EDTA effects on seedling emergence and growth of *Chenopodium album* (L.) in Pb contaminated soil

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Abstract

The study was conducted to investigate the effects of ethylenediaminetetraacetic acid (EDTA) on seedling emergence, morphological characteristics, and photosynthetic pigments of *Chenopodium album* (L.) in lead (Pb) contaminated soil. The Pb contaminated soils were taken from Hirmand River. The soil samples mixed with 0.71 g Li^{-1} Pb $(PbNO_3)_2$ to increase the Pb concentration. EDTA was added to the soil in concentrations of 1.5, 3 and, 9 mg kg^{-1} after sowing the seeds. Results showed that EDTA significantly decreased seedling emergence. The highest reduction in germination was related to 9 mg kg^{-1} EDTA. With increasing EDTA dosage, mean seedling emergence time increased. The highest seedling vigor index (17.29) was related to the control treatment while the lowest seedling vigor index (2.15) was measured in 9 mg kg^{-1} EDTA. With increasing EDTA concentration, plant biomass decreased. The highest reduction was observed in 9 mg kg^{-1} EDTA. The maximum and minimum pedicel length, radical length, and tolerance index were measured in 1.5 and 9 mg kg^{-1} EDTA treatments, respectively. The highest amounts of chlorophyll a, b, total chlorophyll, and carotenoid were observed in 1.5 mg kg^{-1} EDTA. Increased EDTA from 1.5 to 9 mg kg^{-1} led to a decrease in photosynthetic pigments. In general, results showed that EDTA at high concentrations had deterrent impacts on the plant growth. Therefore, high concentrations of this compound are not suggested to increase remediation efficiency of Pb contaminated soil.

Keywords: Aminopolycarboxylic acids; tolerance index; seedling vigor index; lead; *Chenopodium album* (L.)

Ebrahimi , M.. 2018. EDTA effects on seedling emergence and growth of *Chenopodium album* (L.) in Pb contaminated soil'. Iranian Journal of Plant Physiology 8(2), 2361-2370.

Introduction

Soil contamination with heavy metals is one of the major environmental problems in human societies that apart from deleterious effects on soil fauna and flora and ground water contamination, reduces the performance and quality of plants, the health of people, and other living organisms (Shahid et al., 2014). Although heavy metals can naturally be accumulated in soil through weathering rocks and minerals and through soil forming processes, these natural resources are less significant compared to the contamination caused by human activities such as industrial factories, mining, fossil fuels, chemical fertilizers, industrial waste ,and sewage sludge (Li et al., 2014; Li and Yan, 2012). Among heavy metals, Pb has unknown role in living creatures and in high concentration, it is harmful for plants and humans (Ahmad et al., 2007).

Soil contamination with Pb in Iran has been reported in many areas such as Uremia

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Received: May, 2017
Accepted: November , 2017

(Rashid Shomali and Khodaverdiloo, 2012) Karaj, Anzali, Isfahan, and Ramsar (Rahmani et al., 2001).

Chenopodiaceae plants are predominant in arid and semi-arid regions. Most of the studies have focused on distribution of these plant species (Parka et al., 2009) and their resistance to environmental stress such as salinity and drought (Heklau and Wehrdena, 2011) but little studies have been carried out on the resistance of these species under metal stress. The few studies showed that these plant species could grow in contaminated environments (Hu et al., 2012). Therefore, an investigation of the relationship between these plant species and heavy metals stress seems essential to protect areas located in arid and semi-arid regions. *Chenopodium album* L. is one of the predominant species of *Chenopodiaceae* that has a great distribution in arid and semi-arid regions (Adim et al., 2010) because of its high growth speed, great biomass, resistance to drought, and high compatibility with environmental conditions (Hekelau and Wehrdena, 2011).

