PHYTOREMEDIATION OF SOIL CADMIUM USING Chenopodium SPECIES

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INTRODUCTION

Heavy metal toxicity is a threat to environment and a looming issue to agriculture especially in the peri-urban areas of the world. This problem arises as a result of discharge of industrial waste in excessive quantities to the environment and the soil, and this problem is more serious in less developed countries (Wahid \textit{et al.}, 2009; Hussain \textit{et al.}, 2010; Sabir \textit{et al.}, 2011). Heavy metals are causing long term risk on environment sustainability by enhancing soil pollution. Soil fertility, crop yields and microbial activities decrease with the passage of time due to pollution caused by heavy metals (Yang \textit{et al.}, 2005). Concentration of heavy metals increases in soils due to atmospheric deposition from sludge, sewage irrigation, utilization of metal-containing farm manures and fertilizers and industrial wastes. Their entry into the food chain has hazardous effects on the biota including human beings (Prasad and Freitas, 2003; Chitmanat and Traichaiyaporn, 2010; Abdollahi \textit{et al.}, 2011). Heavy metals are also very toxic above certain concentrations, affect plant growth, and cause substantial yield reduction (Khan \textit{et al.}, 2007; Jalloh \textit{et al.}, 2009). Heavy metals are found in the form of free or exchangeable ions and complexes (Leyval \textit{et al.}, 1997; Wahid \textit{et al.}, 2009). The metals in the ionic form move to upper parts of plants (Liu \textit{et al.}, 2007). Concentration and duration of exposure and oxidation state are major factors affecting heavy metal toxicity to plants (Cosio \textit{et al.}, 2005).

Plants can easily absorb Cd and move to other parts causing toxicity. Having been absorbed by the plant roots and transported to aerial parts, the Cd may be redistributed to other plants parts via phloem (Unterbrunner \textit{et al.}, 2007; Souza \textit{et al.}, 2008). Leaf chlorosis due to loss of chlorophyll and carotenoids, and plant stunting and root browning are common symptoms of Cd toxicity in plants (Wahid \textit{et al.}, 2008; Perveen \textit{et al.}, 2011; Bahmani \textit{et al.}, 2012). Photosynthetic process especially the CO\(_2\) assimilation is decreased by heavy metals (Iqbal \textit{et al.}, 2010). Applied Cd usually leads to the disruption of membrane functions by increasing the peroxidation of lipids bilayer and increasing the malondialdehyde content (Liu \textit{et al.}, 2011). Cadmium also induces synthesis of secondary metabolites such as phenolics which can bind the heavy metals causes while wall lignification leads to cell death (Wahid \textit{et al.}, 2009).

Some plants species possess potential to extract heavy metals from soil and improve its physico-chemical properties and health for growing successive plants (Chrysafopeoulou \textit{et al.}, 2005; Mangkoedihardjo, 2007).
About 400 species of 66 woody plants are hyper accumulator of heavy metals (Kukkola et al., 2000). Such an approach, referred as phytoremediation of heavy metals, has been utilized as an effective means to remove the heavy metals from field soils using the metal hyper accumulator plants (Lone et al., 2008). Furthermore, this is a cost-effective, environmentally friendly and highly rewarding technology (Ghosh and Singh, 2005).

Wild plants are important components of ecosystems as their distribution helps in keeping the environment clean. Species of genus Chenopodium are common weeds of cultivated fields and widely distributed in many parts of the world (James et al., 2005). Chenopodium species are capable of accumulating the excessive quantity of heavy metals in leaf tissues, thereby reducing their quantities in the soil. While exploring the heavy phytoextractability of Chenopodium species, Bhargava et al. (2008) reported that C. quinoa was a better hyper accumulator of nickel, chromium and cadmium, while C. album could accumulate copper in large quantities. In their study, Gupta and Sinha (2007) found that C. album accumulated greater amounts of heavy metals (chromium, lead and Cd) in leaves followed by stem and root. Several other Chenopodium spp. remain yet to be investigated for their heavy metal responses.

Chenopodium album and C. murale are common weeds of winter crops in Pakistan, and can grow luxuriantly in stressful conditions. However, the comparative physiological mechanism(s) of Cd tolerance and heavy metals phytoextraction potential of both these species are poorly studied. This study was conducted to elucidate some morphological and physiological mechanisms of Cd tolerance and comparative phytoextraction potential of two common wild species of Chenopodium viz. C. album and C. murale using mungbean (Vigna radiata) as successive test crop.

