Caspian J. Environ. Sci. 2017, Vol. 15 No. 3 pp. 249~262

CJES
Caspian Journal of Environmental Sciences

[Research]

Fungi and bacteria as helping agents for remediation of a Pb - contaminated soil by *Onopordum acanthium*

Karimi A., Khodaverdiloo H.*, Rasouli Sadaghiani M.H.

Department of Soil Science, Urmia University, Urmia, Iran

Corresponding author's E-mail: h.khodaverdiloo@urmia.ac.ir

(Received: Feb. 08. 2017 Accepted: July 03. 2017)

ABSTRACT

Phytoremediation is a promising method for remediation of heavy metals (HMs) contaminated environments. However, the main failures are the limited bioavailabilty of HMs such as lead (Pb) in the soil and/or suppressed plant growth in contaminated sites. These limitations specifically occur in semi-arid zone environments such as calcareous soils. Arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) are known to enhance plant growth and survival in heavy metal contaminated soils. The main objective of this study was to evaluate enhancing soil Pb phytoremediation by *Onopordum acanthium* through inoculation with some AMF and PGPR. A calcareous soil was selected and spiked uniformly with different concentrations of Pb (0, 250, 500 and 1000 mg $Pb.kg^{-1}$ soil). The contaminated soils were then sterilized and subsequently inoculated with AMF and PGPR in which *O. acanthium* seeds were grown. Results indicated that inoculation of AMF and PGPR increased bioavailable Pb, shoot and root dry matter yield and Pb uptake by *O. acanthium.* Microbial inoculation increased the amount of Pb extracted by *O. acanthium* up to 2-11 times higher than the control. The amount of Pb stabilized by roots of *O. acanthium* was 1.75-2.71 and 1.25-1.53 times higher than control for AMF and PGPR treatments, respectively. Therefore, it could be concluded that inoculation with AMF and PGPR can be used as a promising strategy for enhancing the phytoremediation of Pb-contaminated soils by *O. acanthium.*

Key words: Heavy metals, Phytoextraction, Phytostabilization, Soil microorganisms, Wild plants.

INTRODUCTION

Worldwide contamination of soils and water with heavy metals (HMs) pose a serious threat to both the ecosystem and human health (Lim & Schoenung 2010). Lead (Pb) is a highly toxic HM that causes a variety of environmental problems, including loss of vegetation, toxicity in plants and animals and a number of severe health effects in humans (e.g. Punamiya *et al.* 2010). Since HMs are non-biodegradable, remediation of HM-contaminated environments is particularly challenging (Lasat *et al.* 2002). Conventional physicochemical techniques are also ineffective and non-practical for remediation of such environments (e.g., Pulford & Watson, 2003). Alternatively, phytoremediation is a promising technology to overcome this problem, because it is relatively inexpensive and environment-friendly compared to traditional engineering practices (Khan 2005; Glick 2010). Phytoextraction, removal and concentration of HM into harvestable plant parts, and phytostabilization, decreased mobility and immobilization of contaminates in soil by plant roots, have been the major phytoremediation strategies used to clean up Pb contaminated soils (Khan 2005).

However, the HM accumulation efficiency of plants is limited because of the low biomass production in hyperaccumulator plants and also low bioavailability of HMs in soils (Ma *et al.* 2011). Although total Pb concentrations are

high in many of the contaminated sites, the soluble fraction of Pb is often very low (typically less than 1%) in the soil environment due to complexation with various organic and inorganic soil colloids, sorption on oxides and clays, and precipitation as carbonates, hydroxides and phosphates (e.g., Ma *et al.* 2011; Zhang *et al.* 2011). Since plants have the ability to extract soluble or free forms of Pb rather than the bound ones, mobilized Pb fraction is a major limiting factor for Pb phytoremediation. Recently, an alternative strategy has been developed that provides plants with certain beneficial soil microorganisms such as arbuscular mycorrhiza fungi **(**AMF) and plant growth promoting rhizobacteria **(**PGPR) (Zhuang *et al.* 2007; Punamiya *et al.* 2010). The inoculation of autochthonous AMF and PGPR have shown to be an effective strategy in bioremediation of sterilized soils artificially spiked by HM (Vivas *et al.* 2003). AMF are one of the major components of the rhizosphere that form symbiotic associations with most plant species (Ma *et al.* 2011). AMF are the most wide spread mycorrhizae found in both natural and agricultural ecosystems, including HM-contaminated sites (Smith & Read, 1997). AMF may improve nutrition (Clark & Zeto 2000) through the formation of extensive extraradical hyphal networks that absorb and translocate nutrients to the roots, and modification of the root system that generally results in more extensive and increased branching, leading to increased efficiency in nutrient absorption (Berta *et al.* 2002). In addition, AMF have also shown the ability to attenuate biotic and abiotic stresses, including stress produced by HMs (Vivas *et al*. 2003; Gohre & Paszkowski 2006; Zarei *et al.* 2010; Karimi *et al.* 2011; Miransari 2011; Orłowska *et al.* 2011). PGPR also can directly improve the phytoremediation process by changing the metal bioavailability through altering soil pH, release of chelators (e.g. organic acids, siderophores), oxidation/reduction reactions (e.g. Wenzel 2009; Dary *et al.* 2010; Rajkumar *et al.* 2010; Ma *et al.* 2011). For example, Sheng *et al.* (2008) investigated the effect of *Pseudomonas fluorescens* inoculation on Pb phytoremediation and found that the siderophores, IAA and ACC deaminaz producing *Pseudomonas fluorescens* was able to solubilize large amounts of Pb in soils solution. They also reported that inoculation of *Zea mays* with *P. fluorescens* increased root elongation assay, shoot and root dry weight, and Pb extracted by the shoots.

The effect of microbial inoculation on HM phytoremediation depends upon various factors such as plant species, microbial species/ecotypes, and physicochemical properties of soils (Rajkumar *et al.* 2012). While researches have been conducted to study these effects, investigations on various AMF/PGPR species are rare for calcareous soils and wild plants and it is a promising research field that needs to be studied further.