Remediation of the soils contaminated with heavy metals is an important practice for preservation of the environment. Phytoremediation is one of the environmentally-friendly methods in which hyperaccumulator plants are used to remove metals from contaminated soils (Bisone et al., 2014; Shahid et al., 2014). Despite the efficiency of phytoremediation in soil remediation, only a part of heavy metals is usually eliminated from the soil. A commonly used approach for increasing the efficiency of phytoremediation is using chelating agents and plants with high biomasses (Ebrahimi, 2014a). One of the most important chelating agents is aminopolycarboxylic acids, EDTA (Zhao et al., 2011; Ebrahimi, 2014a). Although EDTA increases the accessibility of metals in the soil, its high concentrations will be poisonous for the plants and will reduce their growth and germination, and consequently it will reduce the metal concentration in plant tissues (Barocsi et al., 2003). These compounds might even be poisonous for the plants at low concentrations and might lead to necrosis and even plant death (Ebrahimi, 2012). Ebrahimi (2012) reported that an increase in the concentration of EDTA from 1.5 to 10 mM kg^{-1} reduced germination and tolerance indices of *Echinochloa crus galii* (L.) Beave. Arbabi (2013), in a study on phytoremediation of *Prosopis cineraria* and *Eucalyptus camaldulensis* in Pb contaminated soil using EDTA, reported that the maximum germination and biomass of the plants were related to the uncontaminated soils without EDTA. Therefore, the appropriate concentration of EDTA should be selected to reduce the adverse effects of EDTA on the plant growth and Environment (Shibata et al., 2007). The purpose of this study was evaluation of the effect of EDTA on seedling emergence, morphological properties (biomass, root and shoot growth), and photosynthetic pigments of *C. album* L. in the Pb contaminated soil in greenhouse conditions.

Materials and Methods

Pot preparation

Soil samples were taken from Hirmand River (31°02' N-61°50' E). The river was contaminated with heavy metals including Pb (Adim et al., 2010; Rajaei et al., 2012). Amount of Pb in the soil was determined (DTPA extraction method, Garbisu and Alkorta, 2001) and considering the presence of Pb more than the standard level (12.33 mg kg^{-1}), about 0.71 g Li^{-1} $Pb(NO_3)_2$ was added to the soil by spraying before cultivation to increase the concentration of Pb at high concentrations of 450 mg/kg (Diaz-Ravina and Baath, 1996). Soil samples were kept in the greenhouse for two weeks for homogeneous contamination. The soil texture was determined

Table 1
Some soil physical and chemical characteristics used in the pot experiments

Texture	N_{tot} (%)	P (mg kg^{-1})	K (mg kg^{-1})	EC (dS m^{-1})	pH	Pb (mg kg^{-1})
Silt lomay	0.15	5.10	380	3.44	8.5	12.32

by hydrometer method (Day, 1982). Electrical conductivity (EC) was measured using an EC-meter (DDS-307, Shanghai, China) (Rhoades, 1996). Soil acidity was determined in a 1:5 soil to distilled water slurry after one hour of agitation using a digital pH-meter (Model 691, Metrohm AG Herisau Switzerland) (Thomas, 1996). Total soil N was analyzed calorimetrically with a continuous flow ion analyzer following wet digestion in sulfuric acid using Kjeldhal method (Black, 1965). Total phosphorus was determined by the method of Olsen and Sommers (1982). Total potassium was measured by flame photometry method (Berry et al., 1946), (Table 1).

The seeds were collected from Sistan plain (31° 49' N-61° 39'E). A number of 15 seeds were planted in each pot (upper diameter 20 × lower diameter 15 × height 60 cm). Seeds were buried evenly throughout each pot at least 1 to 2 cm from the edge. The pots were watered (tap water) from the top during the germination period so that, soil moisture was kept near 70% field capacity. EDTA was added at 1.5, 3 and 9 mg kg^{-1} concentrations with five replications over the plant growth in spraying form. Uncontaminated soil without EDTA (control) was considered as control treatment. The samples were put behind the glass windows of the greenhouse (minimum and maximum temperatures 25 °C and 30 °C, respectively, relative humidity of 85%, and photoperiod of 10 hours in light and 14 hours in darkness).

The first counting of the germinated seeds occurred 24 hours after the first germination and the seeds whose pedicle was observable were counted as germinated and took out of the pot (Wise and Binning, 1978). Germinated seeds were counted and recorded daily (Farajollahi et al., 2012). This was done each 24 hours until the germination had been completed. On the last counting day (the 14^{th} day), five seedlings were selected randomly and the radicle and pedicel lengths (using a caliper) and the dry and fresh weights of seedlings were measured (digital balance with 0.0001 g precision). In order to determine the dry weight, the samples were washed by distilled water and the radicle and pedicel were separated. Then, they were placed in an oven (model: 711360 Shimaz Company) at 70 °C for 48 hours. After 14 days, having fixed the number of the germinated seeds and at the end of the growth period, the germination indices including the germination rate, germination percentage, germination time, and seed vigor index were measured according to the following equations:

$$GR = \sum Ni/Di$$

where, GR, Ni and Di are germination rate, number of germinated seeds in each day, and counted day, respectively (Mereddy et al., 2000).