MATERIALS AND METHODS

Experimental plan: Two field plot experiments were performed in the year 2011 for the determination of Cd phytoextraction potential of two Chenopodium species. In the first experiment, the comparative responses of C. album and C. murale was determined, while in second experiment comparative phytoextraction potential of both these species was assessed by growing mungbean as secondary crop in the plots from where the plants of C. album and C. murale were harvested.

Seeds of C. album and C. murale were broadcasted in small field plots measuring 1.5 m (long) × 1.0 m (wide) × 0.25 m (deep), lined with polythene sheets containing 600 kg of loam field soil (~30 cm deep). Separate plots were used for each treatment, and sampling was done randomly in triplicate from each plot. The physico-chemical properties of soil were determined with standard methods from dry soil or soil extracts as the case may be (Tandom, 1993). These properties before the experiment were: sand 37%, silt 32%, clay 31%, organic matter 1.03%, pH 7.02, ECe 2.76 dS m⁻¹, CEC 5.76 cmol c kg⁻¹, and Cd contents not detected. After growing Chenopodium species these properties changed to: sand 36%, silt 34%, clay 30%, organic matter 1.24%, pH 7.12, EC, 3.02 dS m⁻¹ and CEC 6.98 cmol, kg⁻¹. Cadmium contents were not detectable in fallow plots or in Cd non-contaminated soil where Chenopodium species were grown. However, Cd was detected in saturated soil extract of the plots where Cd was added. The Cd values were 15.32, 27.13 and 34.54 μmol kg⁻¹ soil for C. album and 17.43, 32.65 and 45.54 μg kg⁻¹ soil for C. murale at corresponding levels of applied Cd.

Cd tolerance by Chenopodium species: After germination, 20 uniform plants of both species in each plot were grown for one month. Rhizospheric soil of both the species was added with 0 (control), 250, 500 and 750 μM Cd levels prepared from CdCl₂·2.5H₂O, based on the saturation percentage of soil (~50%). One set of plots was kept as blank (no plants grown) without Cd and another blank set was mixed with 750 μM Cd level. Both the plots were kept as fallow for growing successive crop. No fertilizers were added since both the species grow wild, but watering was done when soil surface appeared dry. The plants were grown for two months in Cd contaminated soil and then harvested. The data was recorded for growth characteristics (shoot and root length, number of leaves and roots per plant, fresh and dry weight of shoot and root and leaf area per plant) and physiological and biochemical attributes (chlorophylls, carotenoids, soluble phenolics, anthocyanins in leaves) and ionic analysis (K⁺, Ca²⁺, SO₄²⁻, S and Cd²⁺ in the shoot and root).

Comparative phytoextraction potential of Chenopodium species: In this experiment, mungbean (Vigna radiata cv. NM-98) was grown in plots from which both the Chenopodium species were uprooted (with or without Cd) for a period of one month. For this purpose, 50 seeds were dibbled 2 cm deep in each plot followed by watering. Seed germination was recorded for eight days. After recording germination data the plant density was thinned to 20 uniform plants per plot. The determinations were made for germination percentage, shoot and root length, fresh and dry weight, leaf area per plant and shoot and root Cd contents.

Growth, metabolites and minerals analyses: Among growth parameters, leaf area per plant was determined of intact plants following the method of Carleton and Foote (1965). Shoot and root length of each plant was measured of intact plants whereas root length was measured after uprooting them. Number of leaves and branches was counted before harvesting whereas number of roots was counted after uprooting the plants. Dry weight of shoot and root was taken after drying the fresh harvested plant parts in paper bags at 70°C for seven days.
Cd-tolerance and phyto remediation by Chenopodium Sp.

For the analysis of photosynthetic pigments, 0.1 g fresh leaf material was chopped and extracted with 5 mL of 80% acetone using a pestle and mortar and final volume made to 10 mL using 80% acetone. The absorbance of the extract was taken at 665 and 645 nm using spectrophotometer (U-2001, Hitachi, Tokyo, Japan). The quantities of chlorophyll a and b were computed as described by Yoshida et al. (1976). For carotenoids analysis, the absorbance of above extract was taken at 480 nm while the total quantity was calculated as described by Davies (1976). The 80% acetonic extract was used for the estimation of soluble phenolics with the method of Julkenen-Titto (1985). For anthocyanins estimation, the fresh plant material was extracted with acidified methanol, centrifuged and absorbance of the extract was taken at 535 nm using spectrophotometer (Stark and Wray, 1989).