The objective of this study was to evaluate the effect of inoculation with selected AMF (a mixture of *Glomus* species including *G. intraradices*, *G. mosseae* and *G. fasciculatum*) and PGPR (a mixture of *Pseudomonas* species includeing *P. putida*, *P. fluorescens,* and *P. aeruginosa*) on phytoremediation of a calcareous and Pb contaminated soil by high biomass *Onopordum acanthium* (Cotton thistle) in pot culture. *O. acanthium* is a fast-growing, low-demand, vigorous biennial plant with coarse, spiny leaves and conspicuous spiny-winged stems. Also it is widely distributed in disturbed sites and roadsides. These properties make it proper for phytoremediation purposes.

MATERIALS AND METHODS

Soils physicochemical analyses

The experimental soil was collected from the Western Azerbaijan province, Iran. The soil was classified as Typic Endoaquepts according to USDA Soil Taxonomy (Soil Survey Staff, 2014). Subsamples of the soil were air-dried and ground to pass through a 2mm sieve prior to physico-chemical analysis. Particle size was determined using the hydrometer method (Gee & Bouder 1986). The soil pH was determined using 1:5 soil to 0.01 M $CaCl_2$ suspension with a glass electrode. The total carbonate expressed as calcium carbonate equivalent (CCE) was

determined by a rapid titration method (Rayment & Higginson 1992).

Organic matter was determined by dichromate oxidation (Walkley & Black 1934).

Soil electrical conductivity (EC) and soluble ions were determined in a saturated paste extract (Miller & Curtin 2006).

The cation exchange capacity (CEC) was measured using sodium acetate (1 M NaOAc) at pH 8.2 (Chapman 1965). The total amounts of some native metals (Zn, Cu, Fe, Pb, and Cd) in soil was determined by adding 10 ml HNO_3 (1:1) to 2.0 g dry soil and heating for 15 minutes at 95°C on a heating block followed by adding 2 ml deionized water and 3 ml 30% H_2O_2 (Gupta 2000). All measurements were done in three replicates.

Soil spiking with Pb and aging process

Samples of the soil were screened to pass through a 5 mm sieve before spiking with Pb. Then, the soils were thoroughly mixed in plastic pots with $Pb(NO_3)_2$ in powdery form. For each soil, Pb salt was ground and mixed well with a small portion of soil, and this metal/soil mixture was then mixed with a large amount of soil in order to obtain total Pb concentrations of 250, 500, and 1000 $mg.kg^{-1}$ soil dry matter. The spiked soils were subsequently packed into plastic pots in three replicates for each treatment. To attain the quasi-equilibrium distribution of Pb in the spiked soil, the packed soils were incubated in a moisture regime entailing periodic wetting-drying (WD) cycles (without leaching) for about seven months in room temperature (Khodaverdiloo *et al.* 2012) and remained for further 12 months as air-dried.

Microbial inoculation and greenhouse experiment

The Pb-contaminated soils were sterilized in an autoclave (121˚C, 2 cycle × 25 min) and used to fill the pots and inoculated with AMF and PGPR. Every pot inoculated either with AMF or PGPR. For the AMF treatment, each pot was inoculated with 20 g of mixed *Glomus species* (including *G. intraradices*, *G. mosseae* and *G. fasciculatum*) inoculum containing 90–100 fungal spores. The AMF were provided by the Department of Soil Microbiology of Iranian Soil and Water Research Institute (SWRI). For the PGPR treatment, each pot was inoculated with 20 ml of consortium of three pre-selected metal-tolerant strains of *Pseudomonas* species (including *P. putida*, *P. fluorescens,* and *P. aeruginosa*) inoculum.

The selected strains were able to tolerate and growth well in the media containing elevated concentrations of the metal.

The bacterial strains were provided by the Department of Soil Microbiology of Iranian Soil and Water Research Institute (SWRI). *Pseudomonas* species were grown in Nutrient Broth for 48 h at 28˚C.

Population of this bacteria was 1.3×10^8 CFU. ml^{-1} (CFU = Colony Forming Unit). The control treatment received the same (but sterile, i.e. nonmycorrhizal and nonbacterial) amounts of inoculum to provide a similar condition.

In a greenhouse, seeds of *Onopordum acanthium* were grown in pots containing 3 kg of the soils. After four weeks, the emerged seedlings were thinned to keep the seven strongest seedlings per pot. Fertilizers ($MgSO_4.7H_2O$, $K_2HPO_4.3H_2O$, FeEDTA, $MnSO_4.4H_2O$, H_3BO_3, $(NH_4)_6MO_{24}.4H_2O$, $ZnSO_4.7H_2O$, and $CuSO_4.5H_2O$) were added with the irrigation water to prevent nutrient deficiency. Furthermore, during fertilizer application, the increasing inputs of nitrogen resulting from different concentrations of applied $Pb(NO_3)_2$ was taken into account and adjusted by adding appropriate amounts of urea.

The plants were harvested by cutting shoots at the soil surface and separating roots from soil, four months after their germination. Ammonium nitrate-extractable soil Pb concentrations were quantified on air-dried samples collected at the end of the experiment. This extractable soil Pb is considered to be the main source of phytoavailable soil metal (Langer *et al.* 2009).

Plant shoots and roots were carefully washed with distilled water in order to remove any surface soil or dust deposits and then oven-

dried at 75 ˚C for 72 h, then dry weights were recorded. For Pb analysis of plants, 2.0 g aliquots of ground shoots or roots were digested in 30 ml HNO_3, $HClO_4$, and H_2SO_4 mixture (40:4:1) followed by adding 20 ml deionized water (Gupta 2000). The Pb concentrations in soil and plant extracts were measured by atomic absorption spectroscopy (AAS, Shimadzu 6300).

Assessment of root AMF colonization percentage

To determine the root colonization by AMF, after harvest of plant, approximately 2 g of fine roots (diameter ≤ 1 mm) from each plant was washed with tap water, and then fully rinsed with distilled water.

Then they were cut into segments around 1 cm long, cleared by soaking in 10 % KOH, stained and analyzed for their AMF colonization according to the method of Grace & Stribley (1991).

Stained root samples were examined microscopically to assess the percentage of mycorrhizal colonization using the grid-line intersect method (Giovannetti & Mosse 1980).

