week to the saucer, so soil gets wet by capillary.

At the end of growth, the chlorophyll content of leaves was measured using a chlorophyll meter (Minolta Spad-502) and three readings were taken of leaves of middle third of hemp shoots.

After 65 days, in flowering stage of growth of the hemp, harvest began and shoots and roots were separated. A part of the plant roots was stained with trypan-blue dye and the percentage of root mycorrhizal colonization was estimated using grid-line intersect method [28].

After plant harvesting, shoots and roots were separated, washed with distilled water, oven-dried at 70°C for 72 h, weighed, powdered, and digested and extracted with 4 M HNO_3 at 95°C [29] and Pb was measured in the filtrate. The concentration of Pb in the soil samples was determined according to [30]: 3 g soil was pre-digested with 28 mL 3:1 ratio of 37% HCL: 70% HNO_3 mixture for 16 h at room temperature and then was digested at 130°C for 2 h in a reflux condenser. DTPA extraction of Pb was carried out by shaking 5 g of soil samples with 10 mL of a solution of 0.005 M DTPA, 0.1 M triethanolamine (TEA) and 0.01 M $CaCl_2$ in an end-over-end shaker for 2 hours and then filtering the leachate [31]. The Pb concentration in solutions was determined by atomic absorption spectrophotometry (AAS) (Varian, spectra 220).

Lead uptake by shoots from each pot was estimated from the product of the shoot Pb concentration and shoot bimass. Lead translocation factor was estimated from dividing Pb concentration in the shoot tissue by the root Pb concentration [32]. Enrichment factor was estimated from dividing Pb concentration in shoot tissue (mg·kg^{-1} plant dry weight) by lead concentration in soil (mg·kg^{-1} soil dry weight) [33].

Olsen phosphorus [34] of the soil was measured, and also phosphorus concentration of shoot and root biomass were measured in digest extracts of plant using [35] method.

This study was conducted as a factorial experiment in a completely randomized design with two factors, soil Pb level (0, 600, 1200 and 1800 mg·kg^{-1}) and three fungal species (*G.mosseae*, *G.etunicatum* and *G.constrictum*), with three replications. Data processing and statistical analyses were performed with SAS software. Duncan's multiple range test was performed at 0.05 level of significance to compare the treatments.

3. Results and discussion

ANOVA of effect of Pb and fungus treatments on plant properties:

The ANOVA results in Table 2 show significant changes in chemical and biological properties due to plant inoculation with fungus. Also, significant changes in all properties except total colonization, arbuscule, hyphae and spore number due to soil treatment by Pb have been seen. Although the interaction of Pb pollution and fungus produced different responses.

Table 2. ANOVA of the mean squares of chemical and biological properties

	Pb	Fungus	Pb*Fungus	error
Df	3	3	9	32
Shoot dry W	5.20***	4.70***	0.21 ns	0.21
Root dry W	0.18***	0.21***	0.02 ns	0.02
Shoot Pb con.	60341.04***	398.80***	90.86**	23.64
Root Pb con.	345801.95***	894.84***	47.89 ns	42.00
Pb uptake by shoot	1.19***	0.11***	0.01*	0.47×10^{-2}
Translocation F	0.22***	0.73×10^{-2}*	0.15×10^{-1}***	0.23×10^{-2}
Enrichment F	0.10***	0.67×10^{-3}**	0.14×10^{-3} ns	0.10×10^{-3}
Soil olsen phosphorus	1.77***	0.43***	0.02 ns	0.05
Shoot Phosphorus con.	19.76***	4.50***	0.48**	0.12
Root Phosphorus con.	10.52***	1.60***	0.03 ns	0.04
Total Colonization	0.21 ns	39.78***	0.08 ns	0.20
Arbuscule	0.16 ns	31.94***	0.03 ns	0.16
Vesicle	0.11***	15.82***	0.03*	0.01
hyphea	0.20 ns	29.83***	0.25 ns	0.13
Spore number	10.10 ns	3468.00***	2.18 ns	7.04
ChlorophyII	112.88***	22.30***	6.15***	1.18

Significant are noted by: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, $p > 0.05$

Pb total and DTPA concentrations;

Figure 1 shows the changes in the soil concentrations of total and DTPA extractable Pb after addition of soluble Pb to the soil. It is observable that DTPA-extractable Pb concentration increased with increase in Pb levels, but not at the same rate as total Pb concentration. This may be related to decrease of DTPA extractable Pb due to its precipitation and immobilization in soil in 90 days of soil incubation.

