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Improved phytoextraction capacity of *ProsopisCineraria* (L.) Durce grown on contaminated soil: Roles of EDTA and DTPA treatment time

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Abstract
Pot experiment was conducted to investigate phytoextraction of lead and zinc by *Prosopiscineraria* following EDTA (ethylenediaminetetraacetic acid) and DTPA (diethylenetrinitrilopentaacetic acid) applications and to study the effects of harvest time as a suitable dose of chelating agents. Before the chelating agents were added, Pb level was decreased in the order of shoot > soil > root and Zn content occurred in the sequence of shoot > root> soil. As second step, contaminated soils were treated with EDTA and DTPA (1.5, 3, 6, 9 mmolkg^{-1}). Results demonstrated that chelating agents enhanced the content of metals in *P.cineraria*. The greatest bioaccumulation in EDTA and DTPA treatments was observed in 9EDTA and 9DTPA respectively. With respect to non-significant difference between 9EDTA and 6EDTA and between 6DTPA and 9DTPA, low doses were used in the third step for the highest metal uptake for 60, 90 and 120 days. Results revealed that chelating addition did not increase metals concentration in the plant organs as time passes. Results indicated that *P. cineraria* had the potential for phytoextraction of metal-contaminated soils but it should not be used unless the biomass containing the accumulated metals is removed for disposal.Chelates assisted the phytoextraction should be used cautiously because of their environmental leaching risks into ground water.

*Keywords:*Chelating agent, Copper, Lead; Phytoremediation, *Prosopis cinerari.*

1. Introduction
Heavy metals from mine exploitation, vehicle emissions and irrational use of chemical fertilizer seriously contaminate soil and environment [1].This situation has become a critical environmental issue owing to the potential adverse ecological effects of the pollutants [2].
As compared with physical and chemical techniques of remediation, phytoremediation is a developing technology that aims to extract or inactivate metals and it has attracted much attention because it is an environmentally friendly and relatively cheap technique [3; 4; 5; 6].
Phytoremediation can be categorized into two different approaches: (i) phytoextraction, metal accumulating plants are planted on the contaminated soil and later harvested in order to remove metals from the soil [7; 8] and ii) phytostabilization, metal-tolerant plants are used to reduce the mobility of metals; thus, the metals are stabilized in the substrate [9; 10; 11].
Although phytoremediation can be applied for the reclamation of elevated concentrations of heavy metals present in the contaminated soils, just a fraction of soil metal content is readily available for plant uptake and a large portion is generally present as insoluble compounds unavailable for the absorption by roots, restricting the absorption of hyperaccumulating plants [12].
A commonly used approach of enhancing phytoremediation has employed the chelating agents such as EDTA (ethylenediaminetetraacetic acid) and DTPA (diethylenetrinitrilopentaacetic acid) [13; 14] but the excessive addition of chelating agents in field conditions may pose secondary pollution of soils and the leaching of chelating agents may risk groundwater contamination by the uncontrolled metal solubilization and leaching as well as the increasing cost of phytoremediation [15].
The biodegradation and toxicity of the chelating agents and their metal complexes in soils need careful assessment and evaluation [16] to avoid possible metal chelate movement into groundwater and the effects of remaining them on soil microorganisms while the amount and process of chelate application are important for novel irrigation technique and time control of chelate application. A comprehensive approach to phytoremediation should consider strategies in relation to the potential risk that may affect the ecosystem [4].

However, some plants such as *Prosopis cineraria*(*P. cineraria*(the common name of plant is long tree) is a species of floweringtree in the pea family, Fabaceae. It is native to arid portions of Western and the Indian Subcontinent, including Afghanistan, Iran, India, Oman, Pakistan, Saudi Arabia, the United Arab Emirates, and Yemen. *P. cineraria* is a small tree, ranging in height from 3 – 5 m. Leaves are bipinnate, with 7 – 14 leaflets on each of 1 – 3 pinnae. Branches are thorned along the internodes. Flowers are small and creamy-yellow, and followed by seeds in pods. The tree is found in extremely arid conditions, with rainfall as low as 150mm annually; but is indicative of the presence of a deep water table. It is a beneficial forage for sheep, goat and camel [17]) appeared to have potential for effective wind erosion control; some hazardous waste sites have large areal expanses of the contaminated and severely degraded soil. Reclamation and revegetation of these soils will reduce wind and water erosion and subsequent dispersal of contaminated soil as well as promote restoration of local ecosystem [18].
This study aims (1) to investigate the remediation potential of *P. cineraria* in Pb-Zn contaminated soils; (2) to identify the impacts of application of different concentrations of EDTA and DTPA on the phytoextraction efficiency of plant species and recognize the optimum chelator dosage; and (3) to consider the effects of treatment time on the phytoextraction of Pb and Zn contaminated soils.

