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ORIGINAL ARTICLE

Phytoremediation of Lead and Cadmium Contaminated Soils using Sunflower Plant

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Phytremediation has emerged as a practical approach to clean up metal-polluted soils. In this study the role of sunflower (*Helianthus annuus* L.) plants as a potential phytoremediator to soils contaminated with cadmium (Cd) and lead (Pb) was investigated. Our results showed that the effect of Cd was stronger on the growth of the roots, while the effect of Pb was stronger on the shoots of sunflower seedlings. At the physiological level, Cd treatment was found to induce low levels of lipid peroxidation and membrane leakage with less affected photosynthesis in the leaves of the treated sunflower seedlings compared to the effects of Pb. The results presented here showed that a high amount of the total absorbed Cd (88.84%) was accumulated in roots, while a high amount of the total absorbed Pb (71.39) was tranlocated to shoots of sunflower seedlings. Similar trends of Cd and Pb allocation between roots and shoots at the yield stage were recorded. We suggest here that sunflower plants may remediate Cd contaminated soils through phytostabilization, while may remediate Pb contaminated soils through phytoextraction. Finaly, the trace amounts of Cd and Pb that were accumulated in seeds recommends sunflower plants to be used safely and economically for cleaning up soils contaminated with Cd and/or Pb.

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Metal pollution has become one of the most severe environmental problems today as a consequence of increasing environmental pollution from human activities such as mining and smelting of metals, electroplating, gas exhaust, fuel production, fertilizer, sewage and application of pesticides, etc (Kabata-Pendias and Pendias, 1989; reviewed by Ben Ammar *et al*., 2007).

Heavy metals are known to have harmful effects on plant growth and soil microflora leading to big losses in plant productivity (Roy *et al*., 2005). Heavy metals have been shown, especially in non tolerant plant species, to affect a wide range of plant cellular activities including photosynthesis, respiration, mineral nutrition, membrane structure and function, and gene expression (Maksymiec , 1997; Prasad, 1999; Peixoto *et al*., 2001; Hall, 2002; reviewed by Nouairi *et al*., 2006).

Cd, Cr, Cu, Hg, Pb and Ni represent the most common heavy metal contaminants (Yan-de *et al*., 2007). Cadmium (Cd) and lead (Pb) gained more attention because of their widespread and high toxicity to plant functions (Öncel *et al*, 2000). Cd toxicity is a major factor limiting plant growth (Sandalio *et al*. 2001; Fediuc and Erdei 2002). It has severe effects on the chloroplast functions and structures and affects chlorophyll contents (Krupa and Baszynski, 1995). In addition, high concentrations of Cd cause oxidative stress which is related to lipid peroxidation of cellular membranes (Verma and Dubey, 2003; reviewed by Andrade *et al*., 2008).

Lead is one of the main sources of environmental pollution. Many studies have shown that lead inhibits metabolic processes such as nitrogen assimilation, photosynthesis, respiration, water uptake, and transcription (Kurepa *et al*., 1997; Boussama *et al*., 1999). Lead may inactivates various enzymes by binding to their SH-groups (Rauser, 1995), and can intensify the processes of reactive oxygen species (ROS) production leading to oxidative stress (Cuypers *et al*., 1999; Prasad *et al*., 1999). In addition, lead can negatively affect mitochondria structure by decreasing the number of mitochondrial cristae, which in turn can lower the capability of oxidative phosphorylation (reviewed by Malecka *et al*., 2001).

Excessive heavy metal concentration in the environment pose significant hazard not only to plants, but also to animal and human health because of their cytotoxic, mutagenic, and cancerogenic effects (Shevyakova *et al*., 2008). Due to the potential toxicity and high persistence of metals, soils polluted with heavy metals represent a serious environmental problem that requires an effective and reasonable solution. Methods of environmental restoration of metal-polluted soils using a plant-based technology have attracted increasing interest since it was proposed by Chaney (1983) as a technology for reclaiming metal polluted soils. In this regard, phytoremediation has been developed as a remediation method of contaminated soils with more cost-effective and fewer side effects than physical and chemical approaches. (Nascimento and Xing, 2006; Lone *et al*., 2008).

