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Trends in Biotechnology Research
An International Peer-Reviewed Journal
Online and Print Version
www.sciencejournal.in

PHYTOREMEDIATION OF METAL CONTAMINATED SOILS WITH SPECIAL REFERENCE TO *BRASSICA JUNCEA* (L.) CZERN., *MACROTYLOMA UNIFLORUM* LAM VERDC. (*DOLICHOS BIFLORUS)* AND *MEDICAGO SATIVA* L.

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ABSTRACT
Soil Pollution due to various anthropogenic activities is harmful to human health and phytoremediation is one of the best and cheap way to remove heavy metal s from soil because heavy metals must be trans located and accumulated in the easily harvested part of the plants and Microbial activity in the rhizosphere of plants is several orders of magnitude greater than that in the bulk soil. Several strains of *Bacillus* and *Pseudomonas* increased the total amount of Cd accumulated by *Brassica juncea* seedlings (Salt *et al.*, 1995). Increase in bioavailability and enhancement in root absorption of metals have been reported owing to soil microorganisms.We selected three locally available plants, Brassica juncea (L.) Czern., Macrotyloma uniflorum Lam Verdc..(Dolichos biflorus) and Medicago sativa L. The initial experiments have shown the promise of these plants for the accumulation of cadmium, chromium and nickel. Index of metal tolerance (IT), translocation factor (TF) and metal accumulation appeared be effective parameters for the identification of efficient plants for phytoextraction trials. Soil conditions such as pH and presence of chelating agents have shown to vary the solubility thus availability of metals. Change in availability /solubility of metals influence the overall metal uptake by plants. In order to increase the bioavailability of metals in soil accumulation trials have been conducted in presence of EDTA (0.5 g/kg soil) and microbial inoculum. Increase in cadmium accumulation was achieved by either addition of EDTA and/or microbial cultures when compared with control pots. EDTA and microbial culture have shown synergistic effect on metal accumulation by *Dolichos biflorus* and *Medicago sativa*. Field trials were carried out with *Dolichos biflorus* and *Medicago sativa.*
On the basis of % survival, amount of metal accumulated and translocation factor *Macrotyloma uniflorum* Lam Verdc..(*Dolichos biflorus*) was appeared to be the most efficient plant and suitable candidate for phytoremediation technology.

KEY WORDS: Phytoremediation , IT , TF , Brassica juncea (L.) Czern., Macrotyloma uniflorum Lam Verdc..(Dolichos biflorus) and Medicago sativa L.

INTRODUCTION

Agricultural soils contaminated with heavy metals have posed a major threat to environment and human health due to various anthropogenic activities. The situation demands immediate attention of scientists and technologists to remove heavy metals from contaminated soil. Phytoremediation of heavy metals refers to the use of pollutant-accumulating plants to extract and accumulate contaminants to the harvestable parts and is increasingly being considered as an environmentally friendly, easy and cost-effective solution to clean up soils contaminated by heavy metals (Han *et al*., 2007).

For effective phytoremediation, heavy metals must be translocated and accumulated in the easily harvested part of the plants (Sridhar *et al*., 2005). The research on phytoextraction has been mainly focused on plants known as hyperaccumulators. However, phytoremediation potential may be limited by these plants due to the slow growth rate, low biomass production and a reasonable time frame by remediation with little known agronomic characteristics (Zhuang *et al.*, 2005). In addition, the efficiency of phytoextraction depends on the characteristics of the soil and the contaminants. Phytoextraction is applicable only to sites that contain low to moderate levels of metal pollution as plant growth is not sustained in heavily polluted soils. Soil metals should also be bioavailable (Thangavel and Subbhuram, 2004). Therefore many plants with higher biomass, such as maize and sunflower have been also tested for their phytoextraction potential (Lombi *et al*., 2001; Liphadzi *et al*., 2003). Together with the application of chemical amendments, including chelators, soil acidifiers, organic acid, ammonium, these high biomass plants could partially eliminate these limiting steps (Chaney *et al*., 1997; Salt *et al*., 1998; Dushenkov *et al*., 1999). It has been recognized that selection of appropriate plant materials and appropriate chemical amendments is still very important even today for promoting phytoremediation efficiency (Zhuang *et al*., 2005).

Microbial activity in the rhizosphere of plants is several orders of magnitude greater than that in the bulk soil. Several strains of *Bacillus* and *Pseudomonas* increased the total amount of Cd accumulated by *Brassica juncea* seedlings (Salt *et al.,* 1995). Increase in bioavailability and enhancement in root absorption of metals have been reported owing to soil microorganisms. Plant growth promoting rhizobacteria have been identified as relatively resistant to heavy metals and remain active in moderately acid soils and contribute to phytoremediation indirectly by increasing overall fertility of soil (Kamnev and van der Lelie, 2000). Dual usage of plants, profuse root system, higher metal tolerance, fast growth, high biomass production, economic interest, resistance to disease and pests, unattractive to animal minimizing the risk of appearance of metal in food web and ability to withstand the regional conditions are some of the main criteria applied for the selection of hyperaccumulators (Thangavel and Subbhuraam, 2004). In the present study, a survey has been made for the proper selection of plants satisfying most of the criteria of ideal system to work on. Three plants viz. *Brassica juncea Dolichos biflorus* and *Medicago sativa* have been identified as plants with economic value, withstanding the regional conditions, fast growing and with metal accumulation capacity (Singh *et al.,* 2009; Muratova *et al.,* 2010).