2. Materials and methods

2.1. Soil characterization

The tested soil (sandy loam texture, hydrometer method) [19] was collected from agricultural fields in the University of Zabol (located in Sistan and Baluchistan province, Iran). Surface (0–30 cm) soil samples which were ground to pass through a 2mm mesh were used in the pot-culture experiments. Characteristics of the soil are listed in Table 1. Soil CEC (Cation Exchange Capacity) was measured by the method of Bower and Hatcher [20]; pH 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) [21]; electrical conductivity (ECe) using an EC-meter was estimated (DDS-307, Shanghai, China) [22]; total soil N was analyzed calorimetrically with a continuous flow ion analyzer followed by wet digestion in sulfuric acid [23]; organic carbon was measured by the Walkley-Black method [24]. The $CaCO_3$ equivalent was determined by neutralizing with HCl and back titration with NaOH [25]. The concentration of Pb and Zn extractable with 1M ammonium acetate EDTA (pH 4.60) was below detectable range.

2.2. Pot preparation and metal content analysis

After sieving (4mm), 2kg of dried soil were stored in plastic pots (20cm×15cm). Two days later, the soil was spiked with Pb ($PbNO_3$) 450mgkg^{-1}and Zn ($ZnSO_4$) 450mgkg^{-1}and then, they were mixed thoroughly (solutions were made out of the Pb and Zn salts and then the solutions mixed into the soil). The soil was then allowed to equilibrate for two weeks in the greenhouse.
Seeds of the plant were purchased from agricultural and natural resources research center in Sistan and Baluchistan province, Iran. In all treatments, 10 seeds of the plants were buried evenly throughout each pot at least1 to 2cm distance from the edge (April, 2014) and the pots were placed in the greenhouse (University of Zabol) with the environmental conditions, temperature of 25±5°C, humidity of 60% and moisture content of 70% water-holding capacity (weight of pot and wet soil was 2560g). After seeds germination, in each pot, five uniform seedlings were retained and the others harvested. When the plants had been growing for 30days (May, 2014), the seedlings were harvested at the end of growing trial. The plants were separated into root and shoot. Plant organs were washed before conducting the analysis and samples were baked at 70°C to a constant weight for approximately 48h and ground into fine powder in an agate mortar. Metals were analyzed after the mineralization of 400mg dry matter of shoots and roots in a microwave oven (model: MEMMERT UNB 400, Germany) with 5ml of nitric acid (69% v/v), 5ml deionized water and 2ml H_2O_2 (30% v/v). The digest was made with 25 ml final volume with the deionized water, filtered (0.45 mm, millipore) and then, analyzed for Pb and Zn using ICP/OES (Inductively Coupled Plasma/Optical Emission Spectometry) (model: KONIK (WON 300) BURKE, Barcelona, Spain).
Dried soil samples were passed through a 2mm diameter sieve. About 100mg dry soil was digested with HNO_3 and HCl (3:1) in a microwave oven. After mineralization, the samples were diluted, filtered and analyzed using ICP/OES. Metal concentrations of soil samples were measured as described for the plant samples.
As second step, to recognize the effects of EDTA and DTPA on phytoremediation efficiency of *P. cineraria*, seedlings of the plant were placed throughout each pot (July, 2014) and two chelator solutions were added to