Estimating Pb extraction, Pb stabilization and Pb translocation

For estimating the potential of plant and effect of the microbial inoculations, the extraction and stabilization of Pb by plant were calculated, respectively, as:

Pb Extraction ($mg.pot^{-1}$) = shoot yield ($kg.pot^{-1}$) × Pb concentration in shoot dry matter ($mg.kg^{-1}$) **(1)**

Pb stabilization ($mg.pot^{-1}$) = root yield ($kg.pot^{-1}$) × Pb concentration in root dry matter ($mg.kg^{-1}$) **(2)**

For estimating the potential of plant and effect of the microbial inoculations on root-to-shoot translocation of Pb, translocation factor (TF) was calculated by dividing the Pb concentration in shoot dry matter (C_p^{shoot}) to Pb concentration in root dry matter (C_p^{root}) (Gupta *et al.* 2008; Khoramivafa *et al.* 2012).

$$TF = \frac{C_p^{shoot}}{C_p^{root}} \quad \textbf{(3)}$$

Estimating relative increasing of Pb phytoextraction and phytostabilization affected by microbial inoculation

The relative increase of Pb phytoextraction (RI_{ext}) and Pb phytostabilization (RI_{stab}) affected by microbial inoculation (AMF and/or PGPR) were calculated, respectively, as:

$$RI_{ext} = \frac{Pb_{ext}^{t} - Pb_{ext}^{c}}{Pb_{ext}^{c}} \quad \textbf{(4)}$$

where Pb_{ext}^{t} and Pb_{ext}^{c} are Pb extraction amounts ($mg.pot^{-1}$) in microbial inoculation (AMF and/or PGPR) treatments and control, respectively.

$$RI_{stab} = \frac{Pb_{stab}^{t} - Pb_{stab}^{c}}{Pb_{stab}^{c}} \quad \textbf{(5)}$$

where Pb_{stab}^{t} and Pb_{stab}^{c} are Pb stabilization values ($mg.pot^{-1}$) in microbial inoculation (AMF and/or PGPR) treatments and control, respectively.

Statistical analyses

This experiment was conducted as a factorial experiment based on randomized complete block design with two factors (Pb concentrations at four levels and microbial inoculation at three levels) with three replications. Statistical analysis was performed using SAS (9.1).

Comparison of means was done by the Duncan's test at significant level of 5% using SAS (9.1) software.

RESULTS

General properties of soil

Table 1 presents some physico-chemical properties of soil.

The soil used for this study is classified as calcareous (pH = 8.1), nonsodic and non-contaminated with HMs (Cariny 1995).

Root AMF colonization and soil bioavailable Pb

No root colonization with AMF was observed in the PGPR and control treatments. As shown in Fig. 1, By increasing Pb concentration in the soil, root colonization rate (%) in AMF treatment was decreased significantly ($P \leq 0.05$). Table 2 shows the soil bioavailable Pb in control, PGPR and AMF treatments at different levels of soil Pb contamination. Soil bioavailable Pb increased significantly ($P \leq 0.05$) in all treatments by increasing in soil Pb concentration (Table 2). Inoculation of AMF and PGPR increased significantly ($P \leq 0.05$) the amount of soil bioavailable Pb, compared to the control. There were no significant ($P \leq 0.05$) differences in soil bioavailable Pb between AMF and PGPR treatments.

Table 1. Some physico-chemical properties of the soil used for this study.

Properties	Unit	Value
textural class		loam
Sand	g kg^{-1}	323
Silt		403
Clay		274
OM*		26.9
CEC*	$cmol_c$ kg^{-1}	22.1
EC*	dS m^{-1}	2.5
ESP*	%	3.0
CCE*		30.5
pH		8.1
Total Pb	mg kg^{-1}	21.42 ± 2.81
Total Cd		1.47 ± 0.08
Total Fe		29505 ± 394
Total Zn		62.0 ± 3.60
Total Cu		14.11 ± 1.61
Soluble Ca^{2+}	mg L^{-1}	1.2
Soluble Mg^{2+}		0.4
Soluble Na^{+}		23.8
Soluble K^{+}		0.0
Soluble CO_3^{2-}		0.8
Soluble HCO_3^{-}		5.6
Soluble Cl^{-}		15.2
Soluble SO_4^{2-}		3.8

*: OM: organic matter, CEC: cation exchange capacity, EC: electrical conductivity, ESP: exchangeable sodium percentage, CCE: calcium carbonate equivalent.

Table 2. Concentration of soil bioavailable Pb in control, AMF or PGPR treatments at different levels of soil Pb contamination.

Total Pb added to soil (mg.kg^{-1})*	Soil bioavailable Pb		
	Control	PGPR	AMF
0	1.09 ± 0.14^{d,b}	2.57 ± 0.16^{d,a}	2.52 ± 0.25^{d,a}
250	4.01 ± 0.25^{c,b}	6.1 ± 0.20^{c,a}	6.32 ± 0.32^{c,a}
500	6.19 ± 0.28^{b,b}	9.28 ± 0.14^{b,a}	9.36 ± 0.53^{b,a}
1000	9.02 ± 0.23^{a,b}	12.22 ± 0.14^{a,a}	12.33 ± 0.10^{a,a}

*: native Pb was 21.42 mg.kg^{-1}. Within each data, mean ±standard error followed by the first and second same letter is not significantly different among level of Pb in soil and control, AMF, PGPR treatment respectively, by Duncan's -test at the 5% level, n = 3.

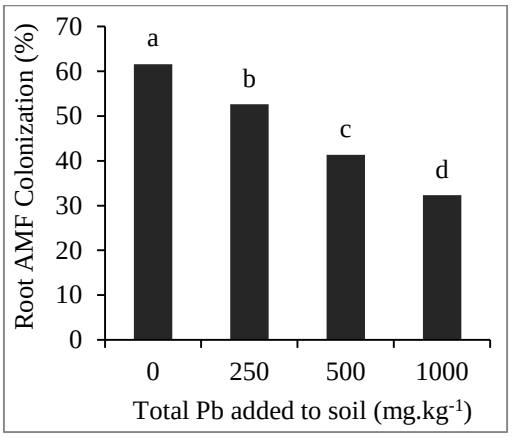

Fig. 1. Root colonization rate (%) in inoculation of AMF treatment at different levels of soil Pb contamination. Different letters above columns indicate significant differences level of Pb concentration in soil by Duncan's -test at the 5% level, n=3.

Plant growth responses

Data in Table 3, show that, adding Pb concentration resulted in substantial decrease in shoot and root dry matter yield in plant in all treatments, particularly in non-inoculated, compared to the inoculated plants. The highest concentrations of Pb resulted in the lowest shoot and root dry matter. Inoculation of AMF and PGPR increased shoot and root dry matter yield compared to the corresponding controls. Both shoot and root dry matter yield of AMF plants were significantly ($P \leq 0.05$) higher than those of the non-inoculated (control) and PGPR inoculated ones.