To analyze the contents of mineral ions, the dried ground plant material (0.5 g) was digested in concentrated HNO₃ by gradually increasing the temperature of heating block in a fume-hood. A blank, in duplicate, was always run for measurement of various mineral elements. After digestion, and making final volume to 25 mL, the extract was used for the estimation of K⁺ and Ca²⁺ using flame photometer (Jenway PFP7, UK). A graded series of 10–50 mg L⁻¹ was run for making the standard curve for both these ions using KNO₃ and CaCl₂, respectively. Amounts of Cd in the shoot and root were measured using atomic absorption spectrophotometer (AAS 3000, Norwich, CT, USA). For the estimation of sulfur (S), the above extract was taken in 50 mL volumetric flask. The extract was swirled after adding 1 mL of 6 N HCl and 1 mL of 0.5% gum acacia. BaCl₂ crystal (0.5 g) were added and kept for 1 min, swirled until the crystals were dissolved. Transmittance of samples was measured using spectrophotometer (Tandom, 1993).

Statistical analysis: All the determinations were made in triplicate from these completely randomized experiments. The presence or absence of significant differences among different factors was ascertained by performing analysis of variance (ANOVA) while the differences among the treatments were determined by using Duncan’s multiple range test (Steel et al., 1997). Computer software MSTAT-C was used for the statistical analysis.

RESULTS

Cd tolerance by Chenopodium species: Both the species differed significantly (P<0.01) for shoot length under control and Cd-stress conditions. Under Cd stress in C. album, the shoot length increased over control at 250 μM Cd, declined to the level of control at 500 μM Cd but further declined at 750 μM Cd. However, in C. murale, the shoot length consistently declined at all Cd levels (Fig. 1a). Root length, although declined consistently in both species at all Cd treatments, it was significantly (P<0.01) greater in C. album than in C. murale (Fig. 1b). C. album, showing greater number of branches and roots per plant than C. murale under control condition, was less affected than C. murale under Cd stress for both these attributes (Fig. 1c-d). The number of green leaves per plant was greater than control in C. album at 250 μM but declined at subsequent Cd levels, whilst C. murale indicated a consistent decline in this number at all the levels of Cd (Fig. 1e). Leaf area per plant under control condition was lower in C. album, but it showed a little decline even at the highest Cd level. However, C. murale showed a marked decline in this attribute at all Cd levels (Fig. 1f). For shoot dry weight, C. album exhibited no change between control and 250 μM Cd treatment but displayed a decline at higher Cd levels. However, shoot dry weight was gradually reduced in C. murale at all Cd levels (Fig. 1g). Root dry weight was higher in C. album than in C. murale under control conditions and was not different (P>0.05) from control up to 500 μM Cd level. However, C. murale manifested a substantial decline (P<0.01) in root dry weight at all Cd levels (Fig. 1h).

In Cd treated plants of C. album, Chl a increased at 250 μM level compared to control plants, which declined at 500 and 750 μM Cd. However, Chl a consistently declined in C. murale at all Cd levels (Fig. 2a). Although Chl b declined in both the Chenopodium spp., this reduction was greater in C. murale (Fig. 2b). In C. album, Chl a:b ratio indicated a little rise at 250 μM Cd, which then declined at higher levels. Contrarily, in C. murale this ratio decreased at 250 μM Cd, which then came closer to control values at 500 μM Cd and increased at 750 μM Cd level (Fig. 2c). In C. album, the carotenoids contents increased at 250 and 500 μM Cd and then a decrease (at 750 μM Cd), while C. murale displayed a decrease in this character at all Cd levels (Fig. 2d). Leaf soluble phenolics although declined in both the species, the decline was much higher in C. murale than in C. album with increased Cd stress (Fig. 2e). In C. album, the anthocyanin contents increased up to 500 μM Cd followed by a decline at 750 μM Cd level. However, in C. murale, the anthocyanins declined gradually at all Cd levels (Fig. 2f).