the soil (after seeds germination). EDTA (disodium salt dehydrate of EDTA (C_{10} H_{14} N_2 Na_2 $O_8.2H_2O$) and DTPA ($(HO_2C_2H_2)_2NC_2H_4$)-$NC_2H_3O_2$) solutions were prepared at concentrations of 1.5, 3, 6, 9mmolkg^{-1} soil. The control pots were prepared at the same levels of spiked heavy metal concentration with no EDTA and DTPA (C). Plants were harvested after 30 days of adding chelator solutions (August, 2014) and dissected in roots and shoots to recognize the different bioaccumulation capabilities and optimum chelator dosage.
As third step, the plant was treated with the most optimum dosage of chelating agents for the highest heavy metal uptake for 60, 90 and 120 days, respectively and at the end of each period, the plants were harvested and trace elements analysis in the plants was performed with ICP/OES (KONIK (WON 300) BURKE, Barcelona, Spain). The chelating agents were added at one time and then sampled the plants at different times after the addition of the chelating agents. In order to determine heavy metals concentrations (the soluble form of the heavy metals in the soil) in the plant organs and soil samples, the sequential extraction technique by Du Laing et al. [26] was used. The methodology for metal concentrations in soil was referenced using the SRM 2711 (Institute of Standard and Technology, USA, Bureau Drive Stop 1070 Gaithersburg) and methodology for metals concentration in plant was referred using BCR-060 (Institute for Reference Materials and Measurements, Geel, Belgium). All the analyses were performed in five replicates.

2.3. Calculation and statistical analysis
The bioconcentration factor (BCF) and translocation factor (TF) were calculated to determine the heavy metal phytoextraction efficiency [27; 28]: BCF= heavy metal concentration in the harvested plant material (mg kg^{-1})/heavy metal concentration in the soil (mg kg^{1}), TF= heavy metal concentration in the aerial plant (mgkg^{-1})/heavy metal concentration in the root (mg kg^{-1}) [29].
All experimental results were statistically analyzed using the SPSS 18. package. Data in the text were expressed as means±standard error. The statistical significance of the differences between groups was evaluated by analysis of variance (ANOVA).Duncan t-test for the means was calculated only if F-test was significant at the 0.05 level of probability. A probability of 0.05 or lower was considered as significant.

3. Results and discussion

3.1. Evaluation of heavy metals removal efficiency before application of chelating agents
Data shown in Table 2 indicated low root metal concentrations for *P. cineraria* as compared to metal concentrations found in the aerial part. In general, the Pb level decreased in the order of shoot > soil > root.
The level of Zn in *P. cineraria* shoot exceeded the level of roots' Zn while the level of Zn in the root was significantly higher in the soil. Zn content occurred in the sequence of shoot> root> soil (Table 2). The decreasing trend of metal concentrations in both root and shoot was Zn> Pb.

Table 1: General properties of soil samples were collected for the greenhouse treatments

Texture	CEC(meq)	N(%)	OC(%)	EC(dSm^{-1})	pH	$CaCO_3$(%)	Pb(mg kg^{-1})	Zn(mgkg^{-1})
Clay loam	36.00	0.14	0.15	3.55	8.30	11.00	ND (0.02>)	ND (0.002>)

*Soils were sampled from 0 to 30 cm depth with a 5.5-cm-diameter hand-driven corer.
ND= NOT Detected/Below detectable range.

Table 2: Concentration of Pb and Zn(mgkg^{-1}) in the plant organs before application of chelating agents

Metals	Pb(mgkg^{-1})	Zn(mgkg^{-1})
Shoot	30.72 ± 0.82^{a}	150.11 ± 4.15^{a}
Root	21.04 ± 0.74^{c}	89.56 ± 2.09^{b}
Soil	29.33 ± 1.04^{b}	74.21 ± 1.17^{c}
sig	0.05^{*}	0.00^{**}

Mean values are reported with SE (Standard Error). Values within a column followed by the different letter indicate significant difference ($p<0.05$, post hoc Duncan test) (r=5, n=5).* and** means significant at 5% and 1% level respectively.