$$GP = (n/N)\ 100$$

where, GP is germination percentage, n is the total number of the germinated seeds during counting, and N is the total number of the germinated seeds in each pot (Behbodian et al., 2005).

$$MGT = \frac{\sum nd}{\sum n}$$

where, MGT, n, d, and ∑n are mean germination time, number of germinated seeds during the day, number of days since the beginning of germination, and total number of germinated seeds, respectively. It is to be mentioned that the required mean time for germination was counted as an index of germination rate and speed (Ellis and Roberts, 1981).

SVI = mean of initial stem length + the mean of initial root length) × viability

The seedling vigor index (SVI) was determined at the end of growing trial after calculating the pedicle and radicle lengths (Saravanakumar et al., 2007). In this respect, viability is the final germination percentage.

In order to study the effect of EDTA on photosynthetic pigments, in each pot three seedlings were selected and three leaves from each plant were considered (Shahsavan Markade and Chamani, 2014). Measuring the level of chlorophyll a , chlorophyll b, and cartenoids were done by rubbing 0.5 g leaf sample in acetone (80%) and reading the optical density at wavelengths 663, 645, and 470 nm (Arnon, 1949). Total chlorophyll was calculated by adding up chlorophyll a and chlorophyll b.

Chlorophyll a = (19.3 × A663 – 0.86 × A645) V/100W

Table 2
Results of analysis variance of germination and morphological properties of *C. album* L. in Pb contaminated soils under different EDTA application

Source of Variation	Degree of freedom	Mean square							
		Germination rate	Germination percentage	Mean germination time	Tolerance Index	Radical length	Pedicel length	Root dry weight	Shoot dry weight
Treatment	3	142.75**	76.44**	0.43**	192.91**	192.91**	0.049**	14.31**	81.78**
Error	16	10.68	62.22	0.44	300.97	300.97	0.006	1.94	7.73
CV (%)	-	4.45	9.75	2.95	2.33	4.78	4.56	2.32	5.42

** significant at the 0.01 probability level.

Table 3
Comparison of the mean effects of EDTA on seedling emergence of *C. album* L. in Pb contaminated soil

EDTA treatment (mg kg^{-1})	Seedling vigor index	Mean germination time	Germination percentage (%)	Germination rate (number/day)
Control	17.29±1.10[a]	1.14±0.19[a]	100.00±5.00[a]	15.00±6.21[a]
1.5	16.23±1.32[a]	1.14±0.19[a]	96.21±4.33[a]	14.00±6.11[a]
3	4.05±0.50[b]	3.14±0.26[b]	40.25±3.24[b]	8.00±2.40[b]
9	2.15±0.27[c]	4.38±0.40[c]	38.47±3.05[b]	6.00±2.40[c]

Control: Uncontaminated soil without EDTA
Values within a column followed by the different letters are significantly different (P<0.05, mean ± SE)

Chlorophyll b = (19.3 × A645 – 3.6 × A663) V/100W)

Carotenoides = 100 (A470) – 3.27(mg chl.a) – 104 (mg chl.b)/227

where, V is volume of the filtered solution (above solution obtained from centrifuge), A is the light absorption at wavelengths 663, 645 and 470 nm, and W is the fresh weight of samples (g).

The remaining herbaceous plants in the pot were collected when reaching the maximum growth (25 days) and their biomass was calculated. In addition, the tolerance index (TI) of the plant was calculated according to the following equation (Lombi et al., 2001).

$$TI = \frac{Wc}{Wu}$$

where Wc is the dry weight of plants grown in contaminated soils and Wu is the dry weight of plants grown in uncontaminated soils.

Data Analysis

Statistical analyses of the data were performed using the SPSS software (ver. 18.0). The experiment was conducted in completely randomized design. All reported results are the means of five replicates, and deviations were calculated as the standard error of the mean (SEM). The statistical processing was mainly conducted by analysis of variance (ANOVA). Duncan test post hoc analysis was performed to define which specific mean pairs were significantly different. A probability of 0.05 or lower was considered as significant level.