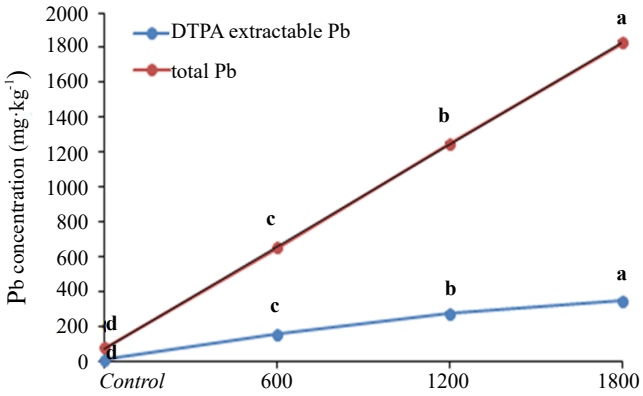


Figure 1. DTPA-extractable and total Pb in soils treated with different levels of Pb

Plant growth and performance;

Shoot and root dry weights showed sharp response to soil Pb pollution (Table 3). Shoot mean dry weight decreased

from 6.04 g·pot^{-1} for hemps planted in control soil to 5.74, 5.18 and 4.55 g·pot^{-1} for hemps planted in soils treated with 600, 1200 and 1800 mg·kg^{-1} Pb respectively. Root mean dry weight decreased from 0.82 g·pot^{-1} for hemp in control soil to 0.63, 0.59 and 0.55 g·pot^{-1} for hemps planted in 600, 1200 and 1800 mg·kg^{-1} Pb soil.

Table 3. Means (± SD) of soil Olsen phosphorus and some plant properties

Pb	fungus	Soil Olsen P	Shoot DW (g·pot^{-1})	Root DW (g·pot^{-1})	Shoot P con. (mg·g^{-1})	Root P con (mg·g^{-1})	Chlorophyll index (spad)
0	G.mosseae	11.52ab (0.26)	5.86bcd (0.45)	0.79b (0.21)	6.44de (0.15)	5.37abc (0.19)	36.00b (0.80)
	G.etunicatum	11.55ab (0.20)	6.93a (0.31)	1.12a (0.06)	8.00a (0.45)	5.39ab (0.29)	38.17a (1.60)
	G.constrictum	11.87a (0.15)	6.26ab (0.39)	0.74bc (0.32)	7.60ab (0.41)	5.56a (0.32)	38.27a (1.22)
	Non-inoculated	11.55ab (0.14)	5.13def (0.18)	0.62bcde (0.09)	6.03ef (0.25)	4.75d (0.16)	34.53bc (1.51)
600	G.mosseae	11.25bc (0.19)	5.48bcde (0.46)	0.46de (0.09)	6.74cd (0.38)	5.00cd (0.21)	34.23bcd (1.37)
	G.etunicatum	11.28bc (0.18)	6.19abc (0.34)	0.78b (0.09)	6.46de (0.68)	5.12bcd (0.20)	32.37d (0.55)
	G.constrictum	11.70a (0.25)	6.12abc (0.83)	0.73bc (0.10)	7.10bc (0.36)	5.07bcd (0.22)	33.70cd (1.15)
	Non-inoculated	11.23bc (0.15)	5.16def (0.38)	0.55 bcde (0.06)	5.45f (0.58)	4.28e (0.17)	33.00cd (1.00)
1200	G.mosseae	10.98cd (0.16)	4.81ef (0.47)	0.50 cde (0.12)	5.51f (0.13)	4.36e (0.20)	29.13ef (1.33)
	G.etunicatum	11.17bc (0.15)	6.28ab (0.31)	0.70bcd (0.10)	5.61f (0.22)	4.12e (0.29)	32.37d (0.61)
	G.constrictum	11.30bc (0.25)	4.90ef (0.72)	0.68bcd (0.13)	5.54f (0.26)	4.14e (0.17)	32.47d (0.93)
	Non-inoculated	10.93cd (0.13)	4.72ef (0.33)	0.46de (0.04)	4.69g (0.23)	3.50f (0.15)	27.63f (1.26)
1800	G.mosseae	10.70de (0.34)	4.51fg (0.61)	0.51cde (0.09)	4.60g (0.37)	3.48f (0.15)	29.03ef (1.15)
	G.etunicatum	10.71de (0.31)	5.36cdef (0.62)	0.63bcde (0.12)	4.32g (0.22)	3.30f (0.17)	33.10cd (0.36)
	G.constrictum	11.02cd (0.22)	4.55fg (0.36)	0.62bcde (0.09)	4.36g (0.21)	3.24f (0.20)	29.77e (0.95)
	Non-inoculated	10.45e (0.21)	3.78g (0.34)	0.42e (0.08)	3.15h (0.14)	2.61g (0.16)	28.93ef (0.70)

Means within each column with the same letter are not significantly different at the 5% level

Hemp growth was greatly influenced by AM inoculation. Shoot and root biomasses compared with those for the non-inoculated hemp significantly were higher. The highest mean dry weight of shoot (6.19 g·pot^{-1}) was observed in hemp inoculated with *G.etunicatum*, and followed by *G.constrictum* inoculated (5.46 g·pot^{-1}), *G.mosseae* inoculated (5.17 g·pot^{-1}) and the non-inoculated (4.70 g·pot^{-1}) hemps. Root dry weights decreased to 0.81, 0.70, 0.57 and 0.51 g·pot^{-1} for *G.etunicatum*-, *G.constrictum*-, *G.mosseae*-inoculated and the non-inoculated hemps, respectively. Other researchers reported the decrease in root biomass with increase in Pb contamination and also the increase of root and shoot dry weights by application biofertilizer of *Glomus mosseae* [36]. The decrease in plant growth due to heavy metal stress could be because of damage of root cell walls and decrease in the absorption of nutrients [37]. Furthermore there are some physical and chemical reactions between heavy metals and soil components (for example organic matter) that lead to reduced soil fertility [38].

