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Effects of salicylic acid, humic acid, and EDTA chelate on the increasing Pb concentration in the barley inoculated with PGPR

Amir Hossein Baghaie[1,✉], **Aminollah Aghilizefreei**[2]

1. Department of Soil Science, Arak Branch, Islamic Azad University, Arak, Iran
2. Department of Chemical Engineering, Isfahan University of Technology, Isfahan, Iran

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ABSTRACT

The present study aimed to investigate the effects of salicylic acid (SA), humic acid (HA), and EDTA chelate on the increasing Pb concentration in a plant inoculated with plant growth-promoting rhizobacteria (PGPR). Treatments consisted of applying two levels of EDTA (0 ($EDTA_0$) and 3 ($EDTA_3$) mmol/kg soil) and soil application of HA (0 (HA_0) and 200 (HA_{200}) mg/kg soil). In addition, foliar application of SA at the rates of 0 (SA_0) and 1.5 ($SA_{1.5}$) mmol/lit was also sprayed on the inoculated plant with and without PGPR cultivated in the Pb-polluted soil. After 9 weeks, barley plants were harvested and plant Pb concentration was measured using atomic absorption spectroscopy (AAS). The soil Pb concentration and plant Pb biomass was also measured. The least significant difference (LSD) test was used to determine the differences between the means (P=0.05). The results indicated that application of HA or EDTA had significantly (P=0.05) increased the Pb phytoremediation efficiency, as, applying 3 mmol EDTA/kg soil increased the Pb phytoremediation efficiency by 14.1%. In addition, a significant increasing (P=0.05) in plant biomass and Pb phytoremediation efficiency was observed by 12.2 and 13.6%, respectively, in the inoculated plant cultivated in the soil that received the greatest rates of EDTA and HA together with the highest rate of SA foliar application. Plant growth regulators such as SA or humic acid can increase plant resistance to Pb toxicity and help to increase Pb phytoremediation efficiency that is important in environmental studies.

Keywords: Phytoremediation, Pb, Plant biomass, Humic acid

Introduction

Heavy metal contamination of soil is widespread in many parts of the world which can toxic even in low concentration. Heavy metals enter the soil via the various ways such as industrial, agricultural, wastewater, combustion of fossil fuels, industrial processes and atmospheric resources enter the environment.[1,2] However, most of the heavy metal pollution in the environment is the result of industrial activities.[3] Among this, Lead (Pb) is one of the major sources of environmental pollution, because it affects consumer health through the food supply. Therefore, entering the high concentration of heavy metals in to the body has negative effect on human health.[4] Generally, Pb is a non-essential and poisonous element for human body that can accumulate in the kidneys and liver. In addition, Pb could enter in the food chain from contaminated soil. Pb toxicity can affect the nervous systems especially in adults. Thus, heavy metal remediation from polluted soils is necessary.[5,6]

Phytoremediation is one of the biodegradation methods that have received much attention in recent decades. In-situ and ex-situ methods are the two suitable ways that are being practiced for decreasing soil heavy metal concentration. The ex-situ remediation of contaminated soils carried by conventional methods is expensive.[7] However, in situ remediation of heavy metal such as phytoremediation is useful in environment studies. The Phytoremediation efficiency is

✉ *Amir Hossein Baghaie*
a-baghaie@iau-arak.ac.ir

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depended on the plant biomass, plant heavy metals concentration, and soil heavy metal availability. Pb has a widespread metal contamination in soils. However, it has low availability due to its insoluble formation.[8]