MATERIALS AND METHODS

Metal accumulation in pot assay

Fertile soil was collected from the agricultural field. The soil was air dried, homogenized with mortar and pestle and sieved through 2 mm mesh. Physicochemical parameters such as pH of soil, water holding capacity, soil texture, EC, $CaCO_3$, total organic carbon and sodium adsorption ratio (SAR) were studied as per the standard procedures (Singh, 1989; APHA, 1998; Amacher, 2003). The physicochemical properties of soil were as follows: pH, 7.51; EC, 0.63; $CaCO_3$ (%), 2.37; TOC (mg/l), 31.24; WHC (%), 54.46; CEC (mEq/100g), 22.06; and SAR, 5.81. Air dried and sieved soil (300 g) was filled in 0.4 l capacity polyethylene pots. Plastic pots were prewashed with dilute nitric acid to remove any adsorbed metal. *Brassica juncea* (Indian mustard), *Medicago sativa* and *Dolichos biflorus* were selected to study metal accumulation in pot assay. The soil pots were irrigated to field capacity with deionized water throughout the growth period. Prior to sowing, the pots were saturated with deionized water and allowed to stand for 2 weeks for equilibration with applied metals. The pots were introduced with 20 ml solution containing 150 and 375 mg/l of copper, chromium, cadmium, lead, zinc and nickel. The final metal concentrations in plastic pots were 10 and 25 mg/l. Each combination of metal and plant was run in triplicates. Stock metals solutions were prepared by adding appropriate amounts of their nitrate salts. Seeds (2 g) of above crops were sown in each pot, before sowing seeds were treated with 4% formalin to avoid fungal growth separately. The plants were harvested 2- 5 weeks after germination and washed with distilled water.

Estimation of chlorophyll and total soluble phenolics

The concentration of total soluble phenolics in plants was determined using Folin–Ciocalteau reagent (Singleton and Rossi, 1965) after extraction using 80% methanol. Soluble phenolics content in samples was estimated using a standard curve obtained for gallic acid. Metabolic response of hydrophytes to metal exposures was assessed by measuring chlorophyll *a*, *b* and total chlorophyll. The chlorophyll contents were estimated by extracting fresh plant biomass with 80% chilled acetone following the methods of Arnon (1949).

Digestion of plant material

Plant samples collected at different time intervals were rinsed thoroughly with distilled water, blotted dry and weighed for fresh weight. Plants were then oven dried at 70°C for 48 h for dry weight measurement. In order to estimate the total metal content plant samples were digested with aqua-regia (3:1, hydrochloric acid + nitric acid) at 150°C for 2 h in the COD digester (Model: 2015M, Make: Spectralab Inst. Pvt Ltd., India). The digested samples were cooled and filtered through Whatman No.1 filter paper and then the volumes were made up to 50 ml using volumetric flasks.

Metal analysis

Metal analysis of all digested samples was performed by atomic absorption spectrometry (Model: S2, Make: Thermo, USA) using the 'SOLAAR' software. Analyses were performed using hollow cathode lamps for cadmium (Cd), chromium (Cr), nickel (Ni), lead (Pb), copper (Cu) and zinc (Zn) at 228.8, 357.9, 232.0, 217.0, 324.8 and 213.9 nm, respectively. Air-acetylene flame was generated using a fuel flow rate of 0.8 to 1.1 l/min. All analyses were replicated three times. All the reagents used were analytical grade (Thermo Fischer Sci. India Pvt. Ltd., and HiMedia Lab. Pvt. Ltd., India).

Metal tolerance and translocation factor
Index of tolerance: The index of tolerance (IT) was calculated according to Wilkins (1957).

$$\text{Index of tolerance} = \frac{\text{Average length of roots in test}}{\text{Average length of roots in the control}} \times 100$$

To evaluate the extent of metal translocation from roots to shoots the translocation factors (TF) for different metals with in the plants were determined as the ratio of average metal accumulation (mg/kg) in shoot (stem + leaves) to the metal accumulation in roots (Das and Maiti, 2007).

$$\text{Translocation factor} = \frac{\text{Average metal accumulation in stem+ leaves (mg/kg)}}{\text{Metal accumulation in roots (mg/kg)}}$$

Effect of soil pH on metal accumulation by plants
For measurement of soil pH, a suspension of 10 g soil in 20 ml deionized water was prepared and incubated on rotary shaker (120 rpm) for 1 h (Eckert and Sims, 1995). The pH of soil suspension was measured using pH meter (LabIndia, Mumbai). In order to vary the pH of soil varying amounts sulfur powder (1, 2, 3 and 4 g) was added to 1 kg soil in separate container (Wang *et al.,* 2006). On mixing the contents the soils were watered every alternate day to keep moist. After incubation of 6 weeks the soils were dried and pH was measured. These soil samples were for metal accumulation assay conducted in pots. Seeds of three plants viz. *Brassica juncea, Dolichos biflorus* and *Medicago sativa* were sown in pots containing the soils conditioned to various pH.

Metal accumulation by plants in presence of EDTA and microbial inoculum
A two factorial design (Table .1) was prepared for experimental set up to test the effect of metal chelating agent, EDTA and microbial inoculum on soil properties as well as plant growth. Effect of these parameters was checked using sterilized soil (SS) as well as nonsterilized soil (NS). Soil amendments were done three days prior to sowing the seeds in the pots. EDTA was applied as a solution at a concentration of 0.5 g/kg soil.

Table 1. Factorial experimental designs for testing effects of EDTA and microbial inoculation on accumulation of cadmium

Treatments	Soil (100 g)	Bacterial inoculum (1×10^6 cfu/ml)	EDTA (0.5 g/kg)
T_1	Non Sterile	-	-
T_2	Non Sterile	✓	-
T_3	Non Sterile	-	✓
T_4	Non Sterile	✓	✓
T_5	Autoclaved	-	-
T_6	Autoclaved	✓	-
T_7	Autoclaved	-	✓
T_8	Autoclaved	✓	✓

Prior to sowing, the pots were saturated with deionized water and allowed to stand for 2 weeks for equilibration. The pots were introduced with 20 ml solution containing 375 mg/l of cadmium so as to have the final metal concentrations of 25 mg/l. Bacterial cultures viz. MJ-Pb-13 and MJ-Cr-6 were grown in 1 lit Erlenmeyer flasks containing growth medium containing 5 g/l yeast extract, 10 g/l glucose, 2 g/l $NaNO_3$, 0.5 g/l KCl and 0.5 g/l $MgSO_4.7H_2O$. Bacterial cells were harvested by centrifugation at 5000 × *g* for 10 min. The cell pellet was washed twice with sterile distilled water. The pots were inoculated with 10 ml bacterial cell suspension containing equal volume of both cultures with final cell count of 1 x 10^6 cfu/ml.