The chlorophyll content of leaves decreased with the increase in Pb level in soil. Other researchers reported a decrease in chlorophyll content of plants grown in soils with contamination of heavy metals, especially with Cu and Pb pollutions [39-42]. This could be because of competition of Pb with nutrient elements, change in the permeability of cell membranes, and replacement of Mg with Pb in chlorophyll structure that resulted in reduce in synthesis of photosynthetic pigments [43].

The inoculation of hemp with AM fungi increased the chlorophyll content of plant. The effect of fungi on chlorophyll content was in the following order: *G.etunicatum* = *G.constrictum* > *G.mosseae* > non-inoculated. The highest chlorophyll content (38.27 spad) was observed in the hemp inoculated with *G.constrictum* planted in the control soil and the lowest contents were observed in hemps inoculated with *G.mosseae* and non-inoculated hemps planted in 1200 and 1800 mg·kg^{-1} Pb polluted soils. This could be related to the role of mycorrhizal fungi in providing phosphorus

as an energy source during photosynthesis [44].

On the average, phosphorus concentration in shoot tissues of hemp decreased with the increase in Pb level in soil. The highest phosphorus concentration in shoots (8.00 $mg \cdot g^{-1}$) measured in hemp inoculated with *G.etunicatum* planted in control soil and the lowest (3.15 $mg \cdot g^{-1}$) was observed in shoots of non-inoculated hemp planted in soil with 1800 $mg \cdot kg^{-1}$ Pb. The phosphorus concentration of roots also decreased with the increase in Pb level in soil and changed from 5.27 $mg \cdot g^{-1}$ for control to 4.87, 4.03 and 3.16 $mg \cdot g^{-1}$ for 600, 1200 and 1800 $mg \cdot kg^{-1}$ Pb respectively. [45] illustrated that great amounts of Pb in soil can cause phosphorus to precipitate, so its absorption by plants will be reduced.

Inoculation of hemp roots with AM fungus, increased the phosphorus concentration in shoot and root tissues. Fungus treatments affect the phosphorus concentration in shoot tissues of hemp in this manner *G.constrictum* > *G.etunicatum* > *G.mosseae* > non-inoculated. This manner for phosphorus concentration in root tissues was *G.mosseae* = *G.constrictum* = *G.etunicatum* > non-inoculated. Mycorrhizal fungi can directly uptake phosphorus through its hyphae and transfer it to plant and furthermore it may contribute to decreased soil pH that resulted in solubilizing of insoluble phosphates in the soil and subsequently more uptake of phosphorus by plant roots [46].

AM fungal colonization status;

Spore numbers in soil treated with *G.constrictum* is higher than other fungi treatments. A few spore numbers counted in the non-inoculated pots is related to spores that retained their shape during the soil sterilization, and also some spores are from fungus pollution during the growth period. It is in accordance with the microscopic observation of the root sections. Low root colonization percentage for the non-inoculated roots shows fungi pollution of hemps in these pots.

No significant difference in spore numbers was found as a result of Pb pollution (Table 2). In other studies by planting Zea mays in contaminated soils with heavy metals, no changes were reported in mycorrhizal spore numbers due to increase of heavy metals concentration [47].

Figure 2 shows mycorrhizal vesicles and hyphae in hemp root. The percentage of root colonization of hemps inoculated with *G.mosseae* and *G.constrictum* increased with increasing Pb level in soil from 0 to 1200 $mg \cdot kg^{-1}$ and then it decreased (not significantly) at 1800 $mg \cdot kg^{-1}$ Pb pollution.

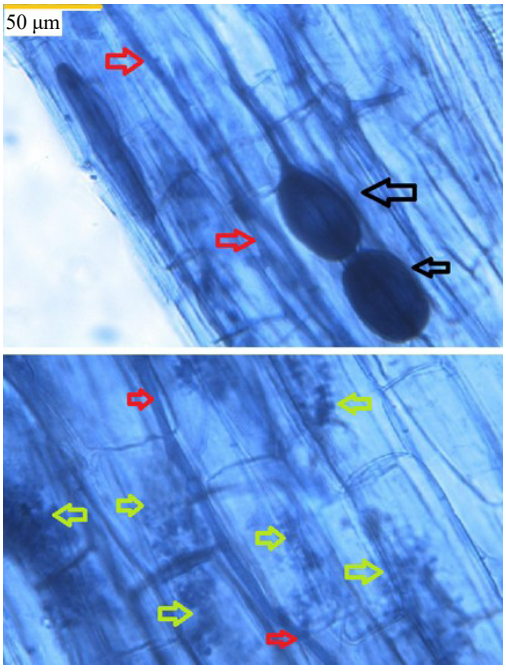


Figure 2. Stained roots of hemp showing fungal organs in the cortex. Black, green, and red arrows showed mycorrhizal vesicles, arbuscules, and hyphae respectively

But the percentage of root colonization of *G.etunicatum* inoculated hemps, decreased (not significantly) with increasing Pb level in soil (Table 4).