In order to increase the soil Pb availability and its translocation from root to shoot, organic chelates such as diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid (NTA) and ethylenediamine-N,N'-disuccinic acid (EDDS) have been introduced.[9] Among this, EDTA is the most effective on increasing soil heavy metal concentration.[10] EDTA can increase soil Pb availability and improve heavy metals uptake.[11] It can persist in the soil as a soluble form, because of its low degradability. However, the time and the amounts of chelate application has effective role on increasing soil heavy metals availability. [12] Liu *et al.* investigated the role of EDTA on increasing Pb phyto-availability efficiency and concluded that applying EDTA chelate has important role on increasing heavy metals availability by potato. Furthermore, they mentioned that plant and chelate type has important role on the amount of plant Pb uptake.[13] Positive effects of organic chelates such as EDTA or humic acids (HA) on improving phytoremediation efficiency have been mentioned.[14] Piri *et al.* investigated the role of humic acid on the changes in soil heavy metal availability and concluded that applying these organic amendments such as HA had significant effect on increasing phytoremediation efficiency.[15] Generally, HA is natural and non-toxic chelate and its ability to mobilize the heavy metals represents a novel and appropriate method to be employed in enhanced phytoextraction techniques.[16] Houshyar *et al.* investigated the effect of DTPA chelate on increasing Cd uptake by corn and concluded that applying DTPA chelate had significant effect on increasing Cd phytoremediation in a soil treated with sewage sludge.[17]

The important point is that increasing the heavy metals availability does not always lead to increased phytoremediation efficiency.[18] Generally; increasing heavy metal availability can decrease plant biomass and thus decrease heavy metal phytoremediation efficiency. Therefore, selecting the plants with high biomass or finding the suitable way to increase plant biomass may help to increase heavy metal phytoremediation efficiency.[19]

On the other hand, much attention has been paid to the use of plant growth regulators (PGRs) due to the useful roles of several plant hormones, i.e. gibberellins, auxins, abscisic acid, ethylene and other PGRs on plant stress tolerance.[20] It appears that PGRs has an important role on decreasing the negative effects of abiotic stresses produced by heavy metals and thus increases heavy metal concentration.[21] Janmohammadi *et al.* investigated the influence of PGPR inoculation and lead stress on the physiological of wheat cultivars and reported that PGPR inoculation had significant effect on decreasing Pb stress.[22] Practical effects of certain PGRs under toxicity due to mercury (Hg) and nickel (Ni), chromium (Cr), and Cd have been studied.[9] Hence, Phytoremediation in the presence of organic amendments such as organic chelates and PGRs has been introduced as an appropriate technique for the heavy metals remediation from contaminated soils.[23] Therefore, this research was done to investigate the effect of soil application of humic acid (HA) or EDTA chelate and foliar application of salicylic acid (SA) as a plant growth regulator on enhancing Pb phytoremediation in a barley plant cultivated in a Pb polluted soil.

Materials and Methods

To investigate the effects of synthetic chelates and plant growth regulators on Pb phytoremediation efficiency, a factorial experiment in the layout of randomized completely block design was done. Treatments (48 treatments in three replication) consisted of applying (soil application) two levels of EDTA (0 ($EDTA_0$) and 3 ($EDTA_3$) mmol/kg soil) and HA (0 (HA_0) and 200 (HA_{200}) mg/kg soil) two weeks after plant cultivation. foliar application of SA at the rates of 0 (SA_0) and 1.5 ($SA_{1.5}$) mmol/lit was done two weeks after barley

(*Hordeum vulgare* L. cv. Makoei) seedling growth in the presence (PGPR+) and absence of PGPR (PGPR-). On the other hand, soil polluted with Pb at the rates of 0 (Pb_0), 800 (Pb_{800}) and 1600 (Pb_{1600}) mg Pb/kg soil and incubated for two weeks to equilibrium. Selected soil physic-chemical properties are shown in Table 1.

Table 1. Physicochemical properties of selected soil in this research

Characteristic	Unit	Amount
pH	----	7.1
EC	dS/m	0.8
$CaCO_3$	%	5
Soil Pb availability	mg/kg	ND*
Soil Cd availability	mg/kg	ND
Soil Ni availability	mg/kg	ND

Not detectable by atomic absorption spectroscopy (LOI and LOD are 0.5 and 0.1 ppm, respectively)

The bacteria used in this study (Bacillus mycoides) were previously isolated from barley rhizosphere, identified, and tested. Thereafter, the seeds of barley (Makoei cultivar) were surface-sterilized in 70% ethanol for one minute followed by dipping seeds in 5% sodium hypochlorite solution for 10 min and rinsed several times with sterilized distilled water. Then half of the seeds were inoculated by immersion in the appropriate PGPR suspension (at 109 CFU mL^{-1}) for 2 h on a rotary shaker at 80 rpm, air dried, and immediately were transferred to the treated soil in each pot (10 seeds per pots). the non-PGPR inoculated seeds were also transferred to the half of remained pot (10 seeds per pots) filled with treated soil.[24]