Mechnisms for heavy metal phytoremediation include: 1) phytostabilization; immobilising the heavy metals within the rhizosphere, so that they neither leach nor enter the plant shoots, 2) phytovolatilization; using the plants to take up the contaminating heavy metals and translocate them into the shoots, where they may be volatilized, or 3) phytoextraction; the use of plants to remove metals from soils and to transport and concentrate them in above-ground biomass; then the heavy metals are removed from the site when the plants are harvested (McGrath *et al*., 2002; Nascimento and Xing, 2006). Phytoextraction appear to be the most promising technique that has received increasing attention from researchers as a technology for remediating heavy metal polluted soils (Robinson *et al*., 2009)

It was reported that more than 400 plant species have been acknowledged to have potential for soil and water remediation (Lone *et al*., 2008). Among wild plant species, a specific group of heavy metal hyperaccumulators was found to be capable of accumulating up to 1000 mg and more of heavy metals per 1 kg of dry weight in their shoots without any visible signs of damage (Salt *et al*., 1998). However, for a wide usage of plants in phytoremediation, plant species should be selected, which can not only accumulate high concentrations of heavy metals in their shoots but also grow actively producing large biomass in order to remove a great amount of metal (Shevyakova *et al*., 2008). In this concern, an ideal plant for metal phytoextraction from contaminated soil should possess certain characteristics including: 1) tolerance to high metal concentrations; 2) short life cycle and highly effective metal-accumulating biomass; 3) concentration of metals in the aboveground plant parts; and 4) easy harvest (Schwitzguebel *et al*., 2002; Nehnevajova *et al*., 2007). Moreover, phytoextraction will be more economically feasible if, in addition to metal removal, plants also produce biomass with an added value (Vassilev *et al*., 2004). For example, energy crops such as oilseed plants, fibers crops, and fragrance-producing plants could be used to produce these valuable products (Schwitzguebel *et al*., 2002). Accordingly, sunflower is a promising plant to remove heavy metals (Gallego *et al*., 1996; Van der Lelie *et al*., 2001; Lin *et al*., 2003; reviewed by Nehnevajova, *et al*., 2005)

In this study, we aimed at studying the role of sunflower plants in phytoremediating soils contaminated with the most common and toxic heavy metals; Cd and Pb.

MATERIALS AND METHODS

Growth conditions and heavy metal treatments

For seedlings stage, sunflower seeds (Sakha 53) were surface sterilized in 50% commercial bleach for 5 minutes and washed three times with distilled water. Then after, sterilized seeds were sown on nutrient free sand (washed with conc. H_2SO_4 and washed three times with distilled water) and irrigated with distilled water for 6 days (day 28±2°C day/ 23±2°C night) before heavy metal treatment. For yield stage, sunflower seeds were sown in plastic pots containing soil (60% clay and 40% sand) and placed in open environment at the green house. Two weeks old seedlings were treated with heavy metals.

Heavy metals treatments: Cadmium Chloride ($CdCl_2$) and Lead Nitrate ($Pb(NO)_3$) salts (both are water soluble) were used for cadmium and lead treatments. The sub-lethal concentration of both elements were determined via treating one week-old seedlings with a series of different concentrations (5, 10, 20, 40 and 80 mM of cadmium chloride and lead nitrate). Cadmium concentration of 10 mM and lead of 40 mM were selected to be used for further experiments. For yield stage, two week-old seedlings were watered a once with 0.5 mM $CdCl_2$ or 5 mM $Pb(NO)_3$. Then after, plants were irrigated with tap water till the end of the season.

Measurements

Growth criteria: After three days of heavy metal treatments (0, 5, 10, 20, 40 and 80 mM of $CdCl_2$ or $Pb(NO)_3$), seedling were removed carefully from the sand soil, washed thoroughly with tap water and divided into roots and shoots. Fresh weights and lengths of both roots and shoot were used as an indicator for studying the effect of both Cd and Pb on sunflower growth at seedling stage (one week-old).

Lipid peroxidation quantification: Malondialdehyde (MDA) content, as a measure of lipid peroxidation level, was determined by the method of Heath and Packer (1968). One gram of seedling leaves, collected two days after treatment, were extracted in 5 ml of 5% (w/v) trichloroacetic acid. The homogenate was then centrifuged at 12,000 rpm for 10 min. The supernatant (2 ml) was mixed with 2 ml of 0.67% (w/v) thiobarbituric acid, then incubated in boiling water bath for 20 min, and cooled immediately. MDA content was calculated using the extinction coefficient of 155 mM-1 by measuring the absorbance at 532 and 600 nm.