Pb accumulation in root and shoot

Table 4 show that the inoculation with AMF and PGPR enhanced the concentration of Pb in *O. acanthium*. Pb concentration in root and shoot increased significantly ($P \leq 0.05$) in all treatments by increasing Pb concentration in soil (Table 4). Although non-significant differences were observed among treatments in Pb 0 concentration in soil, but in other concentration of Pb (250, 500 and 1000 mg.kg^{-1}) inoculation with AMF and PGPR increased significantly ($P \leq 0.05$) the Pb concentration in root and shoot respectively, compared to control. The shoot Pb extraction and root Pb stabilization in each treatment was calculated and are shown in Table 4. The highest soil Pb concentration caused higher accumulation of Pb in both root and shoot (Table 4). Inoculation of AMF and PGPR increased significantly ($P \leq 0.05$) the amounts of shoot Pb extraction and root Pb stabilization, compared to the control in all Pb concentration levels of soil (Table 4). There were no significant ($P \leq 0.05$) differences in shoot Pb extraction between AMF and PGPR treatments, while the root Pb stabilization in AMF inoculated plants was higher significantly ($P \leq 0.05$) than that in PGPR inoculated plants. Translocation factor (TF) values can describe

movement and distribution of heavy metals in plants. As shown in Table 4, Pb translocation from root to shoot (TF) decreased by increasing Pb concentration in soil at all treatments. The highest (2.21 at 0 mg.kg^{-1} Pb) and lowest (1.03 at 1000 mg.kg^{-1} Pb) amounts of TF were observed in plant inoculated with PGPR and AMF respectively (Table 4).

Relative increasing of Pb extraction and Pb stabilization affected by AMF and PGPR inoculation

The effects of AMF and PGPR inoculations on relative increase in Pb extraction and Pb stabilization are presented in Fig. 2. Relative increase in Pb extraction using PGPR inoculation decreased by increasing in soil Pb concentration up to 250 mg.kg^{-1} , while at higher concentrations, no significant ($P \leq 0.05$) changes were observed by elevating the soil Pb contamination (Fig. 2a).

The relative increase in Pb extraction using AMF inoculation showed no significant changes ($P \leq 0.05$) by elevating in the soil Pb concentration. It was also found that relative increase in Pb extraction using PGPR inoculation was higher than that of AMF (Fig. 2a). Furthermore, relative increase in Pb stabilization using AMF increased by elevating the soil Pb contamination up to 500 mg.kg^{-1} , while decreased at 1000 mg.kg^{-1} (Fig. 2b). Although, relative increase in Pb stabilization using PGPR showed no significant changes ($P \leq 0.05$) by increasing in the soil Pb concentration. Relative increase in Pb stabilization using AMF inoculation was significantly ($P \leq 0.05$) higher than that of the PGPR.

Table 3. Shoot and root dry matter yield in control, AMF or PGPR treatments at different levels of soil Pb contamination.

	Shoot dry matter yield (g.pot^{-1})		
Total Pb added to soil (mg.kg^{-1})*	**Control**	**PGPR**	**AMF**
0	3.60 ± 0.13^{a,b}	4.04 ± 0.09^{a,b}	4.68 ± 0.42^{a,a}
250	3.19 ± 0.19^{b,b}	3.44 ± 0.14^{b,b}	4.25 ± 0.28^{a,a}
500	2.11 ± 0.25^{c,b}	2.45 ± 0.06^{c,b}	3.24 ± 0.09^{b,a}
1000	1.70 ± 0.06^{d,b}	1.95 ± 0.07^{d,b}	2.67 ± 0.29^{c,a}
	Root dry matter yield (g.pot^{-1})		
0	1.13 ± 0.07^{a,b}	1.37 ± 0.11^{a,b}	1.78 ± 0.18^{a,a}
250	0.88 ± 0.14^{ab,b}	1.21 ± 0.11^{ab,b}	1.60 ± 0.14^{a,a}
500	0.59 ± 0.25^{b,b}	1.04 ± 0.12^{ab,b}	1.37 ± 0.09^{b,a}
1000	0.47 ± 0.06^{c,b}	0.93 ± 0.22^{a,ab}	1.18 ± 0.05^{b,a}

*: native Pb was 21.42 mg.kg^{-1} within each data, mean ± SE followed by the first and second same letter is not significantly different among level of Pb in soil and control, AMF, PGPR treatment respectively, by Duncan's -test at the 5% level, n = 3.

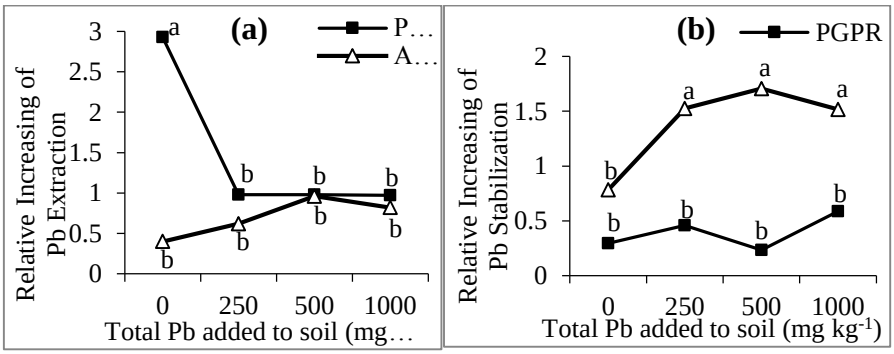

Fig. 2. Relative increase in Pb extraction (a) and Pb stabilization (b) affected by AMF and PGPR inoculation. Different letters above columns indicate significant differences between PGPR and AMF treatment in plant by the Duncan's -test at the 5% level, n = 3.

Table 4. Shoot and root Pb concentration, Shoot Pb extraction, Root Pb stabilization and translocation factor in control, AMF or PGPR treatments at different levels of soil Pb contamination.