Although some researchers reported a decrease in root colonization by mycorrhizal fungi with increasing heavy metal pollution [48-50], others reported the reverse results same as our observation [51-52]. [14] concluded that fungus and plant species as micro and macrosymbionts, heavy metal concentration, and soil properties are all important, and have specific effects on the root colonization percentage.

Table 4. Means (std division) of root mycorrhizal colonization and spore numbers of soil

Pb	fungus	arbuscules (%)	vesicles (%)	hyphae (%)	Colonization (%)	Spore number (num·g^{-1})
0	G.mosseae	22.84d (3.35)	2.20c (0.30)	6.05 (2.08)de	29.58e (6.19)	45.15c (2.72)
	G.etunicatum	23.68cd (4.56)	8.80a (0.30)	18.98a (0.50)	48.87bc (4.84)	46.59bc (3.41)
	G.constrictum	29.73abc (3.65)	1.93cd (0.15)	18.70a (3.93)	48.43bc (0.58)	49.22abc (2.81)
	Non-inoculated	0.56e (0.96)	0.00f (0.00)	0.00e (0.00)	0.56f (0.96)	14.13d (4.49)
600	G.mosseae	29.03abcd (3.51)	1.63de (0.50)	11.93bcd (3.10)	41.88d (2.20)	44.43c (2.75)
	G.etunicatum	30.03abc (2.90)	8.14b (0.25)	14.55abc (5.91)	46.40bcd (3.14)	47.07abc (4.35)
	G.constrictum	31.20ab (2.55)	1.57de (0.38)	17.85abc (4.20)	49.95b (3.38)	49.10abc (3.06)
	Non-inoculated	1.31e (1.14)	0.00f (0.00)	0.71e (1.23)	2.02f (2.14)	14.61d (2.90)
1200	G.mosseae	27.33bcd (1.80)	1.23e (0.25)	18.30ab (2.07)	46.03bcd (3.03)	47.43abc (1.07)
	G.etunicatum	27.33bcd (2.08)	7.92b (0.18)	13.70abc (4.72)	46.86bcd (2.85)	48.27abc (0.90)
	G.constrictum	35.06a (5.39)	1.63de (0.42)	19.17a (7.68)	55.69a (3.09)	51.98a (0.91)
	Non-inoculated	1.06e (0.92)	0.00f (0.00)	0.52e (0.90)	1.58f (1.57)	14.01d (3.43)
1800	G.mosseae	29.24abcd (4.16)	1.27e (0.15)	11.58cd (3.61)	42.03d (2.92)	47.67abc (0.55)
	G.etunicatum	22.92d (3.89)	8.08b (0.39)	13.56abc (2.42)	43.32cd (2.90)	48.15abc (0.36)
	G.constrictum	31.27ab (6.82)	1.33e (0.15)	18.74a (2.81)	50.98ab (4.97)	51.38ab (0.72)
	Non-inoculated	1.10e (0.97)	0.00f (0.00)	0.00e (0.00)	1.10f (0.97)	14.13d (2.39)

Means within each column with the same letter are not significantly different at the 5% level

The highest percentage of root AM colonization (55.69%) was observed in hemp inoculated with *G.constrictum* planted in 1200 mg·kg^{-1} Pb polluted soil and the lowest values (0.56 to 2.02%) were observed in the non-inoculated hemps. This low root colonization of the non-inoculated hemps may be due to the imperfect sterilization of soil and/or infection of soil during the growth period of hemps by irrigation with tap water and aeration with unsterile air of the greenhouse.

Arbuscules percentage in plant roots was the lowest one (19.20%) in control soil and it increased with increasing the levels of Pb in soil. It was 22.89% and 22.69% in soil treated with 600 and 1200 mg·kg^{-1} Pb respectively. Arbuscules percentage in plant roots decreased in soil treated with 1800 mg·kg^{-1} Pb (21.13%). Arbuscules percentage in all of the inoculated plants was higher than that in the non-inoculated plants. Plant inoculated with *G.constrictum* had the highest arbuscules percentage in roots (31.81%).

Vesicles percentage in roots of hemp was lower than the arbuscules and hyphae percentage may be due to the harvesting time. It was at the flowering stage of plants. Vesicles percentage decreased with increase in Pb level in soil from control (3.23%) to 600 (2.83%), 1200 (2.70%) and 1800 mg·kg^{-1} Pb level (2.67%). Vesicles percentage in roots of plants inoculated with *G.etunicatum* (8.23%) was higher than that in roots of plants inoculated with *G.constrictum* (1.62%) and *G.mosseae* (1.58%). Vesicles percentage in roots of non-inoculated plants was 0.00%.

In contrast to arbuscules and vesicles percentages, hyphae percentage in roots of hemp decreased, though not significantly, with increasing of Pb level in soil. It was significantly higher in inoculated plants compared with that

of non-inoculated plants. Plants inoculated with *G.constrictum* had the highest hyphae percentage (18.62%). Plants inoculated with *G.etunicatum* (15.20%) and *G.mosseae* (11.97%), and non-inoculated plants (0.31%) had lower hyphae percentage.

[36] and [54] reported higher root colonization due to the increase of heavy metals concentration in soil.