The plants were irrigated to keep soil moisture at approximately 80% of field capacity. After 9 weeks, plants were harvested and soil and plant Pb concentration was measured using atomic absorption spectroscopy (AAS) (Perkin Elmer, model: 3030) according to the Baghaie *et al.* method.[25] The bio-concentration factor (BCF) was also calculated according to Eq. 1.[26]

$$BCF=C_{Plant}/C_{Soil} \qquad \text{Eq. 1}$$

where, C_{Plant} and C_{Soil} are the plant and the soil Pb concentration, respectively.

Plant Pb phytoremediation was also calculated according to the plant Pb concentration in the treated plant relative to non-treated plant.

Statistical analysis

The statistical analysis was done using SAS V. 9.1. The least significant difference (LSD) test was used to determine the differences between the means. The 95% (P=0.05) probability value was considered to determining the significant difference.

Results and Discussion

Soil properties

The simple effects of EDTA and HA on increasing soil Pb concentration was significant, as, applying 3 mmol/kg soil EDTA chelate significantly increase the soil Pb concentration by 12% that is similar to the study of Gabos *et al.*[27] However, based on the results of our study, the effectiveness of EDTA on increasing soil Pb concentration was more significant relative to HA (Fig. 1-a). basic structural units of HA are rings aromatic and alkyl chains that have a wide range of functional groups such as COOH, OH and NH_2. These bands have a high adsorption capacity to binding to metals that can increase soil Pb availability and thereby increasing Pb phytoremediation efficiency.[28]

On the other hand, plant inoculation with PGPR had significant (P=0.05) effect on increasing soil Pb concentration (Fig. 1-b) that maybe related to their ability for organic acid production as a secondary metabolite and thereby decreasing soil pH.[29] The results of Rasouli-Sadaghiani *et al.* confirm our results clearly.[28]

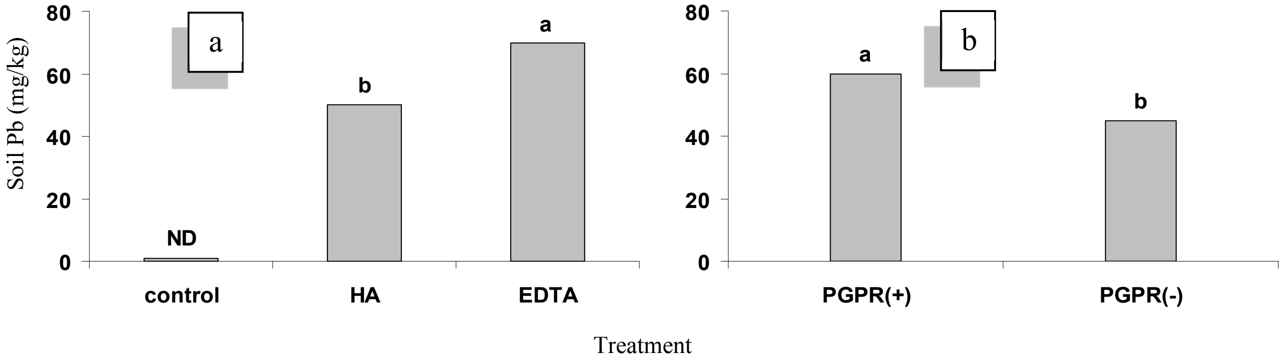

Fig. 1. The simple effect of organic chelates (a) and soil bacterial (b) on soil Pb concentration. HA, ETDA, PGPR (+) and PGPR (-) are humic acid, EDTA chelate and the presence and absence of plant growth-promoting rhizobacteria, respectively.