Depending on the ability of plants used as a phytoremediation to accumulate and tolerate heavy metals, plants are classified into three categories of hyperaccumulators, indicators and excluders [30]. Hyperaccumulators can accumulate very high metal concentrations in their aerial tissues, besides normal levels found in most species. Indicators can uptake and transport heavy metals to aerial tissues regulatory, so that tissue concentrations are proportional to environmental concentrations [30]. Excluders can restrict uptake and transport of elements between roots and shoots, maintaining low metal levels inside plant body over a wide range of external

concentrations [31]. However in the plant species the concentration of Pb was low to consider phytoremediation, Zn level was above the phytotoxic range indicating the plant species as an indicator plant.
Results in Table 3 indicate that bioconcentration factor (BCF) of the plant species followed the sequence of Zn> Pb. The bioconcentration factor (BCF) of Pb ranged from 0.47 and 0.76 in the root and shoot, respectively and the bioconcentration factor of Zn in the root and shoot was 2.02 and 3.02, respectively (Table 3). In particular, BCF_{shoot} values were higher than BCF_{root} indicating that the accumulation of heavy metals in the shoot is higher than the root. Plants with BCF_{shoot} values >1 are accumulators while plants with BCF_{shoot} values <1 are excluders [31]. The results showed that the plant species had the potential for use as an accumulator and the BCF_{shoot} values of >1 indicate high efficacy in the phytoextraction of metal-contaminated soils. However, the concentration of Pb accumulated by the plant was low to consider phytoremediation of Pb (30-300 $mgkg^{-1}$ Pb) and Zn concentration was above the phytotoxic range (100-400$mgkg^{-1}$ Zn) in the plant species[32].
The calculated translocation factors (TF_{Pb} =1.46 and TF_{Zn} =1.63) generally indicate movements of Pb and Zn from the soil to the roots (Table 3) and the results showed that *P. cineraria* would be effective as an accumulator. Zu et al. [33] reported that TFs> 1 were found in metal-accumulating plants whereas they are typically <1 in metal-excluding plants.

Table 3: Bioconcentration factor and translocation factor of *P. cineraria* before application of chelating agents

Metals	BCF_{shoot}	BCF_{root}	TF
Pb	$0.76\pm0.02^{a**}$	$0.47\pm0.02^{b**}$	$1.46\pm0.03^{a(n.s)}$
Zn	$3.02\pm0.07^{a**}$	$2.02\pm0.05^{b**}$	$1.63\pm0.03^{a(n.s)}$

Mean values are reported with SE (Standard Error). Values of BCFs within a row followed by different letters indicate significant difference and values of TFs in a column followed by the same letter do not differ significantly ($p<0.05$, post hoc Duncan test) (r=5, n=5).** means significant at 1% level. n.s means non significant.

3.2. Metals concentration in plant organs and soil after chelating agents application
A gradual increase in EC and available metals' content was observed with increasing concentration of EDTA and DTPA (Table 4). A slight decrease in pH was observed with the addition of chelating doses to the soil. The ability of chelating agents to increase concentration of metals in soil solution is influenced by a number of factors including concentration of metals and chelating agents, presence of competing cations, soil pH, adsorption of free and complexed metals onto charged soil particles and the formation constant of metal–ligand complexes. When chelating agents applied at high concentrations, they have the potential to affect the release of metals from solid phases by forming the dissolved complexes. The formation of metal–chelating agent complexes in soil solution may shift precipitation and sorption equilibrium towards the increased dissolution of metals [34]. In addition, some chelating agents significantly enhance mobilization of metals by plants [35]; therefore, metal uptake can be affected by the application of chelating agents due to low acidity. Some studies have illustrated that pH and EC are important in extraction and uptake of metals by plants. Mossop et al. [36] in their study on the effects of EDTA on the fractionation and uptake by *Taraxacum officinale* showed that pH of the soil leachates was initially lower than that of the EDTA solution added (pH=7.0) due to buffering by the soil. Tandy et al. [37] reported that Pb extraction by EDTA depends on soil pH and shows a strong positive relation up to a soil pH of 6.0.
Treatment of soil with the chelating agents increased the mobility of target metals in the soil solution (Table 4) and the maximum extractable metals were observed in 9 $mmolkg^{-1}$EDTA and 9 $mmolkg^{-1}$DTPA treatments. With respect to non-significant difference between 9 $mmolkg^{-1}$EDTA and 6 $mmolkg^{-1}$EDTA treatments and between 6 $mmolkg^{-1}$DTPA and 9 $mmolkg^{-1}$DTPA, low doses (6$mmolkg^{-1}$) were used in third step of the pot experiment. It should be considered that long-lived chelating agents such as EDTA are inappropriate for being used in the enhanced phytoextraction; its longevity will cause the elevated metal mobility even after harvesting plants [38]. Hence, although the concentration of metals increased with the increase of chelating agent concentration, the application of higher dose of EDTA/DTPA to metals-contaminated soils may be of environmental concerns because of the increased risk of groundwater contamination via metal leaching [39; 40].The bioconcentration factors (BCFs) in roots and shoots of *P. cineraria* showed that all levels of chelating agents enhanced BCF in the roots and shoots of the plant significantly (Table 5).The greatest bioaccumulation capacity in EDTA and DTPA treatments was observed in 6 $mmolkg^{-1}$EDTA (4.25) and 9 $mmolkg^{-1}$DTPA (2.42), respectively. The level of metal BCF decreased from 6 $mmolkg^{-1}$EDTA to 9 $mmolkg^{-1}$EDTA. However, the increase in the level of metal BCF was observed from 1.5 $mmolkg^{-1}$EDTA to 6 $mmolkg^{-1}$EDTA; the increase was not always significant. Similarly, it was found for DTPA.