Plant Pb uptake and translocation;

The concentration of Pb increased in both the root and shoot tissues of hemps with increasing the Pb levels in soil.

Same as other reports it was higher in the root tissues of hemps compared with that of shoot tissues (Table 5).

Shoot Pb concentrations decreased from 195.25 $mg \cdot kg^{-1}$ for hemp planted in the soil treated with 1800 $mg \cdot kg^{-1}$ Pb to 152.50, 106.81 and 29.47 $mg \cdot kg^{-1}$ for hemps planted in the soils treated with 1200, 600, 0 $mg \cdot kg^{-1}$ Pb, respectively. Similarly root Pb concentrations decreased from 437.55 $mg \cdot kg^{-1}$ for hemp planted in the soil treated with 1800 $mg \cdot kg^{-1}$ Pb to 288.49, 167.98 and 39.86 $mg \cdot kg^{-1}$ for hemps planted in the soils treated with 1200, 600 and 0 $mg \cdot kg^{-1}$ Pb, respectively. The highest Pb concentration in shoots (208.33 $mg \cdot kg^{-1}$) was observed in the hemp inoculated with *G.constrictum* in soil treated with 1800 $mg \cdot kg^{-1}$ Pb (Table 5). Hemps planted in control soil (the untreated soil with Pb) had the lowest shoot Pb concentration.

Table 5. Means (std division) of Pb absorb factors by the hemp

Pb	fungus	Shoot Pb con. ($mg \cdot kg^{-1}$)	Root Pb con. ($mg \cdot kg^{-1}$)	Pb uptake by shoot ($mg \cdot pot^{-1}$)	Translocation F	Enrichment F
0	G.mosseae	28.18g (0.84)	37.72gh (4.72)	0.17g (0.02)	0.75b (0.08)	0.30b (0.01)
	G.etunicatum	30.64g (3.07)	44.11g (0.43)	0.22g (0.03)	0.69bc (0.06)	0.32a (0.03)
	G.constrictum	30.24g (1.04)	46.53g (2.10)	0.19g (0.02)	0.65cd (0.05)	0.32ab (0.01)
	Non-inoculated	28.81g (0.71)	31.08h (3.44)	0.15g (0.00)	0.93a (0.12)	0.30b (0.01)
600	G.mosseae	111.96e (2.90)	166.34ef (7.97)	0.61e (0.04)	0.67bcd (0.05)	0.17c (0.01)
	G.etunicatum	112.55e (3.24)	177.16e (10.91)	0.70de (0.04)	0.64cd (0.03)	0.18c (0.01)
	G.constrictum	107.84e (1.48)	169.59ef (12.73)	0.66de (0.10)	0.64cd (0.05)	0.17c (0.01)
	Non-inoculated	94.90f (3.24)	158.82f (3.36)	0.49f (0.05)	0.60de (0.03)	0.15d (0.01)
1200	G.mosseae	152.82d (4.64)	294.17c (7.87)	0.74de (0.09)	0.52efgh (0.03)	0.12e (0.01)
	G.etunicatum	155.39d (5.38)	293.49c (5.38)	0.98ab (0.08)	0.53efg (0.03)	0.12e (0.01)
	G.constrictum	154.87d (1.93)	296.55c (6.17)	0.76cd (0.11)	0.52efgh (0.01)	0.12e (0.00)
	Non-inoculated	146.92d (4.62)	269.75d (7.37)	0.69de (0.07)	0.55ef (0.03)	0.12e (0.01)
1800	G.mosseae	192.67b (9.71)	438.45a (6.83)	0.87bc (0.11)	0.44hi (0.02)	0.11ef (0.01)
	G.etunicatum	199.67b (5.69)	445.53a (4.22)	1.07a (0.07)	0.45ghi (0.02)	0.11ef (0.00)
	G.constrictum	208.33a (8.62)	441.58a (3.68)	0.95ab (0.04)	0.47fghi (0.02)	0.11ef (0.01)
	Non-inoculated	108.33c (7.64)	424.64b (4.00)	0.69de (0.09)	0.42i (0.02)	0.10f (0.01)

Means within each column with the same letter are not significantly different at the 5% level

Shoot Pb concentrations were significantly higher in the inoculated hemps with AM fungi compared with that of the non-inoculated hemp (112.74 $mg \cdot kg^{-1}$). Shoot Pb concentrations were 125.32, 124.56 and 121.41 $mg \cdot kg^{-1}$ in hemps inoculated with *G.constrictum*, *G.etunicatum* and *G.mosseae* respectively.

The lowest Pb concentration in root tissues (221.07 $mg \cdot kg^{-1}$) was measured in non-inoculated hemps. Among inoculated hemps, *G.etunicatum* inoculated hemp showed significantly more Pb concentration in root tissues and then after, were the hemps inoculated with *G.constrictum* and *G.mosseae*, respectively. In fungal treatments, root Pb concentrations were 240.07, 238.56 and 234.17 $mg \cdot kg^{-1}$ in hemps inoculated with *G.etunicatum*, *G.constrictum* and *G.mosseae* respectively.