Total Pb added to soil (mg.kg^{-1})*	Shoot Pb concentration (mg.kg^{-1})		
	Control	PGPR	AMF
0	2.41 ± 0.28^{d,a}	2.76 ± 0.09^{d,a}	2.59 ± 0.23^{a,a}
250	17.26 ± 1.08^{c,b}	31.66 ± 0.14^{c,a}	21.13 ± 2.89^{a,b}
500	26.91 ± 0.88^{b,b}	45.45 ± 1.86^{b,a}	34.06 ± 2.64^{b,b}
1000	35.64 ± 1.25^{a,b}	61.08 ± 4.08^{a,a}	41.43 ± 1.43^{c,b}
	Root Pb concentration (mg.kg^{-1})		
0	3.59 ± 0.13^{d,a}	3.68 ± 0.21^{c,a}	3.91 ± 0.23^{d,a}
250	27.62 ± 0.23^{c,b}	30.90 ± 1.25^{b,b}	41.12 ± 2.99^{c,a}
500	39.71 ± 0.13^{b,b}	42.40 ± 7.87^{b,b}	68.79 ± 0.95^{b,a}
1000	64.65 ± 0.17^{a,b}	71.39 ± 3.27^{a,b}	92.81 ± 4.54^{a,a}
	Shoot Pb extraction (mg.pot^{-1})		
0	0.008 ± 0.000^{b,b}	0.011 ± 0.000^{b,a}	0.012 ± 0.000^{c,a}
250	0.054 ± 0.001^{a,b}	0.109 ± 0.008^{a,a}	0.089 ± 0.007^{b,a}
500	0.057 ± 0.005^{a,b}	0.111 ± 0.003^{a,a}	0.111 ± 0.011^{a,a}
1000	0.061 ± 0.002^{a,b}	0.119 ± 0.012^{a,a}	0.111 ± 0.009^{a,a}
	Root Pb stabilization (mg.pot^{-1})		
0	0.004 ± 0.001^{d,b}	0.005 ± 0.000^{c,b}	0.007 ± 0.001^{c,a}
250	0.026 ± 0.005^{c,b}	0.037 ± 0.002^{b,b}	0.066 ± 0.010^{b,a}
500	0.035 ± 0.003^{b,b}	0.043 ± 0.009^{ab,b}	0.094 ± 0.007^{a,a}
1000	0.043 ± 0.007^{a,b}	0.066 ± 0.007^{a,b}	0.109 ± 0.018^{a,a}
	Translocation factor (TF)		
0	2.20 ± 0.62^{a,a}	2.21 ± 0.19^{a,a}	1.74 ± 0.09^{a,a}
250	2.13 ± 0.58^{a,b}	2.92 ± 0.23^{a,a}	1.36 ± 0.10^{b,c}
500	1.63 ± 0.28^{a,a}	2.53 ± 0.54^{a,a}	1.17 ± 0.17^{bc,a}
1000	1.41 ± 0.25^{a,a}	1.78 ± 0.42^{a,a}	1.03 ± 0.24^{c,a}

*: native Pb was 21.42 mg.kg^{-1} within each data, mean ±□SE followed by the first and second same letter is not significantly different among level of Pb in soil and control, AMF, PGPR treatment respectively, by Duncan's -test at the 5% level, n = 3.

DISCUSSION

The data from our study showed that increasing in soil Pb contamination caused a reduction in root colonization which could be attributed to the toxicity of Pb for AMF. High concentrations of HMs might inhibite sporulation by AMF (Arriagada *et al.* 2005). This reduction in colonization of AMF in the roots may act as a strategy to protect the plant against the deleterious effect of HMs by decreasing in the accumulation of these metals in the shoots (Malcova *et al.* 2003). AMF symbiosis is known to change physiological and biochemical properties of the host and these changes may alter the composition of root exudates which play a role in the modification of the microbial population in the mycorrhizosphere (Arriagada *et al.* 2005). It is likely that Pb treatment effects on the composition of root exudates by AMF.

Inhibition of mycorrhizal colonization by Pb stress has been reported by several authors (e.g., Hovsepyan & Greipsson 2004; Arriagada *et al.* 2005). Al-Ghamdi & Jais (2012) showed that high concentrations of Pb decreased root colonization rate (%). Lower AMF colonization in the roots of maize (*Zea mays* L.) has been reported by Chao and Wang (1990) and Yang *et al*. (2008) when Pb was added to soil.

Results of this study showed that inoculation of AMF and PGPR increase the bioavailability of Pb in soil. AMF might increase bioavailability of Pb through their effects on increasing root elongation (Ma *et al.* 2009). The metabolites released by inoculated PGPR (e.g., siderophores, organic acids, plant growth regulators, ACC deaminase, etc.) can alter the uptake of HMs indirectly through their effects on plant growth dynamics, and directly, through acidification, chelation, precipitation, immobilization, and oxidation–reduction reactions in the rhizosphere (Ma *et al.* 2011). Similar results were also reported by Abou-shanab *et al.* (2008) and Braud *et al.* (2009).

The toxic effects of Pb have widely been studied in different plant species and Pb is known to reduce or inhibit plant growth by decreasing uptake of water and nutrient element, inhibition of photosynthesis and Cellular respiration (Sharma & Dubey 2005). In the present study, by increasing in the soil Pb, the shoot and root dry matters were decreased, in agreement with the previous reports by Nowak *et al.* (2007) in scarlet sage. We also observed the positive effects of AMF on the growth of *O. acanthium.* Similar results have been reported for other mycorrhizal plant species under metal stress conditions (e.g., Arriagada *et al.* 2005; Andrade *et al.* 2009). The positive effect likely attributed to the improvement of P nutrition, the uptake of water by hyphae and the increase of root length density (Rajkumar *et al.* 2012). The beneficial effects of AMF on the acquisition of nutrients, such as N, Ca, Mg, Mn, Cu and Zn, have also been reported by researchers in various plants under metal stress conditions (e.g., Orłowska *et al.* 2011; Rajkumar *et al*. 2012). In the present study, we used three AMF species. The competition or functional complementarity among AMF species might have impacts on growth and Pb accumulation in host plants. Jansa *et al.* (2008) reported that in *Medicago truncatula* and *Allium porrum*, multispecies mixtures of AMF provided more P and supported greater plant growth than single. This supports our results of the predominant AMF in *O. acanthium* under Pb contamination.

Results of the present study indicated that AMF inoculation alleviate Pb toxicity in *O. acanthium.* (Tables 3-4). In this connection Rabie (2005) suggested that the symbioses with AMF provided the host with nutrients such as phosphorus which may be involved in plant Pb detoxification by means of molecules of phytates that can neutralize excess metals, or P can provide metabolic energy indirectly as ATP for possible compartment- alization within the cell vacuoles by means of molecules such as metallothioneins and phytochelatins (Assuncao *et al.* 2003). Metallothionein and phytochelatins may afford protection against HM-induced stress in plants (Cicatelli *et al.* 2010).