Membrane leakage measurement: A half gram of seedling leaves, collected 3 days after treatment, was cut into small pieces and soaked in 30 ml distilled water. Electrical conductivity (µS/cm) of the solution as a measure of the degree of membrane leakage was measured by an EC meter after one hour from soaking.

Photosynthetic efficiency measurement: A portable fluorometer was used for measuring the photosynthetic efficiency in intact leaves of sunflower seedlings. Fully expanded leaves from two weeks old seedling were used for the measurements. The fluorescence was measured in dark adopted leaves according to the instrument manual.

Heavy metal analysis: For seedling stage, the samples were collected three days after heavy metal treatments. Seedling were removed carefully from sand, washed thoroughly and divided into shoots and roots. Samples were oven dried at 80°C for a week. Dry samples were ground using mortar and pestles into fine powder. 0.3 g of dry sample was digested in a mixture of 5 ml H_2SO_4 and 1 ml $HClO_4$ in a 100 ml boiling flask at 200°C for 30 min on a hot plate (the solutions turned colorless). Before filtration, digestion products were diluted up to 30 ml with distilled water. For yield stage, when plant heads turned dry (about four months from sowing) plants were removed carefully from soil and divided into roots, shoots and Heads. Root and shoot samples were oven dried at 80°C for two week. Heads were air dried for two weeks and seeds were obtained. Dry plant parts were ground into fine powder using electric mile. 0.4 g samples were used for digestion as mentioned above (Chapman and Partt 1961).

RESULTS

Effect of Cd and Pb treatments on growth of sunflower seedlings

Sunflower seedlings (6 days-old) were treated with different concentrations of Cd and Pb to study the effect of these two heavy metals on sunflower growth and to determine the sub-lethal concentration of each metal. Our results in Table 1 show that low concentrations of Cd were highly toxic to sunflower seedlings compared to Pb that inhibited growth at higher concentrations. A concentration of 5 mM of Cd was enough to reduce the fresh weight of the sunflower seedlings to 64.09% of control, while the same concentration of Pb (5 mM) led to inhibiting the seedling fresh weight to only 94.17% of control. Results show also that the growth of sunflower seedlings was reduced to about 50% of control when treated with 20 mM Cd. On the other hand, a 50% inhibition in seedling growth was reached at 80 mM of Pb (Table 1). We did not record the seedling fresh weights at concentrations 40 and 80 mM of Cd because these concentrations were too lethal leading to killing the seedlings afterwards. Similar trends of Cd and Pb effect on seedlings length were recorded at different concentrations (see table 1). To investigate the differential effects of Cd and Pb on roots and shoots of sunflower seedlings, we measured their effects on roots and shoots separately. Data presented in table 1 show that at almost all concentrations the effect of Cd was stronger on roots than on shoots, while the effect of Pb on shoots was stronger than on roots. For example, the concentrations 10 mM of Cd and 40 mM of Pb that inhibited the fresh weight by about 60%, these concentrations led to reducing the roots and shoots fresh weights by different ratios. The concentration of 10 mM Cd led to reduction of root fresh weight by about 52% and shoot by about 40% of control, while 40 mM of Pb caused reduction of root by about 30% and shoot by about 42% of control. Finally, according to the recorded effects of different concentrations of Cd and Pb in Table 1, we selected the concentrations 10 mM of Cd and 40 mM of Pb as sub-lethal concentrations to be used for further analyses where both concentrations reduced the growth to about 60% of control.