Other studies [50] have reported that at high Pb concentration (0.1 mM), inoculation with *G.intraradices*

significantly increased the concentration of Pb in the shoot of Z. mays but no changes in root Pb concentration was observed. They also reported converted results about Agrostis, in low Pb concentration (0.01 mM), that mycorrhizal inoculation caused its root Pb concentration to increase but its shoot Pb concentration did not change.

A study [14] on Z. mays showed higher Cu concentration in shoot tissues of plant inoculated with Acaulospora laevis, *Glomus caledonium* and *Glomus manihotis* compared with non-inoculated plant.

In this study, Pb uptake in aerial parts of hemp was calculated. This is an important factor should be study in heavy metal phytoextraction. It was higher than the critical limit (30 $mg \cdot kg^{-1}$ Pb plant dry weight) in shoot and root tissues of hemps planted in Pb polluted soils (600, 1200 and 1800 $mg \cdot kg^{-1}$ Pb). These findings are in agreement with those of [53], that found the uptake of heavy metals in plants close to a mine in China was significantly high (upper than the critical limit) compared with that of plants far from the mine.

Translocation factors (TF) of heavy metals is an index that can be used to evaluate the capacity of plants to translocate heavy metals from roots to shoots. TF, the ratio of Pb concentration in plant shoots to plant roots, was < 1 and decreased with increase in Pb level in soil. On average, TF was the highest one in the non-inoculated hemp and closely followed by the hemps inoculated with *G.mosseae*, *G.etunicatum* and *G.constrictum*, respectively. Although the Pb concentration in root and shoots tissues of hemps increased by AM fungi but the increase of Pb concentration in root of hemps was higher. High TFs (> 1) maybe due to the efficiency of the transport system of plant [55]. However in many studies the TF of Pb by various plant species in soils with different properties was lower than 1 due to it low mobility in plant root and shoots tissues [32].

Enrichment factors (EF) can be used to evaluate the ability of plants to accumulate heavy metals in aerial parts. It is calculated as the ratio of the Pb concentration in shoot tissues to the Pb concentration in soil. It decreased from 0.31 for control to 0.17, 0.12 and 0.11 for 600, 1200 and 1800 $mg \cdot kg^{-1}$ Pb treatments, respectively. Mean EF of all inoculated hemps (0.18) was significantly greater than non-inoculated hemp (0.17). This shows the increase of uptake of Pb by hemp inoculation with AM fungi.

EF is an important index to evaluate a plant's ability to absorb metals and to show the mobility of metals from soil solution to shoot tissues of plant [56]. Plants with high ability to both absorb the metal from soil (EF > 1) and translocate it from root to shoot tissues (TF > 1), are superior for phytoremediation [32, 57]; although some researchers illustrated that some plants with EF < 1 are also accumulators for heavy metals [58].

In this study, both TF and EF decreased with increasing Pb level in soil. This agreed with the findings of [59], who substantiated that EF correlated to bioavailability and concentration of heavy metals in the soil.

Total uptake of Pb by plants increased with the increase in Pb level in soil. The highest value (1.07 $mg \cdot pot^{-1}$) observed in the hemp inoculated with *G.etunicatum* planted in soil treated with 1800 $mg \cdot kg^{-1}$ Pb and the lowest value (0.15 $mg \cdot pot^{-1}$) was observed in the non-inoculated hemp in the control soil. On average, Pb uptake by hemp in the AM fungi treatments followed this order: *G.etunicatum* > *G.constrictum* = *G.mosseae* > non-inoculated.