These results indicated the positive impact of AMF and PGPR in enhancing Pb uptake in *O. acanthium* plant (Table 4 and Fig. 2). It also indicates that Pb concentration in shoot of *O. acanthium* can be modulated by AMF and PGPR when growing in the Pb-contaminated soil. PGPR can potentially improve phytoextraction by altering the solubility, availability, and transport of HM and nutrients by reducing soil pH, release of chelators, P solubilization, or redox changes. Among the various metabolites produced by PGPR, the siderophores play a significant role in metal mobilization and accumulation (Rajkumar *et al.* 2010), as these compounds produced by PGPB solubilize unavailable forms of HM-bearing Fe but also form complexes with bivalent HM ions that can be assimilated by root mediated processes (Braud *et al*. 2009). Braud *et al.* (2009) investigated the release of Pb in soil solution after inoculation of various PGPR and found that the siderophores producing PGPR,

Pseudomonas aeruginosa was able to solubilize large amounts of Pb in soils solution.

Likewise Punamiya *et al.* (2010) investigated the role of vetiver grass [*Chrysopogon zizanioides* (L.)] in association with *G. mosseae* in the phytoremediation of soils polluted with Pb. Low solubility of Pb in the soil is one of the most important factors limiting Pb bioremediation. Colonization with *G. mosseae* significantly increased plant growth as well as Pb uptake by plant roots and its translocation to the plant shoot. In addition, the mycorrhizal plants had a higher rate of thiols with low molecular weight which enhanced tolerance of AMF plant to the Pb contamination (Punamiya *et al*. 2010). However, a pot-culture experiment conducted by Weissenhorn *et al.* (1995) revealed no difference in shoot Pb contents between mycorrhizal and non-mycorrhizal maize seedlings. The observed difference between the present study and Malcova *et al.* (2003) who found that the inoculation with *G. intraradices* isolates significantly reduced Pb concentrations in maize plants. Kaldorf *et al.* (1999) also observed lower Pb concentrations both in the shoot and roots of maize plants inoculated with *G. intraradices* compared to the non-mycorrhizal control.

While there was no significant difference between the PGPR and AMF inoculated treatments in terms of total amount of Pb extracted by the plant, total amount of Pb stabilized by the plant roots was higher in AMF treatments (Table 4). This may be due to the presence of AMF extra-radical mycelium, increasing the root surface area through which soluble minerals particularly Pb can be taken up (Rajkumar *et al.* 2012). AMF might also reduce metal exposure of plants by uptake of metals into their structures such as the chitious cell wall (Zhou 1999), the extra-cellular glycoprotein, glomalin and fungal vesicles (Gonzalez-Chavez *et al.* 2004).

CONCLUSION

This study elucidates the potential of beneficial species of AMF and PGPR in solubilizing Pb, thereby increasing the bioavailability of Pb for uptake by *O. acanthium*. Moreover, the results of this study showed that the inoculation of AMF in *O. acanthium* helped to reduce Pb-induced stress thus improving plant growth and Pb uptake. While there was no difference between the PGPR and AMF-inoculated treatments in terms of total amount of Pb extracted by the plants, total amount of Pb stabilized by the roots was higher in AMF treatments. Therefore inoculation of AMF and PGPR are promising agents to enhance soil Pb phytoextraction by *O. acanthium*. However, AMF inoculation resulted in better phytostabilzation of Pb, which is a major criterion for successful phytoremediation. The present study was a short-term greenhouse study that indicated the beneficial role of AMF and PGPR in enhancing plant growth, Pb stabilization and Pb extraction. However, it is also necessary to conduct longer-term verification of the results. The mechanisms responsible for increased Pb uptake and translocation in *O. acanthium* are still unclear. Further studies are required to test the efficacy of AMF and PGPR in enhancing HM uptake or stabilization in field conditions.

REFERENCES

Abou-Shanab, RAI, Ghanem, K, Ghanem, N & Al-Kolaibe, A 2008, The role of bacteria on heavy-metal extraction and uptake by plants growing on multi-metal-contaminated soils. *World Journal of Microbiology and Biotechnology,* 24: 253-262.

Al-Ghamdi, AAM & Hasnah, MJ 2012, Interaction between arbuscular mycorrhiza and heavy metals in the rhizosphere and roots of *Juniperus procera*. *International Journal of Agriculture and Biology,* 3: 66-76.

Andrade, SAL, Gratao, PL, Schiavinato, MA, Silveira, APD, Azevedo, RA & Mazzafera, P 2009, Zn uptake, physiological response and stress attenuation in mycorrhizal jack bean growing in soil with increasing Zn concentrations. *Chemosphere,* 75: 1363-1370.

Arriagada, CA, Herrera, MA & Ocampo, JA 2005, Contribution of arbuscular mycorrhizal and saprobe fungi to the

tolerance of *Eucalyptus globulus* to Pb. *Water Air and Soil Pollution*, 166: 31-47.

Assuncao, AGL & Schat, H 2003, *Thlaspi caerulescens*, an attractive model species to study heavy metal hyper - accumulation in plants. *New Phytologist,* 159: 351-360.

Berta, G, Fusconi, A & Hooker, JE 2002, Arbuscular mycorrhizal modifications to plant root systems: scale, mechanisms and consequences In: Gianinazzi S *et al.* (eds.) Mycorrhizal Technology in Agriculture, pp. 71–85. Birkäuser Verlag, Basel, Boston, Berlin.

Braud, A, Jezequel, K, Bazot, S & Lebeau, T 2009, Enhanced phytoextraction of an agricultural Cr and Pb-contaminated soil by bio - augmentation with siderophore-producing bacteria. *Chemosphere,* 74: 280-286.

Cariny, T 1995, *The reuse of contaminated land*. John Wiley and Sons Ltd. Publisher, p. 219.

Chao, CC & Wang, YP 1990, Effects of heavy-metals on the infection of vesicular arbuscular mycorrhizae and the growth of maize. *Journal of the Agricultural Association of China,* 152: 34–45.

Chapman, HD 1965, Cation exchange capacity. In: Methods of soil analysis. Chemical and microbiological properties. Part. 2. American Society of Agronomy. (ed. Black, CA) pp. 891-901. Madison, WI, USA.