Effect of Cd and Pb treatments on various stress markers in sunflower seedlings

To find some of the causes behind the growth reduction of sunflower seedlings treated with Cd or Pb, various stress markers, including lipid peroxidation level, degree of membrane leakage and photosynthetic efficiency, were measured under the treatment of 10 mM Cd or 40 mM Pb. Heavy metal stress like all stresses is known to cause the accumulation of reactive oxygen species in plants which lead to oxidative damage to macromolecules such as lipids and protein. We measured the peroxidation product of lipids; malondialdehyde (MDA), in leaves of sunflower seedlings treated with Cd or Pb. Data in Figure 1A show that Cd treatment led to increasing MDA content in the leaves of the treated seedlings by more than two times compared to control, while Pb treatment led to increasing MDA content by about three times compared to control. To confirm the harmful effect of Cd and Pb on membrane lipids, we measured membrane leakage of leaves from treated seedlings. Our results revealed that there is a significant effect of Cd and Pb on membrane leakage in the leaves of the treated seedlings with a prominent effect to Pb. Cd treatment led to increasing the membrane leakage by about 1.5 times compared to control, while Pb treatment caused an increase of membrane leakage by more than 4 times compared to control as shown in Figure 1B. As the photosysnthesis represents the most sensitive process to environmental stress in plant cells, we studied the photosysnthetic efficiency in the leaves of the Cd and Pb treated seedlings. Our results showed a slightly, but significant, inhibitory effect for Cd and Pb on photosynthesis with a higher effect for Pb compared to Cd (Figure 1C).

The allocation of Cd and Pb in sunflower plant parts

All of the previous results suggest that the toxic effects of Cd are more obvious on roots while the effects of Pb are more evident on shoots of sunflower seedlings. This trend may in turn suggest that the accumulation of Cd is higher in roots, while more Pb is translocated to shoots. To test this suggestion, the allocation of Cd and Pb between roots and shoots of the treated sunflower seedlings was investigated using atomic absorption spectroscopy. Indeed, results in Figure 2 show that large amount of Cd was accumulated in roots compared to shoots of treated sunflower seedling. On contrary, large amount of Pb was accumulated in shoots compared to roots. Table one show that the amount of Cd accumulated in roots of the treated seedling was 88.84% of the total Cd up taken, while the amount of Pb accumulated in the seedlings roots was only 28.61% of the total amount up taken by the whole seedling. Also, at the yield stage, plant roots accumulated 62.97% of the total Cd absorbed from soil, while only 31.77% of the absorbed Pb stayed in the roots (Table 1). Interestingly, data show that sunflower seedlings have the ability to uptake an amount of Cd (491.04 µg/g DW) higher about five times of what they can uptake of Pb (102.55 µg/g DW). Similarly, in the yield stage, sunflower plants were able to accumulate an amount of Cd (43.92 µg/g DW) higher about three times of the amount that they were able to accumulate of Pb (16.97 µg/g DW). Importantly, data show that the amount of Cd or Pb accumulated in sunflower seeds were very little compared to the total amount absorbed by the treated plant and similar to a mount detected in the control untreated plants. An amount of about 5 g/g DW Cd was detected in treated plants, where the amount detected in control untreated plants was 2.6 µg/g DW. On the other side, the amount detected of Pb in seeds of treated plants was 1.87 µg/g DW, where it was 1.1 µg/g DW in the seeds of control plants.