Applied Cd declined shoot and root K⁺ in both the species, although lowly in C. album than in C. murale (Fig. 3a-b). Cd stress did not affect the shoot Ca²⁺ but nominally affected root Ca²⁺ in C. album, which was substantially reduced in both these parts of C. murale (Fig. 3c-d). For shoot and root SO₄²⁻-S content under Cd stress, C. album indicated its steadier level while C. murale showed a reduction in this ion at all Cd levels (Fig. 3e-f). For shoot Cd content under Cd treatment, C. album indicated significantly higher Cd content in the shoot than C. murale (Fig. 3g). With an increase in Cd level, both the species indicated accumulation of Cd in the roots. However, of the two species C. album indicated lesser Cd accumulation in the root than C. murale (Fig. 3h).
Figure 1. Changes in some growth parameters of *Chenopodium* species grown in field plots contaminated with increased Cd levels. CA and CM stand for *Chenopodium album* and *Chenopodium murale*, respectively.
Figure 2. Changes in some metabolite levels of *Chenopodium* species grown in field plots contaminated with increased Cd levels. CA and CM stand for *Chenopodium album* and *Chenopodium murale*, respectively.
Figure 3. Changes in some mineral elemental contents of Chenopodium species grown in field plots contaminated with increased Cd levels. CA and CM stand for Chenopodium album and Chenopodium murale, respectively.
Cd phytoremediation by Chenopodium species: This was determined in terms of changes in germination percentage and post germination seedling mortality in mungbean, and growth attributes of mungbean used as successive crop species. With significant (P<0.01) differences among the treatments, the mungbean seeds sown in Cd contaminated plots indicated much lower germination percentage. The order of final germination percentage of mungbean seeds was from the plots: non-contaminated fallow plots > non-contaminated C. album plots > non-contaminated C. murale plots > 250 µM Cd-C. album plots > 250 µM Cd-C. murale plots > 500 µM Cd-C. album plots > 750 µM Cd-C. album plots > 500 µM Cd-C. murale plots > 750 µM Cd-C. murale plots > 750 µM Cd-fallow plots (Fig. 4a). Likewise, with significant (P<0.01) differences amongst treatments, post-germination seedling mortality was the lowest in non-contaminated fallow plots followed by no-Cd C. album and C. murale plots and C. album plots contaminated with 250 µM Cd, while it was the highest in 750 µM Cd contaminated fallow plots followed by 750 and 500 µM Cd contaminated C. murale plots (Fig. 4b).

Determinations made for various growth attributes and tissue Cd contents indicated significant (P<0.01) differences amongst various treatments in mungbean grown as a successive crop species (Fig. 4a-d). Shoot and root lengths, number of branches per plant and leaf area per plant were the highest in the fallow plots not contaminated with Cd followed by the C. album and C. murale plots not applied with Cd and those applied with 250 µM Cd. These parameters were the lowest in plots applied with 750 µM Cd followed by C. murale plots contaminated with 750 and 500 µM Cd. A highest number of roots and leaves per plant and shoot dry weight was noted in the mungbean grown in C. album plots contaminated with 250 µM Cd followed by the C. album and C. murale plots not contaminated with Cd, while it was the lowest in fallow plots contaminated with 750 µM Cd followed by from the C. album and C. murale plots applied with 750 and 500 µM Cd, respectively (Fig. 4d).

Higher shoot and root Cd was analyzed in mungbean grown in fallow plots applied with 750 µM Cd followed by those grown in C. murale plots treated with 750 and 500 µM Cd. Contrarily, lowest shoot Cd was noted in mungbean grown in fallow plots not contaminated with Cd followed by those grown in from the C. murale and C. album plots not contaminated with Cd.

DISCUSSION

This study on two native wild species of Chenopodium, i.e. C. album and C. murale, revealed that both these species showed great differences in their response to increased levels of Cd. Although both species indicated a reduction in their growth with increased substrate Cd concentrations, the performance of C. album was better than C. murale at all Cd levels. This was evident from the fact that for most of these parameters C. album indicated an increased value at 250 µM Cd and incurred a significantly less reduction at 750 µM Cd (Fig. 1a-h). This suggested that C. album entails some specific mechanism(s) to withstand the Cd toxicity. In this context, Chenopodium species have been reported to manifest great differences for accumulating various heavy metals in the aerial parts (Porębska and Ostrowska, 1999; Bhargava et al., 2008).