The results shown in table 5 indicated that the application of chelating agents increased the translocation factors (TF), However, TF values decreased from 6mmolkg^{-1} EDTA to 9 mmolkg^{-1}EDTA in both cases of Pb and Zn. TF of the metals indicated significant difference upon the addition of 3mmol kg^{1} EDTA and DTPA. The minimum TF was calculated for both metals in the control treatment, and the maximum (2.73) TF was observed for 6EDTA treatment in case of Zn.

In most hyperaccumulators of metals, the harvested plant materials to soil ratio of metal concentration is often greater than 1 [41]. In the study, this ratio was greater than 1 or nearly 1 in the plant species found to be better metal accumulators. In addition to the bioconcentration factors, one of the important factors for selecting the accumulator species is translocation factor. Low levels of the factor show

the potentials of plant to accumulate metals in the underground organs. Data obtained for TF_s indicated that EDTA and DTPA increased the factor. Zhao et al. [42] reported that EDTA and DTPA had approximately the same effects on the Pb content in shoots of ryegrass. Peñalosa et al. [43] showed that increasing the doses of complexing agent EDTA significantly increased the concentration of soluble element (Pb).

Table 4: Soil pH, EC and metals concentration after application of chelating agents

Treatments	Ph	EC (dSm^{-1})	Pb_{soil} (mgkg^{-1})	Zn_{soil} (mgkg^{-1})
Control	8.30±0.01^{a}	3.55±0.01^{b}	32.42±2.16^{d}	77.86±5.53^{d}
1.5EDTA	8.10±0.01^{ab}	3.60±0.01^{b}	76.65±5.66^{c}	120.12±11.52^{c}
3EDTA	7.65±0.01^{b}	4.77±0.01^{a}	120.08±12.31^{b}	158.28±11.39^{b}
6EDTA	7.60±0.01^{bc}	4.82±0.01^{a}	160.11±13.73^{a}	195.94±13.46^{a}
9EDTA	7.40±0.01^{c}	4.91±0.01^{a}	170.22±14.05^{a}	236.57±15.42^{a}
Sig	0.05*	0.05*	0.00**	0.00**
C	8.40±0.01^{a}	3.55±0.01^{b}	30.54±2.00^{d}	75.33±4.12^{d}
1.5DTPA	7.80±0.01^{b}	3.75±0.01^{b}	65.15±4.06^{c}	90.75±4.67^{c}
3DTPA	7.80±0.01^{b}	3.80±0.01^{b}	108.66±8.19^{b}	130.53±5.11^{b}
6DTPA	7.60±0.01^{bc}	4.40±0.01^{a}	129.42±10.64^{a}	155.63±6.25^{b}
9DTPA	7.42±0.01^{c}	4.72±0.01^{a}	132.63±11.37^{a}	194.04±6.25^{a}
sig	0.05*	0.05*	0.00**	0.00**