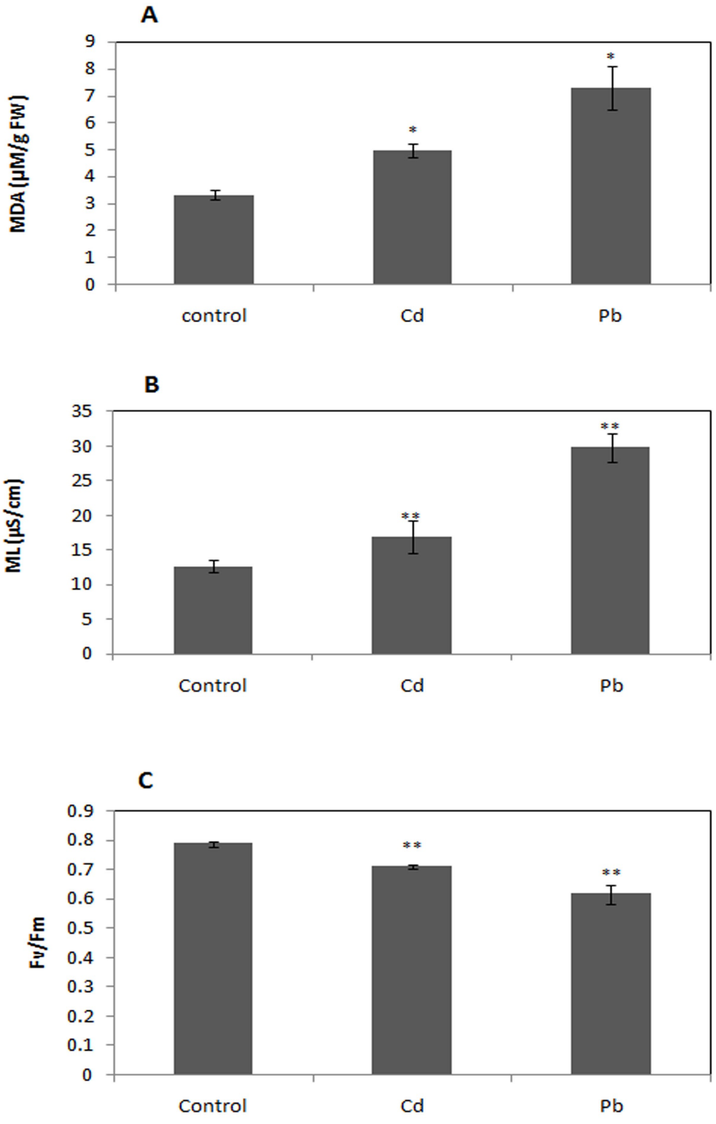

Figure 1 : A) Effect of heavy metal treatments (10mM Cd or 40 mM Pb) on lipid peroxidation level (as malondialdehyde content, MDA), B) membrane leakage (ML, µs/cm), C) photosynthetic efficiency (Fv/Fm). Data represents the average of three replicates. Error bars represent standard deviation. * significant at P<0.05, ** significant at p<0.01.

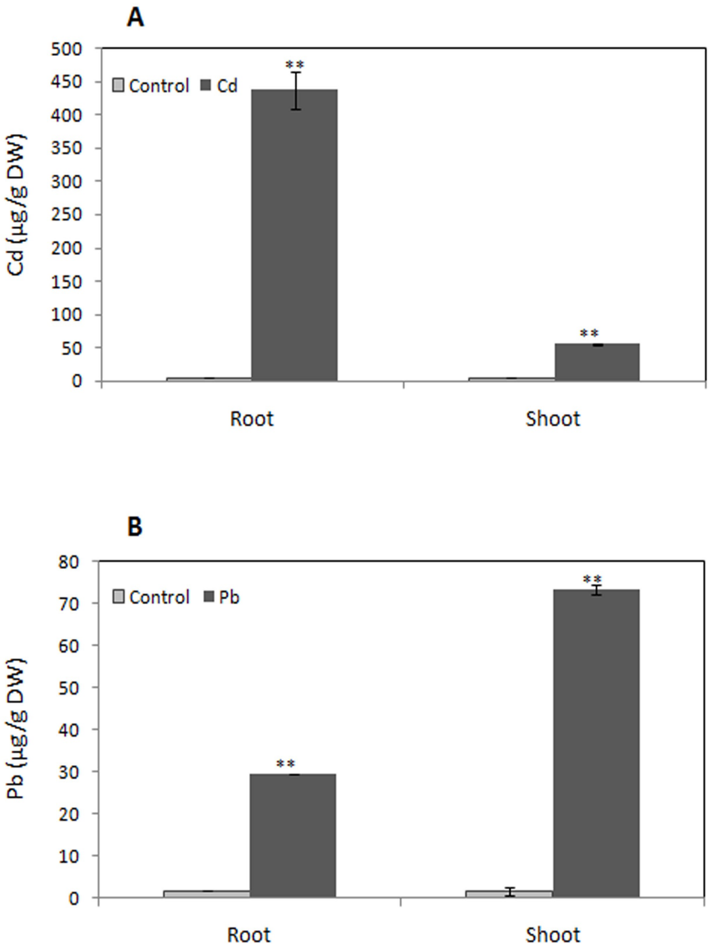

Figure 2 : Accumulation of heavy metals (µg/g dry weight) in Sunflower seedlings treated with 10 mM Cd (A) or 40 mM Pb (B). Heavy metal content was estimated using atomic absorbance spectroscopy. Data represents the average of three replicates. Error bars represent standard deviation. ** significant at P<0.05.