2.8 Field trials

A field experiment was conducted to study cadmium tolerance and accumulation in *Dolichos biflorus* and *Medicago sativa* in Botanical garden of Dayanand College, Solapur. The experiment was done for 6 weeks, from Dec 2006-Jan 2007. Soil plots (6×12 ft) were prepared at each site. Plots were designated at Test-1, Test-2 and Test-3 receiving microbial inoculum, EDTA and EDTA + microbial inoculum, respectively. Appropriate control plots were prepared for comparison with test plots. The plots were irrigated four times each with 72 lit of cadmium solution at a concentration of 50 mg/l at an interval of a week. Seeds of *Dolichos biflorus* and *Medicago sativa* were sown in rows of a set of four plots each. Plants were watered every other day.

All the plants were grown in natural conditions. Bacterial inoculum (1 lit) having 10^6 cfu/ml was mixed with 5 kg soil in a plastic bin. The soil amended with bacterial culture was spread uniformly in the half area of each plot. At each harvest (4-6 weeks), 20 plantlets per row in a plot were carefully taken, washed thoroughly with tap water and rinsed with deionized water, weighed, oven dried at 60°C for 48 h, and the dry weight determined. They were then separated into shoots (leaves and stem) and roots and ground. The ground dry mass was subjected for acid digestion for metal analysis.

RESULTS

Metal accumulation by plants in pot assay

Data on metal accumulation by three plants viz. *Brassica juncea, Dolichos biflorus* and *Medicago sativa* studied in pot assay are presented in Table .2. It can be seen that metal accumulation in these plants increased with increase in initial metal concentration. Highest accumulation, 130.01 mg/kg of zinc was observed in the roots of *Dolichos biflorus.* On the basis of average metal accumulation in these three plants the sequence of metals could be prepared as follows:

Leaves (mg/kg): Pb (58.15) > Zn (57.08) > Ni (37.29) > Cd (31.41) > Cu (27.26) > Cr (23.86)
Stem (mg/kg): Pb (58.15) > Zn (57.08) > Ni (37.29) > Cd (31.41) > Cu (23.17) > Cr (20.50)
Roots (mg/kg): Zn (94.95) > Pb (81.78) > Cu (35.92) > Ni (29.36) > Cd (29.62) > Cr (11.83)

Lead uptake was highest in leaves and stem where as zinc accumulation was highest in roots. Copper and chromium were least preferred metals for accumulation by the plants.

Table 2. Accumulation of metals in plants under pot assay

Plant	Plant part	Metal uptake (µg/g) at the initial metal concentration (mg/kg)											
		Copper		Chromium		Cadmium		Lead		Zinc		**Nickel**	
		10	25	10	25	10	25	10	25	10	25	10	**25**
Brassica juncea	Leaf	15.27[a]	29.28	24.62	26.32	13.11	27.13	32.11	38.11	19.88	36.12	16.13	**28.10**
	Stem	37.18	33.15	18.20	32.09	21.17	35.71	41.13	80.31	16.68	21.44	13.71	**26.08**
	Root	43.12	47.12	19.25	18.13	18.78	21.97	58.10	101.13	26.57	57.49	11.14	**22.72**
Dolichos biflorus	Leaf	16.18	30.20	17.36	31.18	32.15	44.38	56.11	96.17	19.88	79.00	26.85	**47.94**
	Stem	11.79	19.31	21.76	17.68	43.51	58.41	48.67	75.31	16.68	49.0	31.87	**61.39**
	Root	21.14	38.32	8.12	7.09	31.55	43.58	67.81	68.09	36.57	130.01	19.68	**33.25**
Medicago sativa	Leaf	13.18	22.30	11.75	14.09	11.73	22.73	21.10	40.17	12.13	56.13	15.09	**35.83**
	Stem	9.11	17.06	17.35	11.73	22.96	26.62	43.00	29.09	11.10	82.77	26.24	**54.09**
	Root	**11.81**	**22.31**	**8.11**	**9.89**	**15.79**	**23.32**	**65.79**	**76.11**	**48.08**	**97.36**	**16.01**	**32.10**

a, All the figures are the average of three replicates

Table 3. Effect of heavy metals on the growth parameters of *Brassica juncea, Dolichos biflorus* and *Medicago sativa*

Plant	Plant part	Control	Plant growth parameter when exposed to mg/kg of metal											
			Copper		Chromium		Cadmium		Lead		Zinc		**Nickel**	
			10	25	10	25	10	25	10	25	10	25	10	**25**
Brassica juncea	Shoot (cm)	7.3[a]	6.2	7.1	6.5	6.5	5.3	4.8	6.7	5.8	7.1	6.8	6.5	**7.1**
	Root (cm)	2.4	1.8	1.7	2.5	3.5	3.2	2.9	2.3	1.7	1.7	2.9	1.5	**2.5**
	% IT	-	75	70.83	104.17	145.83	133.33	120.83	95.83	70.83	70.83	120.83	62.5	**104.17**
	Biomass (g)	4.6	4.7	4.5	4.5	3.9	4.2	4.1	4.3	3.7	4.5	4.1	4.5	**3.7**
Dolichos biflorus	Shoot (cm)	20.3	15.7	15.8	16.5	15.3	17.6	18.3	17.3	16.7	16.5	17.1	16.2	**16.1**
	Root (cm)	7.1	8.7	7.2	6.5	5.3	6.9	7.1	6.5	5.9	5.7	6.3	7.5	**7.3**
	% IT	-	122.54	101.41	91.55	74.65	97.18	100	91.55	83.10	80.28	88.73	105.63	**102.82**
	Biomass (g)	5.7	4.5	4.2	4.9	4.3	4.7	4.8	4.6	4.1	4.8	4.8	5.5	**5.4**
Medicago sativa	Shoot (cm)	10.5	9.7	8.5	8.6	7.5	6.8	7.5	8.4	7.9	7.5	7.1	6.7	**7.0**
	Root (cm)	6.3	3.7	4.7	4.3	3.7	2.6	3.1	3.7	2.3	2.5	2.6	6.5	**6.3**
	% IT	-	58.73	74.60	68.25	58.73	41.27	49.21	58.73	36.51	39.68	41.27	103.18	**100**
	Biomass (g)	**5.7**	**4.0**	**4.0**	**2.3**	**2.1**	**4.1**	**4.7**	**3.8**	**3.6**	**4.5**	**4.2**	**6.1**	**5.9**

a, All the figures are the average of three replicates; % IT, Index of tolerance

Table 4. Effect of heavy metals on biochemical characters of *Brassica juncea, Dolichos biflorus* and *Medicago sativa* grown in pot assay