Mean values are reported with SE (Standard Error). Values within a column followed by the same letter do not differ significantly ($p<0.05$, post hoc Duncan test) (r=5, n=3).
1.5EDTA, 3EDTA, 6EDTA, 9EDTA= 1.5mmolkg^{-1}, 3mmolkg^{-1},6mmolkg^{-1}, 9mmolkg^{-1} EDTA respectively.
1.5DTPA, 3 DTPA, 6 DTPA, 9 DTPA = 1.5mmolkg^{-1}, 3mmolkg^{-1}, 6mmolkg^{-1}, 9mmolkg^{-1} DTPA respectively.
*means significant at 5% level, ** means significant at 1% level

Table 5: Bioconcentration factor (BCF) and translocation factor (TF) after application of chelating agents.

Treatments	BCF_{Zn} Shoot	BCF_{Zn} Root	BCF_{Pb} Shoot	BCF_{Pb} Root	TF Zn	TF Pb
Control	3.02±0.02^{c}	2.00±0.01^{d}	0.75±0.01^{c}	0.44±0.01^{d}	1.60±0.02^{c}	1.39±0.02^{d}
1.5EDTA	3.22±0.02^{bc}	2.41±0.01^{bc}	0.81±0.01^{bc}	0.53±0.01^{d}	1.72±0.02^{b}	1.43±0.02^{cd}
3EDTA	3.70±0.02^{b}	2.36±0.01^{b}	1.56±0.01^{b}	0.75±0.01^{c}	1.84±0.01^{b}	1.56±0.01^{bc}
6EDTA	4.25±0.03^{a}	2.60±0.02^{a}	1.83±0.02^{a}	0.90±0.01^{b}	2.53±0.01^{a}	2.00±0.01^{a}
9EDTA	3.68±0.03^{b}	2.54±0.02^{a}	1.71±0.02^{a}	1.42±0.01^{a}	2.40±0.01^{a}	1.74±0.01^{b}
sig	0.05*	0.05*	0.04*	0.01*	0.05*	0.05*
C	0.88±0.01^{c}	0.74±0.01^{b}	0.70±0.01^{c}	0.40±0.01^{c}	0.92±0.01^{c}	0.84±0.01^{c}
1.5DTPA	1.07±0.01^{c}	0.89±0.01^{b}	0.83±0.01^{b}	0.52±0.01^{b}	1.21±0.01^{b}	1.07±0.01^{c}
3DTPA	1.55±0.01^{b}	0.97±0.01^{b}	1.04±0.01^{ab}	0.60±0.01^{b}	1.35±0.01^{b}	1.22±0.01^{b}
6DTPA	2.00±0.01^{a}	1.42±0.02^{a}	1.20±0.01^{a}	0.73±0.01^{a}	1.61±0.01^{a}	1.36±0.01^{ab}
9DTPA	2.42±0.01^{a}	1.56±0.02^{a}	1.31±0.01^{a}	0.96±0.01^{a}	1.70±0.01^{a}	1.49±0.01^{a}
sig	0.05*	0.05*	0.05*	0.05*	0.05*	0.05*

Mean values are reported with SE (Standard Error). Values within a column followed by the same letter do not differ significantly ($p<0.05$, post hoc Duncan test) (r=5, n=3). *means significant at 1% level.
1.5EDTA, 3EDTA, 6EDTA, 9EDTA= 1.5mmolkg^{-1}, 3mmolkg^{-1},6mmolkg^{-1}, 9mmolkg^{-1} EDTA respectively.
1.5DTPA, 3 DTPA, 6 DTPA, 9 DTPA = 1.5mmolkg^{-1}, 3mmolkg^{-1}, 6mmolkg^{-1}, 9mmolkg^{-1} DTPA respectively