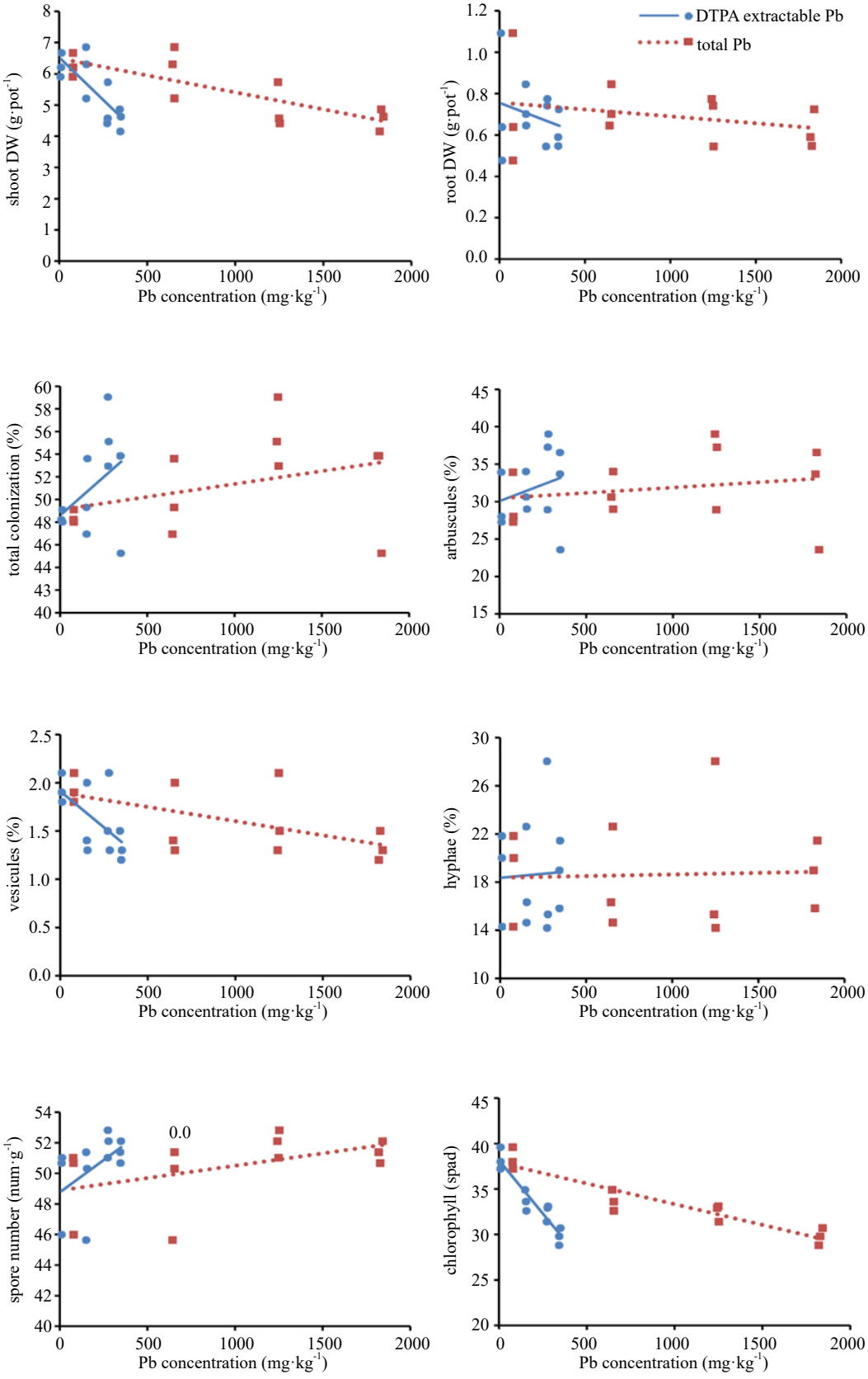
[36] reported that the inoculation of Vetiver grass with *G.mosseae* increased the uptake of Pb and also increased its translocation from root to shoot.

Total Pb uptake into the shoots of hemp had very minute values, so very little amount of soil Pb could be removed by harvesting the shoots at each year. Previous researchers have certified that although at least five years is required for soil remediation, longer time is usually necessary for soil clean-up [60-61].

Uptake of heavy metals by plants does not relate to the total content of heavy metals in soil as a linear equation. It is mainly depends on bioavailability of heavy metals in soils. The active fraction comprises a small amount of heavy metals in calcareous and clay soils. The precipitation and immobilization are two important reactions in soils that can cause the active fractions of heavy metals to decrease, so they can limit the ability of plants to absorb and accumulate these fractions [32].

The result of this study shows that hemp is a tolerant plant that has grown in soils with high concentrations of Pb (1200 and 1800 $mg \cdot kg^{-1}$ of soil) and somewhat translocates Pb from roots to shoot. [39] reported hemp as non-hyperaccumulator but tolerant to Cd, Ni and Cr.

Figure 3 shows simple linear regressions between soil Pb extracts (DTPA and total extracts) and different phytoremediation indices.


DTPA extractable Pb
total Pb
shoot DW (g·pot-1)
root DW (g·pot-1)
total colonization (%)
arbuscules (%)
vesicules (%)
hyphae (%)
spore number (num·g-1)
chlorophyll (spad)
Pb concentration (mg·kg-1)
0.0