Plant	Plant part	Control	Metal concentration (mg/kg)											
			Copper		Chromium		Cadmium		Lead		Zinc		**Nickel**	
			10	25	10	25	10	25	10	25	10	25	10	**25**
Brassica juncea	Chl *a*	27.91[a]	21.11	26.7	22.63	24.99	6.80	9.16	20.7	23.79	9.42	8.13	7.56	**16.45**
	Chl *b*	34.32	13.11	12.7	24.52	32.74	11.25	12.35	32.3	30.20	8.91	6.24	14.35	**13.65**
	Total Chl	49.12	38.7	40.1	50.32	58.11	19.35	21.5	56.42	58.11	13.26	18.0	26.12	**22.17**
	Polyphenols	0.17	0.32	0.56	0.43	0.75	0.40	0.75	0.76	0.35	0.68	0.25	0.30	**0.35**
Dolichos biflorus	Chl *a*	41.07	17.3	21.5	30.70	26.4	19.7	24.7	26.5	23.7	37.12	36.50	29.3	**31.7**
	Chl *b*	38.63	32.6	30.6	38.60	40.12	38.7	40.3	29.7	30.5	41.13	39.15	30.6	**26.8**
	Total Chl	77.34	50.7	50.5	68.70	62.13	63.11	65.3	58..3	55.8	57.19	68.13	58.8	**53.7**
	Polyphenols	0.97	1.35	0.97	0.82	0.65	1.57	1.47	1.38	1.2	1.47	1.37	1.22	**0.79**
Medicago sativa	Chl *a*	21.47	10.23	11.17	0.85	41.35	19.7	24.7	15.4	14.4	13.10	12.75	8.1	**19.9**
	Chl *b*	16.50	22.60	24.11	11.86	12.23	38.7	37.5	33.7	35.7	24.36	26.37	34.2	**18.4**
	Total Chl	31.22	36.8	35.90	9.60	30.68	62.7	63.5	46.70	47.16	41.32	35.32	35.67	**34.31**
	Polyphenols	**0.12**	**0.74**	**0.73**	**2.78**	**0.98**	**1.58**	**4.45**	**0.14**	**0.12**	**0.86**	**1.35**	**0.75**	**0.92**

Chl a, b and Total chl are in mg% fresh weight; Polyphenols are in µg/ml, a, All the figures are the average of three replicates

Metal tolerance by plants in pot assay

The effect of heavy metals on the growth parameters of *Brassica juncea, Dolichos biflorus* and *Medicago sativa* are presented in Table .3. Highest % index of metal tolerance (IT) was seen for chromium (145.83%) at 25 mg/kg initial concentration by *Brassica juncea.* Among these three plants *Medicago sativa* exhibited less % IT values in comparison with *Brassica juncea* and *Dolichos biflorus.* It could be noticed that the %IT alues were not dependent on initial metal concentrations.

Effect of heavy metals on biochemical parameters of plants grown in pot assay

The data on effect of heavy metals on biochemical parameters of plants grown in pot assay are shown in Table .4. The chlorophyll content was found to be decreased in all combinations of plants and metal in comparison with control plants. Chlorophyll *a* and *b* decreased significantly with increased metal concentrations. The total chlorophyll content was also found to be decreased in a similar way. The polyphenol synthesis was found to be stimulatory to the metal concentrations. Increase in metal concentrations increased the polyphenol content of plants. The % increase in polyphenol content was different for various metal-plant combinations.

Metal translocation factor for plants

The translocation factor (TF) of heavy metals relative to *Brassica juncea, Dolichos biflorus* and *Medicago sativa* are shown in Table .5. The highest value of metal translocation factor i.e. 3.45 was obtained for chromium by *Dolichos* *biflorus* at 25 mg/kg initial concentration. The order of metals on the basis of translocation factor could be prepared as follows:

Brassica juncea: Cr (1.36) > Ni (1.27) > Cd (1.17) > Cu (0.64) > Pb (0.61) > Zn (0.60)
Dolichos biflorus : Cr (2.99) > Ni (1.57) > Cd (1.19) > Pb (1.02) > Cu (0.66) > Zn (0.50)
Medicago sativa : Cr (1.55) > Ni (1.35) > Cd (1.08) > Cu (0.91) > Pb = Zn (0.48)

All plants have shown highest TF values for chromium followed by nickel and cadmium. Metal Average TF values obtained for all metals by *Dolichos biflorus*, *Medicago sativa* and *Brassica juncea* were 1.31, 0.98 and 0.94, respectively. On the basis of TF the metals under test could be distributed into two groups; i) TF >1, chromium, nickel and cadmium, and ii) TF<1, lead, copper and zinc. On this ground for further studies group metals were selected to assess the effect other parameters on accumulation by these three plants in pot studies.