3.3. Impacts of treatment concentration on the biomass production

After chelating agents addition into the soil, there were some brown dots on the leaves and the leave became yellow indicating phytotoxicity of EDTA and DTPA. The plants grown on DTPA amended soil exhibited

significantly higher dry weights than those determined for EDTA treatments, but the differences among all treatments were not significant (Table 6).
Vassil et al. [44] reported that the addition of 3 and 6mmol EDTA kg^{1} did not significantly influence biomass production of maize grown in the studied soils as compared to the control. The only statistically significant decrease in maize biomass yield was observed in soils after the addition of 9mmol EDTA kg^{-1}. They suggested that the growth reduction after the 9mmol EDTA kg^{-1} treatment is probably due to high contents of heavy metals mobilized to the soil solution and to some extent, due to the toxicity of free EDTA if present.
Turgut et al. [45] reported that the EDTA level resulted in a higher total metal uptake but high concentrations of EDTA are toxic for plants and ultimately, reduce plant biomass and concentrations of metals in the shoot. Cell membranes of the root tissues might be damaged by the chelants at a threshold concentration of above 10mmol chelant kg^{-1} [39; 46].

Table 6: Effect of treatment concentration on dry weight of *P. cineraria*

Treatment		Dry weight (g)	Treatment		Dry weight (g)
EDTA ($mgkg^{1}$)	Control	18.76 ± 3.09^{a}	DTPA ($mgkg^{1}$)	Control	17.65 ± 3.00^{a}
	1.5	15.45 ± 2.55^{a}		1.5	16.27 ± 3.00^{ab}
	3	10.42 ± 1.74^{b}		3	12.38 ± 1.55^{bc}
	6	8.50 ± 1.56^{c}		6	10.74 ± 1.36^{c}
	9	7.67 ± 1.23^{c}		9	9.07 ± 1.16^{c}

Mean values are reported with SE (Standard Error). Different letters in each column indicate significant differences between treatment concentrations ($p<0.05$, post hoc Duncan test) (r=5, n=3).
*means significant at 1% level.

3.4. Impacts of treatment time on the biomass production and metals concentration
Mean values of total dry weights subjected to different treatment times (60d, 90d and 120d after the chelating agents application) are reported in Table 7. Dry weight of *P. cineraria* decreased significantly ($p<0.05$) as time passed. However, maximum dry weight was observed 60d after the application (10.14g and 8.32g for DTPA and EDTA treatments respectively); no significant difference was seen between dry weights of the plant in days of 90^{th} and 120^{th}.
It can be concluded that chelating addition may affect plant growth with the passage of time because of higher available metals in the soil. As a result, shorter treatment time should be adopted for high Pb-Zn contaminated soils. Wang et al. [12] reported that the shoots of *Sedum alfredii* in 14^{th} day for low Pb soil and in 10^{th} day for high Pb soil could achieve the highest phytoextraction effects. The authors cited that EDDS addition may affect plant growth significantly with the passage of time, especially for high Pb soil because of higher available Pb in soil.

Table 7: Effect of treatment time on dry weight of *P. cineraria*

	Treatment time			
	60d	90d	120d	
	Dry weight (g)	Dry weight (g)	Dry weight (g)	sig
6EDTA	8.32 ± 1.21^{a}	5.54 ± 1.12^{b}	5.00 ± 1.11^{b}	0.05*
6DTPA	10.14 ± 1.37^{a}	9.00 ± 1.25^{ab}	7.32 ± 0.90^{b}	0.05*

Mean values are reported with SE (Standard Error). Different letters in each row indicate significant differences between treatment times ($p<0.05$, post hoc Duncan test) (r=5, n=3).
6EDTA= 6mmolkg^{-1}EDTA, 6 DTPA= 6mmolkg^{-1} DTPA.
*means significant at 5% level.

Data in table 8 indicated that chelating addition did not increase metals concentration in the roots and shoots of *P. cineraria* with passage of time. Results showed that harvesting the shoots of *P. cineraria* in 60th day for Zn and Pb soil could achieve the highest phytoextraction effects. It was found that concentration of Zn and Pb in the soil solution decreased gradually with the passage of time. Soil Zn and Pb under the chelating application decreased in day 120, but there was no significant difference in the metals reduction between the days 90 and 120.