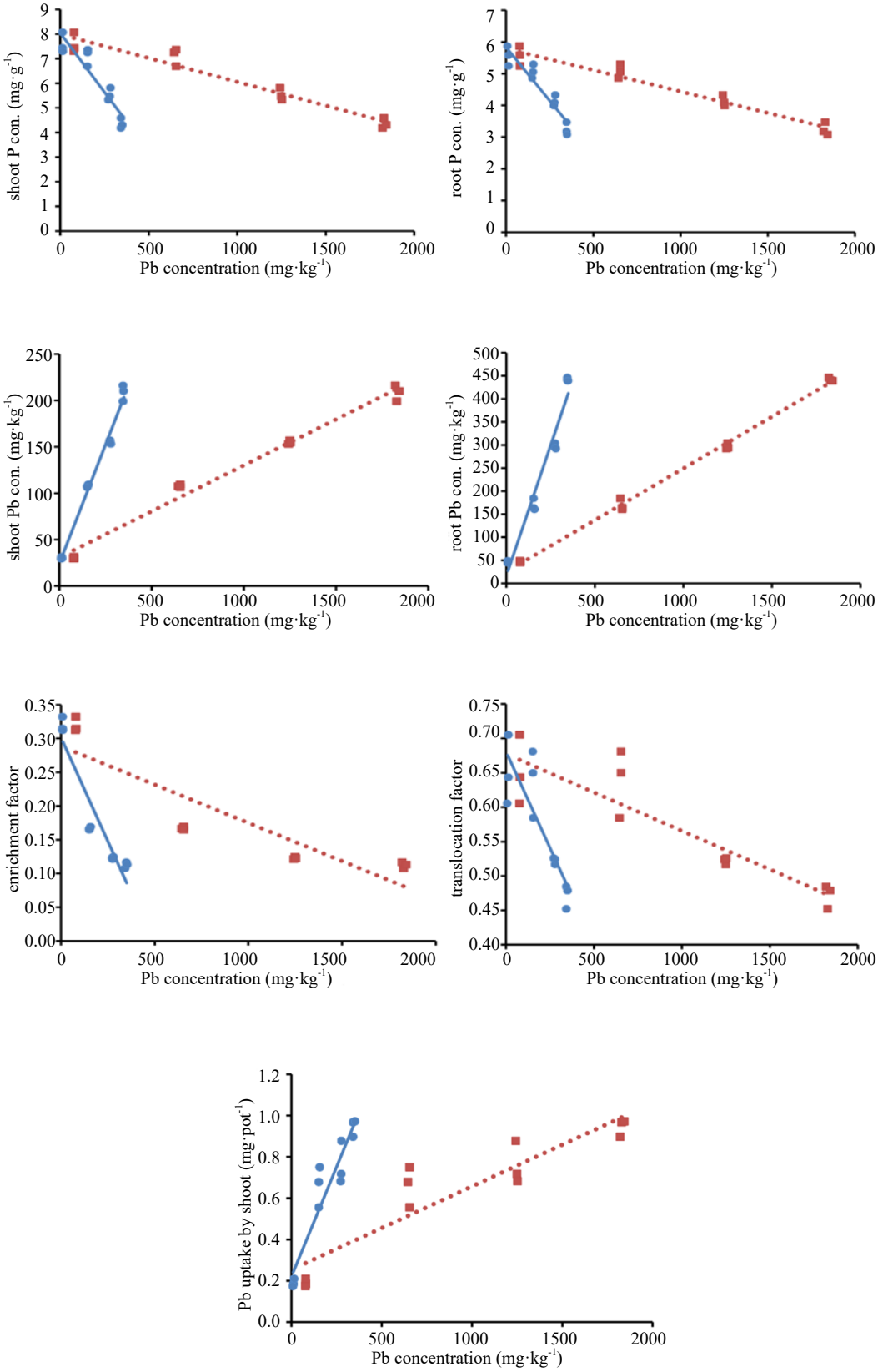


Figure 3. Simple linear regression between total and DTPA extractable Pb in soil and various phytoremediation indices by hemp

As observed in this figure all phytoremediation indices have a steeper linear relation with DTPA extractable Pb rather than total Pb. It indicates that the DTPA extractable Pb is more proper to evaluate Pb toxicity than the total forms in soil. Here with the increase of Pb levels, the negative effects of DTPA extractable Pb on phytoremediation indices are greater than those of total Pb. In Figure 1, it can be seen that DTPA extractable Pb concentration increased with increase in Pb pollution but not at the same rate as the total Pb. Hence, the bioavailable forms of heavy metals are more effective and more important than its total concentration. Due to precipitation of most of the total Pb that was added to the soil, especially in calcareous soils, usually its available form is toxic to living organisms. So DTPA extractable Pb compared with total extractable Pb in soil, has markedly higher direct and indirect effects on the phytoremediation indices. Earlier studies [62] reported the negative effect of different soil Pb fractions on diminishing soil biological properties. Soluble and labile fractions of Pb such as DTPA fraction had more effects on soil biological properties compared with residual and total fractions of Pb in soil.

Shoot and root dry weights, chlorophyll content, shoot and root phosphorus contents and enrichment and translocation factors, all showed negative regressions with soil Pb extracts. However, spore number, total shoot uptake of Pb and Pb concentration of root and shoot had positive regressions with soil Pb extracts. The percentage of vesicules in roots of hemp had negative regression with Pb levels in soil. It may due to harsh condition for plant to prepare sufficient organic carbon for mycobiont to produce and store fatty globules in vesicules. In contrast, arbuscules and hyphae percentage in roots of hemp had positive correlation coefficients with Pb levels in soil. Total root colonization similarly showed positive relation with soil Pb extracts. The higher root colonization and spore formation by AM fungi in hard condition was reported in many studies. Here the addition of Pb levels in soil increased the tendency of hemp for symbiosis with AM fungi and fungi spore formation.

4. Conclusion

The study of simple linear regression shows that DTPA extractable Pb had higher correlation coefficients with soil biological properties and plant indices for Pb phytoextraction. So it is a more proper index to evaluate heavy metals toxicity in soil compared to total concentration of heavy metals.

Pb concentration in shoot and root of hemps increased by AM fungi inoculation. Inoculation the hemp with *Glomus* species caused TF to decrease compared with non-inoculated ones, while resulting to higher dry biomass; so that total Pb uptake increased in inoculated hemps. Although inoculation of the hemp with *G.constrictum* caused higher Pb concentration in shoots, but with regard to the total Pb uptake, *G.etunicatum* is more proper for inoculation of hemp in phytoextraction of Pb from calcareous soils similar to the studied soil.

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