Table 5. Translocation factor for *Brassica juncea, Dolichos biflorus* and *Medicago sativa* when grown in presence of metals

Metal and its concentration (mg/kg)		Metal Translocation Factor		
		Brassica juncea	*Dolichos biflorus*	*Medicago sativa*
Copper	10	0.61	0.66	0.94
	25	0.66	0.65	0.88
Chromium	10	1.11	2.41	1.79
	25	1.61	3.45	1.31
Cadmium	10	0.91	1.20	1.1
	25	1.43	1.18	1.06
Lead	10	0.63	0.77	0.49
	25	0.59	1.26	0.46
Zinc	10	0.69	0.50	0.24
	25	0.50	0.49	0.71
Nickel	10	1.34	1.49	1.29
	25	1.19	1.64	1.40

TF, Translocation factors (TF), the ratio of average metal accumulation (mg/kg) in shoot (stem + leaves) to the metal accumulation in roots

Fig.1 Effect of soil pH on accumulation of chromium in plants and EDTA extraction of metal content, Br, *Brassica juncea*; Me, *Medicago sativa*; Dl, *Dolichos biflorus*

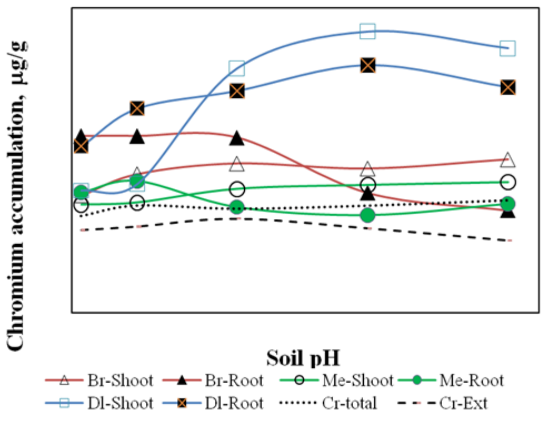

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Fig. .2 Effect of soil pH on accumulation of cadmium in plants and EDTA extraction of metal content, Br, *Brassica juncea*; Me, *Medicago sativa*; Dl, *Dolichos biflorus*

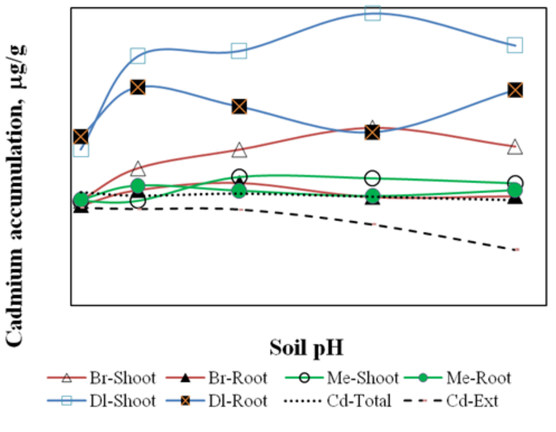
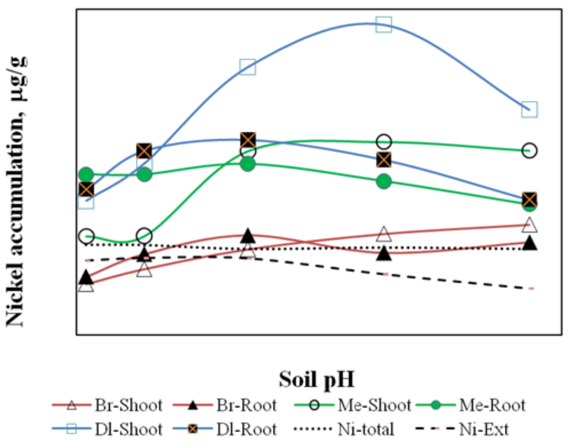


Fig. .3 Effect of soil pH on accumulation of nickel in plants and EDTA extraction of metal content, Br, *Brassica juncea*; Me, *Medicago sativa*; Dl, *Dolichos biflorus*



Effect of soil pH on accumulation of metals

The data on effect of soil pH on accumulation of chromium by three plants and EDTA extracted concentration are depicted in Fig. .1. It could be seen that increase in soil pH from 4.56 to 6.4 has resulted in increase in accumulation of chromium by *Dolichos biflorus* in shoot and roots. However, chromium uptake by roots of *Brassica juncea* was found to be decreased with concomitant increase in soil pH. Marginal rise in chromium accumulation could be noticed in shoots of *Brassica juncea* with rise in soil pH. Similar pattern of decreased chromium uptake in roots and increase in shoots was seen for *Medicago sativa*. No change was observed for total and EDTA extractable chromium concentrations upon change in pH of soil.

The results of cadmium accumulation by three plants as a function of changed soil pH are shown in Fig. .2. Cadmium accumulation by *Dolichos biflorus* was found to be pH sensitive. The cadmium content in shoots was increased when soil pH was changed from 4.56 to 6.4, and decreased with further increase in pH to 7.3, whereas, the cadmium in roots of *Dolichos biflorus* increased with increase in soil pH initially and was found to decrease with further rise. Cadmium accumulation in shoots of *Brassica juncea* was increased by 50% with change in pH from 4.56 to 6.4. No significant rise in cadmium accumulation in roots and both roots and shoots was seen in case of *Brassica juncea* and *Medicago sativa*, respectively. Total concentration of cadmium remained unchanged with change in soil pH. However, drop in % extraction of cadmium with EDTA could be noticed with concurrent ascend in soil pH. Figure 3 shows the data on nickel accumulation by three plants in pot assay at different soil pH values. Nickel accumulation reached a maximum of 76.34 µg/g at 6.4 soil pH. Nickel accumulation in shoots of all the three plants was increased with rise in soil pH. The EDTA extraction concentrations of nickel followed similar pattern as seen for cadmium and chromium.

Table 6. shows the translocation factor (TF) values for chromium, cadmium and nickel with three plants *Brassica juncea, Dolichos biflorus* and *Medicago sativa* at 25 mg/kg initial metal concentration and varied soil pH. Pattern of increasing TF values at increased soil pH was found common for all the metal-plant combinations. Among three plants *Dolichos biflorus* had exhibited higher TF values for cadmium than chromium and nickel. It showed maximum TF of 1.69 for cadmium at pH 6.4. Change in soil pH towards acidity adversely influenced the metal translocation.