Table 1 : Effect of different concentrations of cadmium and lead on the fresh weight and length of sunflower seedlings. Values represent the percentage of the control.

Fresh weight (% of control)

	Cd			Pb	
Concentration (mM)	Root	Shoot	Seedling	Root	Shoot
5	60.42	64.84	64.09	97.96	93.39
10	47.92	60.62	**58.48**	92.52	92.69
20	40.28	51.76	49.82	87.76	76.51
40	NA	NA	NA	69.39	57.67
80	NA	NA	NA	53.88	50.63

Length (% of control)

	Cd			Pb	
Concentration (mM)	Root	Shoot	seedling	Root	Shoot
5	76.44	74.72	75.54	97.26	88.68
10	64.11	60.38	**62.16**	84.38	83.77
20	43.84	49.56	46.82	65.75	77.36
40	NA	NA	NA	63.56	62.89
80	NA	NA	NA	38.36	36.73

NA; not applicable as these high concentrations of Cd were too lethal to the growth of sunflower seedlings.

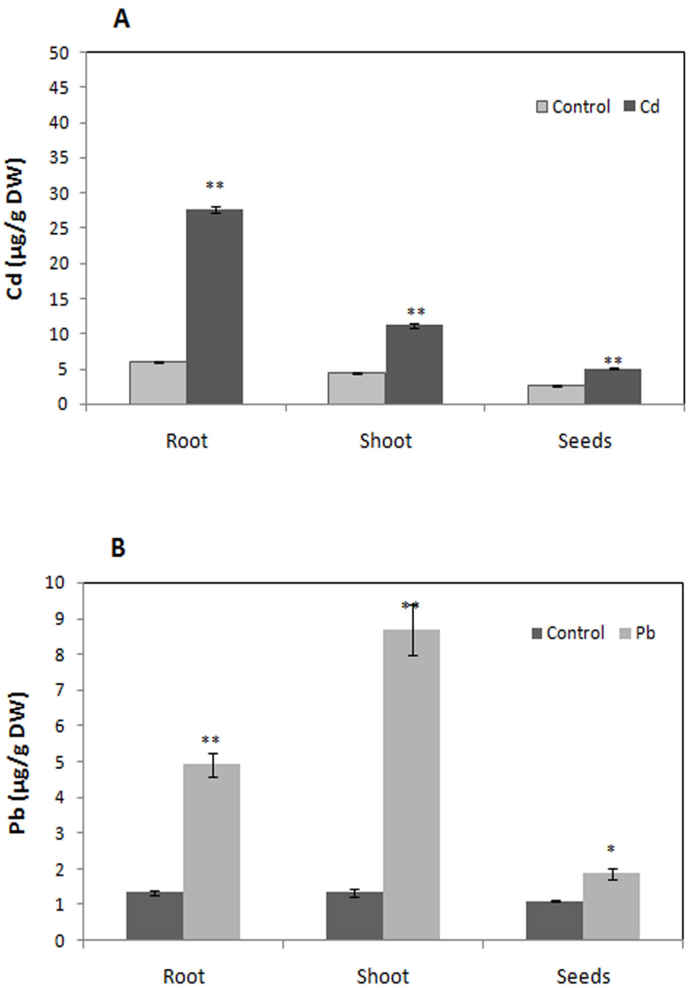

Figure 3 : Accumulation of heavy metals (µg/g dry weight) in Sunflower plants (yield stage) treated with 0.5 mM Cd (A) or 5 mM Pb (B). Heavy metal content was estimated using atomic absorbance spectroscopy. Data represents the average of three biological replicates. Error bars represent standard deviation. * significant at P<0.05, ** significant at p<0.01.

Table 2. Allocation of the up taken heavy metals between roots and shoots of sunflower seedlings/plants treated with Cd or Pb.