Results

Effects of EDTA on germination properties

Results revealed that EDTA had a significant effect ($p<0.01$) on the emergence properties of *C. album* L. (Table 2). Also, EDTA was found to reduce the germination rate of the seedlings significantly ($p<0.01$) (Table 3). The maximum germination rate and the percentage of reduction of the seedlings were related to the 9 mg kg^{-1} EDTA treatment (6 per day and 38.47%, respectively). The maximum germination rate and percentage were related to the control treatment.

Table 4
Compare the mean effects of EDTA on morphological properties of *C. album* L. in Pb contaminated soil

EDTA treatment (mg kg^{-1})	Tolerance Index	Radical length (mm)	Pedicel length (mm)	Root dry weight (g)	Shoot dry weight (g)
Control	1.00±0.00^{a}	32.15±2.00^{a}	35.19±2.19^{a}	29.22±0.92^{a}	50.72±0.92^{a}
1.5	1.00±0.05^{a}	34.16±2.00^{a}	36.16±2.11^{a}	33.10±1.36^{a}	54.41±1.36^{a}
3	0.83±0.01^{b}	16.03±1.10^{b}	18.33±2.00^{b}	17.30±1.54^{b}	30.50±1.54^{b}
9	0.71±0.01^{c}	9.01±1.10^{c}	11.42±1.50^{c}	11.40±1.00^{c}	30.80±1.00^{b}

Control: Uncontaminated soil without EDTA
Values within a column followed by different letters are significantly different (P<0.05, mean ± SE)

Table 5
Results of analysis variance of photosynthetic pigments of *C. album* L. in Pb contaminated soils under different EDTA application

Source of variation	Degree of freedom	Mean square			
		Carotenoid	Total Chlorophyll	Chlorophyll b	Chlorophyll a
Treatment	3	44.03*	0.97*	0.11*	0.43*
Error	16	300.53	0.42	0.04	0.10
CV (%)	6.77	5.23	11.92	12.15	7.28

* Significant at the 0.05 probability level

Table 6
Compare the mean effects of EDTA on photosynthetic pigments of fat hen in lead contaminated soil

EDTA treatment (mg kg^{-1})	Carotenoid (mg.g^{-1} fresh weight)	Total Chlorophyll (mg.g^{-1} fresh weight)	Chlorophyll b (mg.g^{-1} fresh weight)	Chlorophyll a (mg.g^{-1} fresh weight)
Control	83.78±5.26^{a}	1.69±1.32^{a}	0.44±0.01^{a}	1.25±0.30^{a}
1.5	83.99±4.12^{a}	1.71±1.49^{a}	0.45±0.01^{a}	1.29±0.30^{a}
3	52.55±3.20^{b}	1.22±0.90^{b}	0.28±0.01^{b}	0.94±0.20^{b}
9	43.15±3.20^{c}	1.11±0.90^{b}	0.22±0.01^{b}	0.94±0.20^{b}

Control: Uncontaminated soil without EDTA
Values within a column followed by different letters are significantly different (p<0.05, mean ± SE)

However, there was no significant difference between the control treatment and 1.5 mg kg^{-1} concentration of EDTA in terms of germination rate and percentage. The results showed that the increased EDTA concentration in the Pb contaminated soil led to a significant increase in the time required for the seedling emergence (p<0.01). The maximum mean germination time of the seedlings was calculated in 9 and 3 mg kg^{-1} EDTA treatments, respectively. The least mean germination time was related to the control treatment. However, there was no significant difference between the control and 1.5 mg kg^{-1} EDTA treatments. EDTA significantly reduced the plant vigor index (p<0.01).The maximum seedling vigor index (17.29) was related to the control treatment. While the minimum seed vigor index (2.15) was measured in 9 mg kg^{-1} EDTA treatment.