Increasing soil Pb concentration with decreasing soil pH had been mention by many researchers.[30,31] Karami *et al.* investigated the heavy metal uptake by wheat from a sewage sludge-amended calcareous soil and concluded that applying sewage sludge had significant effects on decreasing soil pH and thereby increasing soil heavy metal availability.[32] According to the results of this study, the simple effects of PGPR inoculated plants on increasing soil Pb concentration was greater relative to non-inoculated plant (Fig. 1-b)

The interaction effects of applying EDTA, SA and HA on increasing soil Pb concentration was significant (Table 2), as, the greatest soil Pb concentration belonged to the soil that amended with EDTA and HA chelate under cultivation of inoculated sunflower plant with PGPR that received 1.5 mmol/lit SA as a foliar application, while the lowest that was belonged to the soil under sunflower plants with receiving any soil or foliar chelate application. Applying 3 mmol EDTA and 200 mg/kg HA significantly increased the soil Pb availability by 15.1 and 11.4% in the soil under inoculated plant, respectively. Rasouli-Sadaghiani *et al.* investigated the effect of HA on the phytoremediation efficiency of Pb in the contaminated soils by wormwood plant and concluded that HA has a positive role on increasing soil Pb availability. However, the amount of solubility and availability of metals depends on the type and concentration of the heavy metals and chelates present in soil. Generally, the constant stability of divalent metal cations with EDTA is usually higher than HA.[28] Halim *et al.* reported that synthetic chelates that added to soil are effective in soil Pb availability, as, the results of their studied show that application of 20 mg/kg soil HA significantly increased the soil Pb availability. However, they did not mentioned the other soil chemical properties on the changes in soil heavy metal availability.[33]

Table 2. Effect of treatments on soil Pb concentration (mg/kg)

	Treatment					
	PGPR (+)					
	Pb_0HA_0	Pb_0HA_{200}	$Pb_{800}HA_0$	$Pb_{800}HA_{200}$	$Pb_{1600}HA_0$	$Pb_{1600}HA_{200}$
$EDTA_0SA_0$	ND**	ND	45.2s	48.7r	60.1o	65.6n*
$EDTA_0SA_{1.5}$	ND	ND	50.5q	55.4p	68.7m	71.5kl
$EDTA_3SA_0$	ND	ND	69.4m	72.1k	85.5f	88.4d
$EDTA_3SA_{1.5}$	ND	ND	75.5j	78.4i	93.6b	96.8a
	PGPR (-)					
$EDTA_0SA_0$	ND	ND	40.1t	45.5s	56.5p	60.8o
$EDTA_0SA_{1.5}$	ND	ND	48.7r	51.5q	60.1o	65.4n
$EDTA_3SA_0$	ND	ND	65.4n	68.1m	80.5h	83.4g
$EDTA_3SA_{1.5}$	ND	ND	70.1l	75.2j	87.5e	90.3c

*Data with same letter are not significant (P=0.05), ND**: not detectable by AAS

The results of Guo *et al.* on increasing soil heavy metal availability with the use of synthetic chelates confirms our results clearly.[34] Soil Pb availability in non-Pb polluted soil was not detectable by atomic absorption spectroscopy (AAS).

The greatest plant biomass belonged to the inoculated plants with PGPR cultivated in the non-Pb polluted soil that received the greatest level of HA and SA without any EDTA chelate, while the lowest that was observed in the Pb polluted soil (1600 mg Pb/kg soil) amended with 3 mmol/kg soil EDTA chelate without any application of HA or SA (Table 3).

Table 3. Effect of treatments on plant biomass (g)

	Treatment					
	PGPR (+)					
	Pb_0HA_0	Pb_0HA_{200}	$Pb_{800}HA_0$	$Pb_{800}HA_{200}$	$Pb_{1600}HA_0$	$Pb_{1600}HA_{200}$
$EDTA_0SA_0$	3.78s	5.55c	3.55u	4.72k	3.31w	4.45m*
$EDTA_0SA_{1.5}$	5.71b	5.89a	5.31f	5.45e	4.79j	5.13g
$EDTA_3SA_0$	3.55u	3.82r	3.41v	3.61t	3.17z	3.41v
$EDTA_3SA_{1.5}$	4.01p	4.44m	3.55u	3.86q	3.28x	3.55u
	PGPR (-)					
$EDTA_0SA_0$	3.52u	5.12g	3.31w	4.55i	3.19y	4.31n
$EDTA_0SA_{1.5}$	5.51d	5.55c	5.12g	5.31f	4.55i	5.00i
$EDTA_3SA_0$	3.32w	3.55u	3.18z	3.28x	2.95c′	3.12a′
$EDTA_3SA_{1.5}$	3.61t	4.14o	3.41v	3.55u	3.00b′	3.41v