	Seedling stage		
	Total (µg/g DW)	Root (% of total)	Shoot (% of total)
Cd	491.04	88.84	11.16
Pb	102.55	28.61	71.39
	Yield stage		
	Total (µg/g DW)	Root (% of total)	Shoot (% of total)
Cd	43.92	62.97	25.66
Pb	15.47	31.77	56.11

DISCUSSION

The increase of heavy metal contamination in soils worldwide causes significant hazard to human, animal and plant health. Cleaning up the contaminated soils through phytoremediation has gained increasing attractiveness, because it is more cost-effective with fewer side effects than physical and chemical techniques. In this study we tested the ability of sunflower plants to phytoremediate soils contaminated with Cd and Pb. Sunflower, as an oilseed plant producing large biomass, was selected for this study. Thus, the large biomass can hold big amount of heavy metals per plant with an added value of oil production that could be used as food or for energy production.

Many studies had reported an inhabiting effect of various heavy metals, including Cd and Pb, on plant growth (Sandalio *et al*., 2001; Seregin *et al*., 2001; Sharma and Dubey 2005; Mishra *et al*., 2006). In agreement with these reports, our results showed that the growth of sunflower seedlings is reduced by Cd and Pb. However, our data showed that Cd and Pb differentially affect the growth of sunflower seedlings. The effect of Cd was stronger on the roots compared to its effect on shoots, while the effect of Pb on shoots was stronger than its effect on roots of sunflower seedlings (Table 1). In accordance with these results, and at the physiological level, Cd treatment was found to induce low levels of lipid peroxidation and membrane leakage in the leaves of the treated sunflower seedlings compared to the effects of Pb (Figure 1). In addition, the photosynthetic efficiency was found to be more affected by Pb treatment compared to Cd (Figure 1C). Similarly, Becerril *et al*. (1989) reported that low cadmium and relatively high lead concentrations considerably decrease net photosynthesis. Together, these results suggest that the recorded high effect of Pb on shoot compared to root growth was mainly because of its higher toxic effects on membrane lipids and photosysnthesis. Furthermore, these results confirm that the harmful effect of Cd was related mainly to its effect on roots, which in turn led to reduction of shoot growth. These results were also confirmed by measuring the heavy metals (Cd and Pb) contents in roots and shoots as shown below.

Interestingly, the results presented here showed that high amount of the total up taken Cd (88.84%) was accumulated in roots with low amount (11.16%) translocated to shoots of sunflower seedlings (Figure 2 and Table 2). On the other side, high amount of the total up taken Pb (71.39) was tranlocated to shoots with low amount (28.61) stayed in roots of sunflower seedlings (Figure 2 and Table 2). Similar trends of Cd and Pb allocation between roots and shoots at the yield stage were recorded (Table 2 and Figure 3). Similarly, it was reported that Cd is accumulated mainly in roots; where about 22% of the total Cd up taken was translocated to the shoots of sunflower plants (Andrade *et al*., 2008). This circumstance may be due to the low Pb absorption and its low mobility compared to Cd (Becerril *et al*., 1989, reviewed by Öncel *et al*., 2000). In addition, it could be due to certain special structural, physiological and molecular characteristics in sunflower roots that enable them to absorb and keep large amounts of Cd, but absorb and keep low amounts of Pb. This suggestion needs deep investigations.

The results presented here suggest that sunflower plants may clean up the soils contaminated with Cd and Pb through different phytoremediation mechanisms. In this regard, it is reported that the technologies for metal phytoremediation include: 1) phytostabilization; immobilising the heavy metals within the rhizosphere, so that they neither leach nor enter the plant shoots, 2) phytoextraction; the use of plants to remove metals from soils and to transport and concentrate them in above-ground biomass; then the heavy metals are removed from the site when the plants are harvested, or 3) phytovolatilization; using the plants to take up the contaminating heavy metals and translocate them into the shoots, where they may be volatilized (Lasat, 2002; Ernst, 2005; Robinson *et al*., 2009). Considering the results presented here (Figures 2 and 3, Table 2) where sunflower seedlings and plants accumulate the bulk of the absorbed Cd in their roots, while the majority of the absorbed Pb is translocated to their shoots, it may be concluded that sunflower plants phytostabilize Cd and phytoextract Pb.

Finally, the trace amounts of Cd and Pb accumulated in the seeds (table 2 and figure 3) recommends sunflower plants to be used safely and economically for cleaning up soils contaminated with Cd and/or Pb. These negligible concentrations in sunflower seeds, and in turn in the oil, should limit the risk of food chain contamination and allow the use of oil for technical purpose (Madejon *et al*., 2003).

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