Table 6. Effect of soil pH on metal transformation factor, total and EDTA extractable metal concentration of soil

Parameter	**TF and concentration of metal at pH**														
	Chromium					Cadmium					**Nickel**				
pH of soil	4.56	4.92	5.56	6.4	7.3	4.56	4.92	5.56	6.4	7.3	4.56	4.92	5.56	6.4	**7.3**
TF (*B. juncea*)	0.86	0.78	0.85	1.20	1.50	1.04	1.19	1.28	1.64	1.46	0.87	0.81	0.86	1.23	**1.19**
TF (*D. biflorus*)	0.73	0.63	0.91	0.88	0.85	0.92	1.14	1.28	1.69	1.21	0.92	0.93	0.73	0.56	**0.60**
TF (*M. sativa*)	0.90	0.84	0.85	0.77	0.83	1.01	0.87	0.89	0.86	1.11	0.72	0.62	0.93	0.80	**0.71**
Total metal (mg/kg)	19.05	21.08	20.45	21.05	22.09	22.84	22.14	22.56	21.94	21.24	22.08	22.03	21.12	21.44	**21.12**
EDTA-extracted metal (mg/kg)	16.22	16.92	18.48	16.56	14.12	19.67	19.45	19..34	16.33	11.24	18.24	18.84	18.76	14.88	**11.35**
% Extraction	**85.15**	**80.26**	**90.36**	**78.66**	**63.92**	**86.12**	**87.85**	**85.72**	**74.43**	**52.91**	**82.60**	**85.51**	**88.82**	**69.40**	**53.74**

a, All the figures are the average of three replicates; TF, Translocation factor; EDTA-ext, EDTA extractable concentration; %Ext, percent extraction of metals with EDTA

Table 7. Effect of EDTA and microbial inoculation on accumulation of cadmium by *Dolichos biflorus* and *Medicago sativa* grown in pot assay

Plant	**Parameter**	**Treatment**							
		T_1	T_2	T_3	T_4	T_5	T_6	T_7	$\mathbf{T_8}$
	Soil pH	7.36[a] (0.25)[b]	7.17 (0.33)	7.22 (0.21)	7.19 (0.42)	7.32 (0.34)	7.12 (0.88)	7.14 (0.28)	**7.21 (0.41)**
Dolichos biflorus	Cd-Shoot	51.05 (2.67)	77.90 (3.24)	65.04 (2.22)	82.17 (3.89)	46.92 (3.45)	61.43 (2.17)	58.26 (1.87)	**73.22 (6.21)**
	Cd in Root	44.66 (3.01)	62.61 (2.19)	58.55 (4.01)	63.83 (2.44)	31.47 (1.76)	60.40 (2.06)	48.05 (2.04)	**51.03 (4.27)**
	TF	1.14	1.24	1.11	1.29	1.49	1.02	1.21	**1.44**
	Total Chl	64.23 (3.48)	72.31 (2.48)	65.30 (2.14)	71.07 (3.06)	63.11 (1.97)	68.77 (2.92)	65.12 (2.11)	**73.82 (3.05)**
Medicago sativa	Cd in Shoot	24.22 (2.88)	41.04 (3.81)	29.55 (2.66)	52.08 (3.42)	24.43 (2.13)	32.92 (1.64)	27.44 (3.06)	**48.00 (2.72)**
	Cd in Root	21.54 (1.76)	28.33 (2.02)	25.43 (2.05)	34.30 (2.49)	21.08 (2.46)	27.41 (2.01)	24.04 (2.03)	**39.46 (3.77)**
	TF	1.12	1.45	1.16	1.52	1.16	1.20	1.14	**1.22**
	Total Chl	**64.88 (2.41)**	**71.40 (2.88)**	**66.19 (1.87)**	**72.01 (3.07)**	**61.22 (2.56)**	**68.02 (2.00)**	**62.99 (3.04)**	**71.47 (2.08)**

a, All the figures are the average of three replicates; b, standard deviation; TF, Translocation factor; Cd-Shoot, Cadmium content in shoot (mg/kg); Cd-Root, Cd content in roots; Chl, chlorophyll

Table 8. Accumulation of cadmium by *Dolichos biflorus* and *Medicago sativa* in the field trials

Plant	Parameter	Control	Test-1	Test-2	Test-3
Dolichos biflorus	Soil pH	7.33[a] (0.22)[b]	7.20 (0.67)	7.22 (0.21)	**7.11 (0.34)**
	Cd-Shoot	77.16 (6.52)	97.22 (7.31)	69.45 (6.54)	**94.02**
	Cd in Root	58.58 (4.05)	64.35 (6.88)	61.94 (4.21)	**70.18**
	TF	1.32	1.51	1.12	**1.34**
	Total metal content	112.94 (8.20)	110.46 (6.66)	114.32 (4.06)	**112.64 (4.32)**
	EDTA-extracted Cd (mg/kg)	75.96	92.58	91.20	**93.41**
	% Extraction	67.26	83.81	79.78	**82.93**
Medicago sativa	Soil pH	7.11[a] (0.34)	7.07 (0.55)	7.14 (0.74)	**6.89 (3.77)**
	Cd in Shoot	64.39 (4.08)	79.53 (6.01)	73.60 (3.70)	**78.18 (8.04)**
	Cd in Root	49.45 (4.61)	71.55 (4.97)	62.73 (4.15)	**75.22 (5.18)**
	TF	1.30	1.11	1.17	**1.04**
	Total metal content	107.00 (6.23)	109.08 (4.88)	115.91 (5.94)	**116.23 (4.19)**
	EDTA-extracted Cd (mg/kg)	73.45	91.86	95.01	**92.30**
	% Extraction	**68.65**	**84.21**	**81.97**	**79.41**

a, All the figures are the average of three replicates; b, standard deviation; TF, Translocation factor; Cd-Shoot, Cadmium content in shoot (mg/kg); Cd-Root, Cd content in roots; Chl, chlorophyll

Effect of chemical and microbial amendments to the soil on metal accumulation

Plants viz. *Dolichos biflorus* and *Medicago sativa* were selected on the basis of TF, tolerance and accumulation of cadmium. Change in availability /solubility of metals influence the overall metal uptake by plants. In order to increase the bioavailability of metals in soil, accumulation trials have been conducted in presence of EDTA (0.5 g/kg soil) and microbial inoculum. The concentration of EDTA was selected on the basis of literature reports. The data on metal accumulation in shoots and roots, translocation factor and chlorophyll content are presented in Table .7. It can be seen that soil amendments such as EDTA and microbial inoculum resulted in decrease of soil pH from 0.04 to 0.24 units. Overall increase in cadmium accumulation by both the plants could be noticed with either addition of EDTA and/or microbial cultures when compared with control pots (T_1). Highest accumulation of 82.17 and 63.83 g/kg cadmium was obtained in shoots and roots, respectively, of *Dolichos biflorus* in presence of microbial culture and EDTA (T_4). Total chlorophyll and translocation factor was found to be increased in presence of bacterial inoculum in both the plants tested. EDTA and microbial culture have shown synergistic effect on metal accumulation by *Dolichos biflorus* and *Medicago sativa*. EDTA + Microbial inoculation was more effective than either EDTA or microbial inoculation alone.