In general, harvest time as a suitable dose of chelating agents is a crucial factor concerning the effectiveness of phytoextraction [12] and there is still a lack of information about the exact timing of the harvest after the application of chelating agents. In this way, Chiu et al. [47]reported that Cu intake in *Vetiver* shoots under HEIDA application reached its maximum in 16th day. In present study, treatment time dependent experiment showed that harvesting the shoots of the plant in 60th day after the first harvest could achieve the highest phytoextraction efficiency. In the experiment of Wu et al.[48] the concentration of DTPA-extractable Pb in soil decreased with increasing the extraction time from 6 to 12h.

Table 8: Effects of treatment time on the metals concentration in soil and tissues of *P. cineraria*.

Treatments	Metals	Soil/Plant organs(mgkg^{-1})	Day			
			60	90	120	sig
6EDTA	Zn	Shoot	234.09±13.14^{a}	210.42±12.10^{b}	194.70±11.18^{b}	0.05*
		Root	195.28±9.09^{a}	184.07±8.14^{ab}	173.29±8.11^{b}	0.05*
		Soil	164.36±10.17^{a}	160.24±10.13^{ab}	157.00±10.00^{b}	0.03*
	Pb	Shoot	180.29±9.00^{a}	175.29±8.71^{ab}	170.25±8.16^{ab}	0.04*
		Root	144.13±6.53^{a}	132.61±6.17^{ab}	127.92±6.05^{b}	0.05*
		Soil	160.35±7.33^{a}	149.29±7.00^{b}	140.22±6.47^{b}	0.04*
6DTPA	Zn	Shoot	196.51±9.05^{a}	182.92±9.04^{ab}	176.27±8.87^{b}	0.03*
		Root	165.37±8.33^{a}	154.28±8.23^{ab}	144.58±8.10^{b}	0.05*
		Soil	147.15±7.14^{a}	134.27±7.11^{ab}	127.19±7.09^{b}	0.05*
	Pb	Shoot	154.32±9.92^{a}	147.09±9.62^{ab}	136.28±9.47^{b}	0.05*
		Root	124.66±7.11^{a}	119.37±7.00^{ab}	115.43±7.00^{b}	0.04*
		Soil	132.18±7.21^{a}	125.16±7.15^{b}	120.05±7.14^{b}	0.03*

Mean values are reported with SE (Standard Error). Values within a row followed by the same letter do not differ significantly ($p<0.05$, post hoc Duncan test) (r=5, n=3).
6EDTA= 6mmolkg^{-1}EDTA, 6 DTPA= 6mmolkg^{-1} DTPA.
*means significant at 5% level.

Conclusion

1. Results revealed that *P. cineraria* had shoot concentrations of metals that were greater than those in the root indicating that the plant species had the potential for being used as an accumulator and it had high efficacy in the phytoextraction of metals-contaminated soils.
2. However, the concentration of Pb accumulated by the plant was low to consider phytoremediation of Pb, Zn concentration was above the phytotoxic range of the plant species.
3. Application of EDTA and DTPA enhanced metals uptake in of *P. cineraria* due to their greater bioavailability
4. At higher doses of EDTA and DTPA, the chelating agents may cause the contamination of groundwater resources and may also exhibit their phytotoxic effects; it is suggested that they may be applied only for metal specific cleaning of soil and lower doses should be used.
5. There were no significant differences between 6 mmolkg^{-1}EDTA and 9 mmolkg^{-1}EDTA treatments, the 6mmol dose of EDTA seemed optimum to enhance the efficiency of plant species. Similarly, it was demonstrated that for DTPA, 6 mmolkg^{-1}DTPA was the most effective dose of the treatment in increasing the solubility of Pb and Zn in the contaminated soils.
6. Treatment time dependent experiment showed weak relationships between the treatment time and remediation of contaminated soils and the maximum remediation can be done 60 days after the plant cultivation.
7. Results showed that *P. cineraria* had the potential for phytoextraction of metal-contaminated soils. The use of phytoextraction, however, raises the concerns about the transfer of contaminants to the broader ecosystem; thus, *P. cineraria* should not be used because it increases the diffusion of heavy metals through grazing the animals and wind erosion due to its considerable BCF in above ground organs unless the biomass containing the accumulated metals is removed for disposal.

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