Cicatelli, A, Lingua, G, Todechini, V, Biondi, S, Torrigiani, P & Castiglione, S 2010, Arbuscular mycorrhizal fungi restore normal growth in a white poplar clone grown on heavy metal contaminated soil, and this is associated with up regulation of foliar metallothionein and polyamine biosynthetic gene expression. *Annals of Botany*, 106: 791–802.

Clark, BR & Zeto, SK 2000, Mineral acquisition by arbuscular mycorrhizal plants. *Journal of Plant Nutrition,* 23: 867–902.

Dary, M, Chamber-Pérez, MA, Palomares, AJ & Pajuelo, E 2010, "In situ" phytostabilisation of heavy metal polluted soils using *Lupinus luteus* inoculated with metal resistant plant-growth promoting rhizobacteria. *Journal of Hazardous Materials,* 177: 323–330.

De Souza, LA, Andrade, SAL, De, Souza, SCR & Schiavinato, MA 2012, Arbuscular mycorrhiza confers Pb tolerance in *Calopogonium mucunoides*. *Acta Physiologiae Plantarum,* 34: 523–31.

Dell'Amico, E, Cavalca, L, Andreoni, V 2008, Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria. *Soil Biology and Biochemistry,* 40: 74-84.

Gee, GH & Bauder, JW 1986, Particle size analysis. In: Methods of soil Analysis. Physical Properties. SSSA, (ed. Klute, A) pp. 383-411. Madison, WI.

Giovannetti, M & Mosse, B 1980, An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist,* 84: 489-500.

Glick, BR 2010, Using soil bacteria to facilitate phytoremediation. *Biotechnology Advances,* 28: 367–74.

Gohre, V & Paszkowski, U 2006, Contribution of the arbuscular mycorrhizal symbiosis to heavy metal Phytoremediation. *Planta,* 223: 1115–1122.

Gonzalez-Chavez, MC, Carrillo-Gonzalez, R & Wright, SF & Nichols, K 2004, The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. *Environmental Pollution,* 130: 317–323.

Grace, C & Stribley, DP 1991, A safer procedure for routine staining of VAM fungi. *Mycological Research,* 95: 1160-1162.

Gupta, PK 2000, Soil, plant, water, and fertilizer analysis. Agrobios, New Delhi, India. p. 438

Gupta, S, Nayek, S, Saha, RN & Satpati, S 2008, Assessment of heavy metal accumulation in macrophyte, agricultural soil and crop plants adjacent to discharge zone of sponge iron factory. *Environmental Geology,* 55: 731-739.

Hovsepyan, A & Greipsson, S 2004, Effect of arbuscular mycorrhizal fungi on phytoextraction by corn (*Zea mays*) of lead-

contaminated soil. *International Journal of Phytoremediation,* 6: 305-321.

Jansa, J, Smith FA & Smith, SE 2008. Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? *New Phytologist*, 177: 779–789.

Kaldorf, M, Kuhn, AJ, Schröder, WH, Hildebrandt, U & Bothe, H 1999, Selective element deposits in maize colonized by a heavy metal tolerance conferring arbuscular mycorrhizal fungus. *Journal of Plant Physiology,* 154: 718-728.

Karimi, A, Khodaverdiloo, H, Sepehri, M & Rasouli Sadaghiani, MH 2011, Arbuscular mycorrhizal fungi and heavy metal contaminated soils. *African Journal of Microbiology Research,* 5: 1571-1576.

Khan, AG 2005, Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. *Journal of Trace Elements in Medicine and Biology,* 18: 355–364.

Khodaverdiloo, H, Rahmanian, M, Rezapour, S, Ghorbani Dashtaki, Sh, Hadi, H & Han, FX 2012, Effect of wetting-drying cycles on redistribution of lead in some semi-arid zone soils spiked with a lead salt. *Pedosphere,* 22: 304–313.

Khoramivafa, M, Shokri, K, Sayyadian, K & Rejali, F 2012, Contribution of microbial associations to the cadmium uptake by peppermint (*Mentha piperita*). *Annals of Biological Research,* 3: 2325-2329.

Langer, I, Krpata, D, Fitz, WJ, Wenzel, WW & Schweiger, PF 2009, Zinc accumulation potential and toxicity threshold determined for a metal-accumulating *Populus canescens* clone in a dose–response study. *Environmental Pollution,* 157: 2871–2877.

Lasat, MM 2002, Phytoextraction of toxic metals: a review of biological mechanisms. *Journal of Environmental Quality,* 31: 109–120.

Lim, SR & Schoenung, JM 2010, Human health and ecological toxicity potentials due to heavy metal content in waste electronic devices with flat panel displays. *Journal of Hazardous Materials,* 177: 251-259.

Ma, Y, Prasad, MNV, Rajkumar, MH & Freitas, H 2011, Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnology Advances,* 29: 248–258.

Ma, Y, Rajkumar M & Freitas, H 2009, Improvement of plant growth and nickel uptake by nickel resistant-plant growth promoting bacteria. *Journal of Hazardous Materials,* 166: 1154–1161.

Malcova, R, Vosatka, M & Gryndler, M 2003, Effects of inoculation with *Glomus intraradices* on lead uptake by *Zea mays* L. and *Agrostis capillaris* L. *Applied Soil Ecology,* 23: 255-267.

Miller, JJ & Curtin, D 2006, Electrical Conductivity and Soluble Ions. In: Carter MR, Gregorich, EG (eds.) Soil sampling and methods of analysis. Second ed. pp. 161-171. CRC Press. Boca Raton, FL.

Miransari, M 2011, Hyperaccumulators, arbuscular mycorrhizal fungi and stress of heavy metals. *Biotechnology Advances,* 29: 645–653.

Nowak, J 2007, Effects of cadmium and lead concentration and arbuscular mycorrhiza on growth, flowering and heavy metal accumulation in scarlet sage (*Salvia Splendens Seelo 'Torreador'*). *Acta Agrobotanica,* 60: 79–83.

Orłowska, E, Przybyłowicz, W, Orlowski, D, Turnau, K & Mesjasz-Przybyłowicz, J 2011, The effect of mycorrhiza on the growth and elemental composition of Ni-hyperaccumulating plant *Berkheya coddii* Roessler. *Environmental Pollution,* 159: 3730–3738.