Accumulation of cadmium by *Dolichos biflorus* and *Medicago sativa* in field trials

The results of field trials on cadmium accumulation by *Dolichos biflorus* and *Medicago sativa* are presented in Table .8. Growth performance of *Dolichos biflorus* in all plots had >95% survival throughout the experiment whereas in *Medicago sativa*, a decrease in survival rate was observed in Test-2 plots. Highest cadmium uptake of 97.22 mg/kg was observed in shoots of *Dolichos biflorus* cultivated in Test-1 plot. Whereas highest cadmium accumulation in roots (75.22 mg/kg) was obtained in *Medicago sativa* grown in Test-3. Decrease in soil pH could be noticed in all test conditions except Test-2 of *Medicago sativa* field. Highest rise of 16.55% in EDTA extraction was obtained when soil was amended with only bacterial culture (Test-1). Microbial addition has raised the translocation factor in Test-1 plot. In other test plots TF values were decreased in comparison with control plots. The TF values for *Dolichos biflorus* and *Medicago sativa* ranged from 1.12 to 1.51 and 1.04 to 1.30, respectively. TF values of *Dolichos biflorus* were higher than *Medicago sativa* in control, Test-1 and Test-2. This indicates that *Dolichos biflorus* accumulated cadmium more efficiently from contaminated soils than *Medicago sativa* under different set of conditions.

DISCUSSION

The main objective of this study was to assess the potential usefulness of *Dolichos biflorus, Brassica juncea* and *Medicago sativa* as phytoextractors of metallic contaminants. To qualify as a hyperaccumulator, a plant must exhibit a concentration of heavy metals in its above ground biomass that is 10-500 times greater than that in normal plants and/or the concentration of heavy metals should reach a hyperaccumulating level (Pb > 1000 mg/kg in the shoots) and/or have an enrichment coefficient (shoot/root ratio, translocation factor >1) (Baker *et al.*, 1994; Shen and Liu, 1998; Shen *et al*., 2004). Based on the critical value proposed by Baker and Brooks (1998) a plant is a cadmium byperaccumulator when the content of Cd in the dry weight of the shoots or leaves is >100 mg/kg. In the present study, highest accumulation in shoots or roots was < 100 mg/kg for any of the six metals tested. Zinc accumulation in the roots of *Dolichos biflorus* reached 130 mg/kg. The less metal accumulation values could be corroborated to low initial concentration of metals (10 and 25 mg/kg). Many plants are known to concentrate metals in roots and metal translocation to the shoots is normally very low (Salt and Krämer, 2000). Development of roots and chlorophyll content was found to be sensitive to metal concentrations. The state of pigment system can be considered to be an indicator of healthy photosynthesis and thus physiological status of plant. Abiotic stresses decrease the chlorophyll content in plants. Metals have been reported to inhibit the final reduction stage in chlorophyll formation by interacting with the functional –SH group of the enzyme synthesizing chlorophyll (Prasad and Prasad, 1987). The chlorophyll content of *Brassica juncea*, *Dolichos biflorus* and *Medicago sativa* was decreased when exposed to elevated metal concentrations. Similar effect of metal concentration on chlorophyll content has been reported by Liu *et al.* (2007) in case of *Brassica pekinensis* and *Brassica chinensis* in presence of cadmium.

Plant cells possess powerful antioxidative systems composed of non-enzymatic scavengers of ROS such as ascorbate, glutathione, phenolics, and numerous antioxidant enzymes such as superoxide dismutase, peroxidase (POD), and catalase (Morina *et al.,* 2008). Phenolics have various functions in plants. An enhancement of phenylopropanoid metabolism and the amount of phenolic compounds can be observed under different environmental factors and stress conditions (Michalak, 2006). Antioxidant action of phenolic compounds is due to their high tendency to chelate metals. Phenolics, besides their function as electron donors to hydrogen peroxide in reactions catalyzed by peroxidase, have the ability to scavenge radical species directly (Rice-Evans *et al.,* 1997). Phenolic compounds such as o-diphenols and catechins have been recognized as metal binders and considered as an important class of phytochelators as their metal complexes have been shown to be stable (Sakihama *et al.*, 2002). Plant roots when exposed to heavy metals exude high levels of phenolics that bind to metal ions owing to presence of hydroxyl and carboxyl groups (Morina *et al.*, 2008).
Translocation factor (TF) is defined as the ratio of the metal concentration in the shoots to that in the roots which is used to evaluate the effectiveness of a plant in translocating metal from roots to shoots (Fayiga *et al*., 2004; Sun *et al.*, 2007; Waranusantigul *et al.,* 2008; Adesodun *et al*., 2009). In present study TF values >1 were obtained for Cr, Cd, Pb, Ni by *Brassica juncea, Dolichos biflorus* and *Medicago sativa* indicating that these metals moved more easily in these plants. Blaylock *et al.* (1997) noted that Pb translocation from roots to shoot is very slow, and Cr, Pb, and Hg have least translocation rate to the plant top. (Chaney *et al*., 1997).