*Data with same letter are not significant (P=0.05)

The important point of this study is that the application of HA chelate could significantly increase the Pb plant resistance and thereby increased plant growth. Shafigh *et al.* investigated the influence of plant growth regulators and HA on the corn Pb phytoremediation in a Pb-polluted soil and concluded that SA was the most effective in improving heavy metal phytoremediation through Pb phytostabilization. However, they mentioned that SA application has not significant effect on Pb translocation factor.[35] Hadi *et al.* reported that foliar application of plant growth regulators significantly improved maize shoot and root dry weight in Pb-contaminated soil and significantly enhanced Pb uptake and accumulation in roots.[36]

Although the application of EDTA chelate has also significant effect on plant Pb availability, but the plant biomass shows a decreasing trend that can be attributed to the effects of Pb toxicity on the plant growth.[37] In this regard, Htwe *et al.* conducted Pb-phytoextraction in a Pb treated soil and reported that although soil Pollution with Pb can increase Pb extraction from soil, but it had negative effect on plant biomass that is similar to our study. Thus, it could not affect Pb phytoremediation efficiency.[37] Therefore, it seems that the simultaneous application of EDTA chelate, SA and HA have an important role on enhancing phytoremediation efficiency that can be related to the positive role of SA and HA on plant growth. However, phytoremediation efficiency is highly dependent on the concentration of chelate applied, as, the results of Shafigh *et al.* showed that addition of more than 3 mg/kg soil significantly reduced the corn Pb efficiency.[35] Although they did not mentioned the role of soil sorption properties on the changes plant Pb phytoremediation. Nevertheless, the results of most studies indicate the positive role of HA chelates and plant growth regulators such as SA on increasing plant resistance to heavy metals.[28, 38]

Based on the results of this study, the greatest plant Pb concentration was belonged to the plants that cultivated in the soil that received the greatest level of HA, EDTA and SA, while the lowest that was measured in the plant without and receiving any synthetic chelates in the absence of PGPR (Table 4). The plant Pb concentration in non-Pb polluted soil was not detectable by AAS.

Table 4. Effect of treatments on plant Pb concentration (mg/kg)

	Treatment					
	PGPR (+)					
	Pb_0HA_0	Pb_0HA_{200}	$Pb_{800}HA_0$	$Pb_{800}HA_{200}$	$Pb_{1600}HA_0$	$Pb_{1600}HA_{200}$
$EDTA_0SA_0$	ND**	ND	27.3q	32.3m	36.2j	40.1g
$EDTA_0SA_{1.5}$	ND	ND	29.4o	36.5j	39.1h	45.7e
$EDTA_3SA_0$	ND	ND	32.3m	37.5i	40.4g	56.4b
$EDTA_3SA_{1.5}$	ND	ND	37.1i	40.4g	46.7d	59.3a
	PGPR (-)					
$EDTA_0SA_0$	ND	ND	24.6r	28.6p	34.1k	37.2i
$EDTA_0SA_{1.5}$	ND	ND	27.2q	33.1l	37.3i	40.8g
$EDTA_3SA_0$	ND	ND	30.3n	34.1k	37.5i	45.6e
$EDTA_3SA_{1.5}$	ND	ND	33.4l	37.2i	41.4f	51.3c

*Data with same letter are not significant (P=0.05), **ND: not detectable by AAS

The effect of SA on the prevention of biological and non-biological stresses has also been considered [9] that is similar to our results. Accordingly, applying SA treatment significantly increased the plant biomass and thereby plant Pb concentration. Elhassan *et al.* studied the Pb phytoremediation of corn using SA and concluded that application of plant growth regulator such as SA had a positive effect on the plant's physical properties as well as increasing plant Pb concentration.[39] Generally, SA is an inductive molecule in the plant protection system. This compounds decrease the adverse effects of some abiotic stress such as plant heavy metal stress. This material decreases the negative effects of heavy metals by affecting enzymes, such as catalase or peroxidase, and changes in osmotic systems. In addition, SA can improve the plant antioxidant system and reduces the plant toxicity effects. These changes are needed to increase plant's resistance to the environmental factors. Therefore, using SA can be a benefit factor in increasing Pb phytoremediation efficiency (Table 5).