Pulford, ID & Watson, C 2003, Phytoremediation of heavy metal-contaminated land by trees. *Environment International,* 29: 529–540.

Punamiya, P, Datta, R, Sarkar, D, Barber, S, Patel, M & Da, P 2010, Symbiotic role of *Glomus mosseae* in phytoextraction of lead in vetiver grass (*Chrysopogon zizanioides*

(L.)). *Journal of Hazardous Materials,* 177: 465-474.

Rabie, GH 2005, Role of arbuscular mycorrhizal fungi in phytoremediation of soil rhizosphere spiked with poly aromatic hydrocarbons. *Mycobiology,* 33: 41-50.

Rajkumar, M, Ae, N, Prasad, MNV, Freitas, H 2010, Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends in Biotechnology,* 28: 142–149.

Rajkumar, M, Sandhya, S, Prasad, MNV & Freitas, H 2012, Perspectives of plant-associated microbes in heavy metal phytoremediation. *Biotechnology Advances,* 30: 1562‑1574.

Rayment, GE & Higginson, FR 1992, Australian Laboratory Handbook of Soil and Water Chemical Methods. Inkata Press, Melbourne.

Sharma, P & Dubey, RS 2005, Lead Toxicity in plants. *Plant Physiology,* 17: 35-52.

Sheng, XF, Xia, JJ, Jiang, CY, He, LY & Qian, M 2008, Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. *Environmental Pollution,* 156: 1164-1170.

Smith, SE & Read, DJ 1997, Mycorrhizal Symbiosis, Academic Press, San Diego, USA.

Vivas, A, Azcón, R, Biró, B, Barea, JM & Ruíz-Lozano, JM 2003, Influence of bacterial strains isolated from lead-polluted soil and their interactions with arbuscular mycorrhizae on the growth of *Trifolium pratense* L. under lead toxicity. *Canadian Journal of Microbiology,* 49: 577–588.

Walkley, A & Black, IA 1934, An examination of the detjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Science,* 37: 29-38.

Weissenhorn, I, Leyval, C, Belgy, G, Berthelin, J 1995, Arbuscular mycorrhizal contribution to heavy metal uptake by maize (*Zea mays* L.) in pot culture with contaminated soil. *Mycorrhiza,* 5: 245-251.

Wenzel, WW 2009, Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. *Plant and Soil,* 321: 385–408.

Yang, R, Yu, G, Tang, J & Chen, X 2008, Effects of metal lead on growth and mycorrhizae of an invasive plant species (*Solidago canadensis* L.). *Journal of Environmental Sciences,* 20: 739–744.

Zarei, M, Wubet, T, Schäfer, SH, Savaghebi, GR, Salehi Jouzani, G, Khayam Nekouei, M & Buscot, F 2010, Molecular diversity of arbuscular mycorrhizal fungi in relation to soil chemical properties and heavy metal contamination. *Environmental Pollution,* 158: 2757-2765.

Zhang, YF, He, LY, Chen, ZJ, Zhang, WH, Wang, QY, Qian, M & Sheng, XF 2011, Characterization of lead-resistant and ACC deaminase-producing endophytic bacteria and their potential in promoting lead accumulation of rape. *Journal of Hazardous Materials,* 186: 1720–1725.

Zhou, JL 1999, Zn biosorption by *Rhizopus arrhizus* and other fungi. *Applied Microbiology and Biotechnology,* 51: 686–693.

Zhuang, X, Chen, J, Shim, H & Bai, Z 2007, New advances in plant growth promoting rhizobacteria for bioremediation. *Environment International,* 33: 406–413.

قارچ‌ها و باکتری‌ها به‌عنوان عوامل یاری دهنده برای پالایش یک خاک آلوده به سرب توسط خار پنبه (*Onopordum acanthium*)

کریمی الف.، خداوردی‌لو ح.*، رسولی صدقیانی م.

گروه علوم خاک دانشگاه ارومیه، ارومیه، ایران

(تاریخ دریافت: ۹۵/۱۱/۱۹ تاریخ پذیرش: ۹۶/۰۴/۱۲)

چکیده

پالایش سبز روشی نویدبخش برای پالایش خاک‌های آلوده به فلزات سنگین است. با وجود این، عدم موفقیت آن عمدتاً مربوط به زیست‌فراهمی محدود فلزات سنگین مانند سرب در خاک و یا محدود بودن رشد گیاه در مکان‌های آلوده است. این محدودیت به‌ویژه در محیط‌های مناطق خشک مانند خاک‌های آهکی رخ می‌دهد. قارچ‌های میکوریز آربوسکولار (AMF) و باکتری‌های ریزوسفری افزاینده رشد گیاه (PGPR) در بردباری و رشد گیاهان در خاک‌های آلوده به فلزات سنگین موثر شناخته شده‌اند. هدف از این مطالعه ارزیابی افزایش پالایش سبز سرب توسط خار پنبه با استفاده از مایه زنی برخی قارچ‌های میکوریز آربوسکولار و باکتری‌های ریزوسفری افزاینده رشد گیاه بود. یک خاک آهکی انتخاب شد و به‌طور یکنواخت با غلظت‌های مختلف سرب (صفر، ۲۵۰، ۵۰۰ و ۱۰۰۰ میلی‌گرم بر کیلوگرم خاک) آلوده شد. سپس خاک آلوده شده استریل شد و پس از آن هر یک از بذرهای خار پنبه که رشد کردند، با AMF و PGPR مایه‌زنی شد. نتایج نشان داد مایه‌زنی AMF و PGPR سرب زیست-فراهم، عملکرد ماده خشک ریشه و شاخساره و جذب سرب توسط خار پنبه را افزایش می دهد. مایه‌زنی میکروبی سرب استخراج شده توسط خار پنبه را به بیش از ۱۱-۲ برابر بیش‌تر از تیمار شاهد افزایش داد. مقادیر سرب تثبیت شده در ریشه خار پنبه در تیمارهای AMF و PGPR به‌ترتیب ۲/۷۱ - ۱/۷۵و ۱/۵۳ – ۱/۲۵ برابر بیش‌تر از تیمار شاهد بود. بنابراین می‌توان نتیجه‌گیری کرد که مایه‌زنی AMF و PGPR می‌تواند به‌عنوان روشی نویدبخش برای پالایش‌سبز خاک‌های آلوده به سرب توسط خار پنبه مورد استفاده قرار گیرد.

*مولف مسئول