To compare metabolic and nutritional responses of C. album and C. murale, the determinations were made for changes in chlorophylls, carotenoids, soluble phenolics and anthocyanins (Fig. 2a-f). Loss of chlorophyll as a result of damage to chlorophyll protein complexes in thylakoids are often taken as important criteria of sensitivity to metal stress (Fatoba and Udoh, 2008; Wahid et al., 2008). Carotenoids...
Figure 5. Changes in growth and Cd accumulation properties of mungbean grown as succeeding crop in field plots from where Chenopodium was harvested. Thin dotted bars are control; dark shaded bars are Cd contaminated plots where no Chenopodium was grown; thick dotted bars pertain to C. album and checkered bars represent C. murale. In legends: VR, Vigna radiata, CA and CM are C. album and C. murale, respectively and 0, 250, 500 and 750 are levels of Cd (μM).
have multiple roles in plant stress tolerance as their contents may change depending on the type and severity of stress (Wahid et al., 2008). Accumulation of soluble phenolics and anthocyanins has been regarded as an indicator of stress tolerance in plants (Chalker-Scott, 1999; Hale et al., 2001). Data on photosynthetic pigments for both the species in this study revealed that both chlorophyll a and b species were minimally reduced in C. album than C. murale and that chlorophyll a was increased at 250 μM Cd level in the former species, resulting in increased chlorophyll a:b ratio in the former species. Likewise, carotenoids were reduced lowly in C. album and substantially in C. murale. With wide differences, soluble phenolics although were reduced in both the species, there was markedly greater accumulation of anthocyanins in C. album than C. murale at all levels of applied Cd. These data suggested that C. album could reduce the loss of chlorophyll a and anthocyanins even at higher levels of Cd. Here, the role of anthocyanins in Cd tolerance appears to be exclusive since they are reported to complex with heavy metals and sequester them in the vacuole (Chalker-Scott, 1999; Hale et al., 2001). In addition, maintenance of greater carotenoid content is also advantageous in nullifying oxidative damage caused by heavy metals (Ünyayar et al., 2005). In morpho-anatomical terms, C. album has more elaborate epidermal cells and succulent leaves than C. murale (Ahmad et al., 2009), which may be an adaptive advantage in terms of keeping toxic ions away from physiologically active sites. It is reported that Cd interferes for the transport of ions such as Ca²⁺ and K⁺ at plasmalemma, thus reducing their concentration in the cytosol and causing toxicity after being competitively taken up (Adhikari et al., 2006; Wahid et al., 2009). However, a cooperative uptake of mineral ions has been reported with Cd (Liu et al., 2007). Heavy metals reduced the assimilation of certain macronutrients when present in soluble form (Gill and Tuteja, 2011). Our results revealed that C. album, as compared to C. murale, tended to show minimal reduction in shoot and root K⁺ (Fig. 3a,b) no significant change in shoot Ca²⁺ but a reduction in root Ca²⁺ (Fig. 3c,d) as well as reduction in shoot and root SO₄²⁻ (Fig. 3e,f). Analysis of Cd contents in shoot and root indicated that C. album accumulated greater Cd in the shoot than C. murale. On the other hand, Cd contents of root were greater in C. murale than C. album (Fig. 3g,h). Potassium is metabolically important in cytosol and chloroplast, while Ca²⁺ is found both in the cytosol and as structural part of cell wall and certain macromolecules (Epstein and Bloom, 2005). Sulfur is more important nutrient in heavy metal stress tolerance because it is part of phytochelatins and metallothioneins, which are crucially important in cytosolic binding of heavy metals and thus reducing their toxicities and improving plant growth (Liu et al., 2011, 2012). These findings implied that C. album had greater capacity to absorb and assimilate the available nutrients than C. murale, which appeared to enable it show better growth under Cd stress. From the increased shoot Cd contents in C. album it can be inferred that above-mentioned metabolic changes, especially anthocyanins and plausibly greater synthesis of Cd binding and inactivating proteins could help this species to bind and sequester the excess of Cd in the vacuole (Chalker-Scott, 1999; Hale et al., 2001). From the changes in the shoot Cd contents of both the species, it was noticed that C. album could accumulate excess of Cd in the shoots, which indicates its potential to phytoremediate the soil. To test this possibility, mungbean was grown as successive crop due to its sensitivity to Cd toxicity (Wahid and Ghani, 2008), in those plots in which C. album and C. murale were grown without or with increased Cd levels as well as in the fallow plots not contaminated with and contaminated with 750 μM Cd for comparison purpose. The results regarding germination (Fig. 4a) and post-emergence mortality of seedlings (Fig. 4b) revealed that both these important parameters were far better in the C. album grown plots. Growth data further substantiated that mungbean displayed appreciably better growth in the plots where the C. album and C. murale were grown as compared to 750 μM Cd contaminated plots fallow plots (Fig. 5a-j). A comparison of both these species revealed that C. album was more effective phytoextractor of Cd from soil than C. murale, and this effect was related to exclusion of most of the root absorbed Cd to the shoot followed by either its binding by the phytochelatins or anthocyanins and sequestration into the vacuole.

We conclude from the above that C. album compared to C. murale is more efficient phytoremediator of marginally Cd contaminated soils especially in the peri-urban areas. The possible mechanisms involved are fast growth, distinct morpho-anatomical features, metabolic adjustments, Cd sequestration with anthocyanins and maintenance of greater nutrients contents in C. album, which otherwise were minimally observed in C. murale.

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