Texture	N_{tot} (%)	P (mg kg^{-1})	K (mg kg^{-1})	EC (dS m^{-1})	pH	Pb (mg kg^{-1})
Silt loam	0.15	9.10	300	3.44	8.5	12.32

Effects of EDTA on morphological properties

Results of variance analysis showed that EDTA had significant effects (p<0.01) on the morphological properties of *C. album* L. (Table 2). Results of the comparison of the mean effects of EDTA on morphological properties of *C. album* L. are summarized in Table 4 that shows EDTA had a significant effect on the dry weight of plant parts (p<0.01). The plant dry weight decreased when EDTA concentration increased. The maximum dry weight reduction was observed in 9 mg kg^{-1} EDTA treatment. The maximum dry weight of shoot (54.41 g) and root (33.10 g) were related to 1.5 mg kg^{-1} EDTA treatment. The effect of EDTA on the plant roots was more than shoots. The maximum and minimum radicle and pedicel lengths were measured at 1.5 and 9 mg kg^{-1} concentrations of EDTA. However, there was no significant difference between the control treatment and 1.5 mg kg^{-1} EDTA. Results showed that tolerance index

of the plant reduced in EDTA treatments compared to the control treatment, so that the tolerance index of the plant in the control and 1.5 mg kg^{-1} EDTA treatments were maximum (1). The minimum level of tolerance index (0.71) was related to 9 mg kg^{-1} EDTA treatment.

Effect of EDTA on photosynthetic pigments

Results showed that EDTA had significant effects on the photosynthetic pigments of *C. album* L. ($p<0.05$) (Table 5). Comparison of the means (Table 6) showed that application of EDTA made a significant difference in the chlorophyll pigments of the plants compared to the control treatment ($p<0.05$). The maximum value of chlorophyll a, chlorophyll b, and total chlorophyll were observed at 1.5 mg kg^{-1} of EDTA and the control treatments, respectively. The minimum values of chlorophyll a, chlorophyll b, and total chlorophyll were measured at 9 mg kg^{-1} concentration of EDTA. The increase in EDAT concentration from 1.5 to 9 mg kg^{-1} led to a reduction of 72.86%, 48.88%, and 65.68% in chlorophyll a, chlorophyll b, and total chlorophyll, respectively compared to the control treatment. Results also showed that the maximum and minimum amount of cartenoid contents were observed in 1.5 and 9 EDTA mg kg^{-1} treatments. The increase in EDTA concentration from 1.5 to 9 mg kg^{-1} led to a 51.50% reduction in carotenoid content of the plants under study (Table 6).

Discussion

Plant tolerance to the environmental stress depends on the stage of their growth. Growth and development of a plant start from seed germination stage and, the seeds of a plant need to adapt to the environmental conditions to continue their life and become firm in the soil. If the plant can tolerate this stage successfully, the chance of surviving becomes high (Memon et al., 2010). Results of the present study showed that an increase in EDTA concentration reduced the rate and percentage of the seedling emergence. The reason might be related to the increase in the accessibility of Pb after EDTA application into the soil (Arbabi, 2013). Many studies have showed that EDTA reduces plant growth and germination due to the increase in accessibility of metals in the soil and the toxic effects of EDTA (Ebrahimi, 2012). In addition, despite the possible usefulness of EDTA some studies have expressed concerns regarding the increased risk of ground water contamination associated with EDTA–assisted phytoremediation. In addition, in high concentrations of EDTA, plant biomass, germination, and the contents of photosynthetic pigments significantly reduce (Nasciment et al., 2006).

Results showed that the application of EDTA reduced the dry weight of plant species compared to 1.5 mg kg^{-1} EDTA and the control treatments. However, the morphological properties of the plant had higher values when treated with 1.5 mg kg^{-1} EDTA compared to the control treatment. The amount of Pb absorbed by plant tissues was low in low concentrations of EDTA. Numerous studies have showed that not only Pb has no deterrent effect on the plant growth at low concentrations, but also it stimulates plant growth. Bashmakov et al. (2005) reported that the growth of *Zea mays* in contaminated soil containing 1-5 mM kg^{-1} Pb increased. Even in the presence of Pb in soil, the concentration of nutrients like nitrogen in plant tissues will increase (Bojarczuk, 2004).

The reduction in the dry weight of the plant, radicle, and pedicel lengths and the tolerance index of the plant at high concentrations of EDTA can be related to the increase in the concentration of Pb in the soil which will lead to the reduction of root cell division and reduction in plant germination and biomass (Ebrahimi, 2012). Ebrahimi (2014b) reported that although EDTA application increased the accessibility of Pb in the soil, high concentrations of EDTA are toxic for the plant and reduce plant biomass. The reasons for the reduction of plant biomass is that the root cells are destroyed at high concentrations of chelating agents such as EDTA (Luo et al., 2006). Efficiency of aminopolycarboxylic acids such as EDTA is enhancing the metal mobility in the soil and root cell walls of plant that help the transmission of metals from roots to aboveground plant parts, but plants exposed to the high levels of both free Pb and free chelate produce low biomass due to low seed germination, chlorosis, leaf wilt and necrosis, shoot desiccation and reduced transpiration

(Nascimento et al., 2006). Chen et al. (2004) reported signs caused by toxicity of EDTA including necrosis in sunflowers and Indian mustard.