In addition to translocation factor ‘Index of Tolerance (IT)’ has been considered as another parameter to choose efficient metal accumulating plants (Han *et al*., 2007; Singh *et al*., 2009). The higher %IT values obtained for *Brassica juncea, Dolichos biflorus* and *Medicago sativa* could be attributed to their inherent capacity of metal tolerance. The bioavailability of heavy metals is an important factor in the process of phytoextraction by non-hyperaccumulators, especially in neutral or calcareous soils. The bioavailability of heavy metals in soil can be increased by either lowering soil pH (Salt *et al.*, 1995; Chlopecka *et al*., 1996) or adding synthetic chelates such as EDTA, NTA and DTPA (Blaylock *et al.*, 1997). A decrease in soil pH can be achieved by application of mineral or organic acids or acid-producing fertilizers such as ammonium chloride (Salt *et al*., 1995; Huang *et al*., 1997; Wasay *et al*., 1998). However, these methods have some limitations due to negative effects on soil fertility or soil structure, or they may lead to groundwater pollution. Use of elemental suphur to decrease the pH increases solubility of heavy metals without having adverse effects (Cui *et al.,* 2004). Certain groups of acidophilic soil bacteria, predominantly, the genus *Thiobacillus*, can metabolize sulphur and generate H^+ and SO_4^{2-}, leading to soil acidification (Kayser *et al*., 2000). Thus, first lowering soil pH and then adding EDTA might be an effective strategy to maximize the phytoextraction of Pb and Zn from contaminated soils that are mostly neutral or slightly alkaline (Cui *et al.,* 2004). In present study, soil pH was successfully decreased by addition of sulphur. Increase in EDTA extractable concentration of chromium, cadmium and nickel with increased soil acidity was indicative of elevated solubility of metals.

Numerous reports on the use of synthetic chelators in enhancing uptake and transport of heavy metals by plants have been published in recent years (Epstein *et al.,* 1999; Kambhampati *et al.*, 2003; Li *et al.,* 2008). Ethylenediaminetetraacetic acid (EDTA) is an effective chelate that has been found to increase the solubility of heavy metals in soil and their bioavailability to plants, and the effect can be improved by decreasing soil pH (Blaylock *et al*., 1997). Metals like lead have very limited solubility in soils and thus availability for plant uptake due to complexation with soil organic matter, sorption on oxides and clay minerals and precipitation as carbonates, hydroxides and phosphates (McBride, 1994). Enhancement of lead accumulation by Indian mustard from soils amended with a range of chelates was reported by Blaylock *et al.* (1997), who found that EDTA was the most effective chelate tested and that citric acid was not effective. Use of chelates may help overcome the diffusion limitation. McGrath *et al.* (2001) reported that EDTA and NTA did not increase Zn and Cd uptake by *T. caerulescens*, as EDTA did for the uptake of Pb by *B. juncea*. The variation in the effect of synthetic chelates on metal accumulation could be ascribed to differences in metal binding specificity and hydrophobicity of natural and synthetic chelates in soil environment. This necessitates choosing / optimizing an appropriate chelate structure for maximum phytoextraction of different metals (Wu *et al*., 1999).

The efficiency of phytoextraction of metals has been reported to be influenced by activity of rhizosphere microbes, speciation and concentration of metals deposited into soil (Wang *et al*., 1989; Khan *et al.*, 2009). Lippmann *et al.* (1995) have shown that rhizobacteria *Pseudomonad* and *Acinetobacter* enhances phytoremediation abilities of non-hyper accumulating *Zea mays* L. by increasing its growth and biomass. Plants growing in metal stressed soils can protect themselves from metal toxicity by synthesizing antioxidant enzymes, which scavenges the toxicity of reactive oxygen species generated by plants

The use of various heavy metal resistant bacteria having plant growth-promoting feature has raised high hopes for eco-friendly and cost-effective measures toward reclamation of heavy meal pollution in soil (Sinha and Mukherjee, 2008). Wu *et al.* (2006) demonstrated that the use of rhizospheric bacterium *Pseudomonas putida* not only improved cadmium binding but also alleviated the cellular toxicity of cadmium. Whiting *et al.* (2001) stated that the bacteria did not facilitate an increase in the rate of soluble zinc transport into the roots nor did not enlarge the surface area of roots of *Thlaspi caerulescens* but increased the bioavailability of zinc from the non-available phases in soil. However, various microbial metabolites such as biosurfactants, siderophores and organic acids were reported to increase the metal bioavailability in the soils (Braud *et al.*, 2006). Increase in metal bioavailability in soil, thus enhancing chromium, lead, zinc and copper accumulation by *Zea mays* and *Sorghum biocolor* owing to inoculation of four bacterial isolates have been reported by Abou-Shanab *et al*. (2008).

Phytoextraction is a long-term remediation effort, requiring many cycles to reduce metal concentrations to acceptable levels (Luca *et al.,* 2007). Numerous reports have been published on phytoextraction of heavy metals demonstrated in either hydroponic or pot assays conducted in laboratory conditions. Such reports are not adequate to give results applicable for the future clean-up of contaminated areas (McGrath *et al.,* 2006; Luca *et al*., 2007; Waranusantigul *et al.*, 2008). Field trials would be closer approach for better understanding of metal accumulation abilities of plants. With this intention the field trails in garden have been conducted in the current study. The results have supported the performance of *Dolichos biflorus* and *Medicago sativa* seen in pot assays.

CONCLUSIONS

Three plants viz. *Brassica juncea*, *Dolichos biflorus* and *Medicago sativa* with economic value, withstanding the regional conditions, fast growing and with metal accumulation capacity have been used for metal accumulation in pot and field studies. The initial experiments have shown the promise of these plants for the accumulation of cadmium, chromium and nickel. Index of metal tolerance (IT), translocation factor (TF) and metal accumulation appeared be effective parameters for the identification of efficient plants for phytoextraction trials. Soil conditions such as pH and presence of chelating agents have shown to vary the solubility thus availability of metals. Change in availability /solubility of metals influence the overall metal uptake by plants. In order to increase the bioavailability of metals in soil accumulation trials have been conducted in presence of EDTA (0.5 g/kg soil) and microbial inoculum. Increase in cadmium accumulation was achieved by either addition of EDTA and/or microbial cultures when compared with control pots. EDTA and microbial culture have shown synergistic effect on metal accumulation by *Dolichos biflorus* and *Medicago sativa*. Field trials were carried out with *Dolichos biflorus* and *Medicago sativa.* On the basis of % survival, amount of metal accumulated and translocation factor *Dolichos biflorus* was appeared to be the most efficient plant and suitable candidate for phytoremediation technology.

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