Table 5. Effect of treatments on Pb remediation efficiency

	Treatment					
	PGPR (+)					
	Pb_0HA_0	Pb_0HA_{200}	$Pb_{800}HA_0$	$Pb_{800}HA_{200}$	$Pb_{1600}HA_0$	$Pb_{1600}HA_{200}$
$EDTA_0SA_0$	NC**	NC	0.68q	0.81m	0.90j	1.00g*
$EDTA_0SA_{1.5}$	NC	NC	0.73o	0.91j	0.98h	1.14e
$EDTA_3SA_0$	NC	NC	0.81m	0.94i	1.01g	1.41b
$EDTA_3SA_{1.5}$	NC	NC	0.93i	1.01g	1.16d	1.48a
	PGPR (-)					
$EDTA_0SA_0$	NC	NC	0.61r	0.71p	0.85k	0.93i
$EDTA_0SA_{1.5}$	NC	NC	0.68q	0.83l	0.93i	1.02g
$EDTA_3SA_0$	NC	NC	0.76n	0.85k	0.94i	1.14e
$EDTA_3SA_{1.5}$	NC	NC	0.83l	0.93i	1.03f	1.28c

*Data with same letter are not significant (P=0.05), **NC: not calculated

On the other hand, adding a compound that increase the availability of heavy metal in the soil is required. Therefore, the simultaneous use of EDTA chelates and SA could be a good way to increase the Pb phytoremediation efficiency that is similar to our results. As mentioned before, the greatest plant Pb concentration was belonged to the plants that received the EDTA, SA and HA. Afrousheh *et al.* showed that using SA had a positive effect on the increasing heavy metal phytoremediation efficiency.[40] the Pb phytoremediation efficiency showed the similar trend (Table 5), as, the greatest Pb phytoremediation efficiency was observed in the soil received the greatest level of HA, SA and EDTA chelates in the presence of PGPR.

Generally, bio-assisted phytoremediation or rhizoremediation has an important role in decreasing soil heavy metal availability. Rhizoremediation is the most emerging, ecofriendly and potentially effective process of biodegradation of heavy metals in the soil. Soil microorganism increases the heavy metal phytoremediation efficiency in different ways. According to the results of this study, the presence of PGPR, significantly increased the plant resistance to Pb toxicity, as, the results of this study showed that the greatest plant biomass was observed in the presence of PGPR. Among this, adding organic chelate such as SA, HA or EDTA helped to increase Pb phytoremediation efficiency with increasing plant biomass. Erkovan *et al.* investigated the effect of phosphorus fertilizer and PGPR application on clover dominant meadow and concluded that PGPR help in contributed to the phytoremediation processes through the production of soluble minerals (e.g., phosphorus and potassium), which were required for the increasing of the plant biomass.[41]

Conclusion

According to the results, the application of PGRs such as SA and synthetic chelate (HA) had significant effects on the increasing of the plant biomass, which contributed to lead phytoremediation efficiency as the highest lead phytoremediation efficiency was observed in the plants that were cultivated in the soil inoculated with HA, EDTA, and SA. On the other hand, the presence of PGPR had significant effects on the increasing of the plant biomass and lead phytoremediation efficiency. The findings of the current research indicated that the application of 3 mmol/kg soil of the EDTA chelate had significant effects on the increasing of soil lead availability, which in turn improved soil lead extraction. However, it also had adverse effects on the plant biomass. Therefore, it could be concluded that to increase lead phytoremediation efficiency, the simultaneous application of HA, EDTA, and SA is required. Furthermore, it is necessary to investigate the role of application time of these organic amendments on increasing Pb phytoremediation efficiency. On the other hand, the type and amount of heavy metals has important role on heavy metal phytoremediation that should be considered in the future studies.

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