Results of the investigation of tolerance index (TI) of the plant under EDTA application indicated a reduction in tolerance index of the plant compared to the control treatment. The value of TI is equal to 1 when there is no influence of treatment on the growth, higher than 1 when there is a favorable effect of treatment on the growth, and lower than 1 when the growth is affected negatively by the treatment (Zaier et al., 2010; Ebrahimi, 2012). Shen et al. (2002) reported that although at high concentration of EDTA, the accessibility of metals increase in the soil and plant tissues especially in the roots, due to the negative effect of EDTA on the plant properties and tolerance index of plants and risk of leaching into the ground water, high concentrations of this material is not recommended. In the study on the effects of EDTA and DTPA on Pb and Zn uptake by *Eu. camaldulensis* Dehnh, Ebrahimi (2014a) showed that by the increase in the concentration of EDTA and DTPA, tolerance index of the plant decreased.

Results showed that compared to the control treatment, the EDTA at 1.5 mg kg^{-1} concentration increased the contents of chlorophyll pigments in the plant. However, the increase in EDTA concentration from 1.5 to 9 mg kg^{-1} reduced the level of chlorophyll a, chlorophyll b, the total chlorophyll, and carotenoids. EDTA is widely used for the dissolution of calcareous sediments as a result of evaporation of water. EDTA is an aminopolycarboxylic acid. Its usefulness arises because of its role as a hexadentate ("six-toothed") ligand and chelating agent (Harris, 2007), i.e., its ability to "sequester" metal ions. After being bound by EDTA, metal ions remain in solution, but exhibit diminished reactivity (Holleman, 2001). For example, they create strong complexes with iron (Holleman, 2001) that plays a crucial role in photosynthetic (Kauser and Azam, 1985). Therefore, the increase in nutrients of the plant can be considered the reason for the increase in photosynthetic pigments of the plant and as a result, the increase in the root and shoot length and plant biomass at low concentrations of EDTA (Bojarczuk, 2004). However, at high concentrations of EDTA, it reduces photosynthesis, growth, and plant biomass through lack of nutrients because EDTA creates strong complexes with vital plant elements such as calcium, manganese and iron (Salido et al., 2003). Lack of vital nutrients in plants increases Pb uptake. As an example, Alkotra et al. (2004) showed that lack of Fe in the plant tissues resulted in an increase in absorption of heavy metals in the plants. Deleterious effects of Pb on chlorophyll synthesis are because of the prevention of absorbing vital elements like Fe and Mn. Photosynthetic pigments are also destroyed due to the constraints on protein-ligands of nitrogen and sulfur. The increase in chlorophyllase activity also increases chlorophyll destruction in the presence of Pb (Sharma and Dubey, 2005), so that chlorophyllase decomposes chlorophyll by separating phytol from chlorophyll and separating magnesium from chlorophyllid and forming pheophorbes and consequently, decomposing the four pyrrole ring (Boyer et al., 1987). Apart from decreasing the plant biomass, Pb also decreases chlorophyll b formation, prevents photosynthesis, destroys cells, and damages chromosomes (Estrella-Gomez et al., 2009).

Conclusion

Suitable selection of EDTA with appropriate selection of the plant are prerequisites to reduce the negative effects of this kind of chelant. Results of the present study revealed that low concentrations of EDTA had positive effects on the morphological properties of *C. album* L in Pb contaminated soil. The findings demonstrated that due to the negative effect of EDTA on the morphological properties and plant growth, high concentrations of EDTA are not recommended to increase the efficiency of soil remediation. Considering the fact that this research was carried out in pots and at greenhouse conditions, there is a needs to study the efficiency of this compound at farm conditions and the comparison of the effects of other chelating agents on plant growth. Moreover, considering the fact that the amount of Pb absorption was not measured in the plant tissues and the soil, complementary studies are recommended to compare the effect of different concentrations of EDTA on Pb absorption.

Acknowledgments

The author is thankful to Vice Chancellor for Research and Technology of University of Zabol for providing necessary facilities to undertake this study.

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