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ORIGINAL ARTICLE

Study of Metal Resistance Potential of the Cd, Cr Tolerant Alligator Weed

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Background – Environmental deterioration due to heavy metal pollution is a major global concern for its immense importance in the ecosystem. Indiscriminate use of heavy metals for rapid urbanization and industrial exploration is a pressing threat to human health. Among this Cd and Cr contamination is most dangerous as these metals directly enter into the food chain due to their higher solubility and mobility. Identification of a metal tolerant native plant species would be helpful to decontaminate Cd and Cr polluted land. In our previous study, field investigations were conducted to evaluate the tolerance potential of Alligator weed to Cd and Cr.Alligator weed [*Alternanthera philoxeroides* (Mart). Griseb],is the most widely distributed perennial stoloniferous herb in these contaminated areas in and around Kolkata.

Purpose of the study – To establish metal tolerant capacity of the species , different biochemical parameters assessing its metal accumulation capacity and reflecting its detoxification mechanism were studied. For these purpose, the same plant collected from the highest metal contaminated area was grown under laboratory condition with external application of various concentration of Cd and Cr individually and synergistically (0.5, 0.8, 1.0, 1.2, 1.5, 1.8 mM). To estimate the hazardous effects of Cd and Cr on this weed, membrane damage was quantified in form of lipid peroxidation i.e MDA production. The metal uptake and accumulation potential was estimated by measuring the Cd and Cr concentration in root and shoot. Some soil parameters such as Orgnaic Carbon, Cation exchange capacity were also studied to explain the bio availability of metals. Various biochemical parameters such as free proline content, non protein- thiol content and zymogram analysis of antioxidative isozymes (such as, Guiacol peroxidase, superoxide dismutase, glutathione reductase and ascorbate peroxidase) were studied to assess its metal resistant capacity.

Result: The acidic pH and enhanced Cation Exchange Capacity of soil made both Cd and Cr more bioavailable with increasing metal concentration. Linear increase in metal uptake and accumulation was recorded upto an optimum level at 1.0 mM, 1.2 mM for Cd and Cr respectively, evident from Translocation Factor > 1. Gradual increase in membrane damage reflected the devastating effect of both Cd and Cr. But enhanced free proline content and non protein thiol content provide enough detoxification capacity to tolerate 1.2 mM Cd, Cr after which biochemical defenses declined. Increased activity of glutathione reductase and superoxide dismutase were well documented in 1.2 mM and 1 mM Cd, Cr treated plants respectively. Overexpression of ascorbate peroxidase, superoxide dismutase and glutathione reductase was evident by the appearance of additional bands with respect to control plants which would provide acute detoxification capacity of the plant to cope up with gradual increasing Cd, Cr contamination.

Conclusion: This newly emergent Cd and Cr tolerant plant which can thrive well in highly Cd, Cr contaminated soil under field condition is thought to have the potential for phytoremediation of multiple metal contaminated sites of major polluted cities.

Key words: Alternanthera philoxeroides, Cation Exchange Capacity, Glutathione Reductase, MDA, Non protein thiol contents

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Pollution of soil and water by Cd, Cr is an alarming problem in urban and semi urban areas of India. The sources of pollution are mainly anthropogenic. Being biologically non essential,

these two metals are extremely phytotoxic after a threshold value. According to Agency of Toxic Substances and Disease Registry (2007) Cd and Cr occupies 7 th and 77 th position respectively in the list of most hazardous substances of the world. As these two metals have vast industrial uses (leather tanning, electroplating, mineral fertilizers, Ni- Cd battery production, paints used in glass and ceramics and soft drink), almost 65 % of industrial workers as well as normal population living in polluted areas are regularly exposed to the hazards of these two toxic metals. Cr normally exists in two oxidation states in soils; trivalent Cr (III) and hexavalent (VI). Being more mobile hexavalent Cr is extremely toxic to plants and animals in high doses and is responsible for inducing cancer and teratism, resulting liver and kidney damage. Cd is a non redox metal, unable to participate in Fenton reactions but it leads to the formation of reactive oxygen species indirectly by interfering antioxidative defense system of plants. Cd with a long biological half life causes cancer of lung and prostate, kidney tubule damage, osteomalacia and fragility of bones. Cd is capable of entering the food chain through uptake into plant tissues. Presence of excessive amount of Cd, Cr in soil and water causes a range of plant responses including leaf chlorosis, disturbance in nitrogen metabolism, photosynthesis and respiration, stunted growth and even death. In comparison to crop plants, indigenous weed plants usually display some inherent properties to hypertolerate, strong endurance and higher capacity to absorb heavy metals. The tolerance capacity of plants to heavy metals depends on an interrelated network of physiological and molecular mechanisms. Aerobic organisms as well as plants have evolved antioxidant pathways that are usually sufficient to protect them from oxidative stress induced by Cd, Cr toxicity. The most important are low molecular weight non enzymatic antioxidants such as ascorbic acid, glutathione, thiole, α tocopherol, protective pigments such as carotenoids and accumulation of free proline. The oxidative damage of cell imposed by Cd, Cr and other heavy metal toxicity is ameliorated by the overexpression of antioxidative enzymes such as catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) that serve as the first line of defense system and provide detoxification capacity to the plant. In the present work we would like to assess the metal tolerance capacity of the Alligator weed under Cd and Cr stress. Alligator weed was chosen for two reasons. Firstly the plant was found to grow widely in various habitats; secondly, the plant was found with higher frequency in Cd and Cr contaminated areas.

Different Cd and Cr co-contaminated areas in and around Kolkata, with high population density; were surveyed and screened thoroughly for wildly grown native plants capable of accumulating these metals. Alligator weed (*Alternanthera philoxeroides*), a member of Amaranthaceae was found to be the most abundant with considerable amount of Cd, Cr in the above ground parts. Some of these studied areas showed very high soil Cd (44.62 mg/kg) and Cr (1833.2 mg/kg) concentration that far exceeded maximum allowable limits (1-3 mg/kg Cd, 30-400 mg/kg Cr, ECDGE 2010). A large number of reports are available in the literature regarding the screening of several plant species for the ability to tolerate and detoxify heavy metals under laboratory condition - *Thalspi caerulescens* (Baker *et al*., 2000), *Thalspi praeox* (Torla *et al*., 2006), *Rorippa globosa*, *Arabidopsis halleri* (Kupper *et al*., 2000), *Sedum alfredii* (Yang *et al*., 2004) for

Cd hyperaccumulation; *Leersia hexandra* (Zhang *et al*., 2007) for Cr hyperaccumulation. In the present work, (*Alternanthera philoxeroides*) with highest Cd, Cr tolerance capacity was collected from maximally contaminated area and was potted in laboratory condition with the external application of individual and combined dosage of both Cd and Cr to ascertain its optimum tolerance and detoxification potential. To determine the metal toxicity and tolerance capacity, this palnt was treated with $CdCl_2$ and $K_2Cr_2O_7$ externally under laboratory condition for 10 days and its free proline content, MDA content, GSH content were measured. Activity of SOD, POX, APX, GR were studied both by zymogram analysis and assay to quantify its defense system.

MATERIALS AND METHODS

Plant material: (Alligator weed), *Alternanthera philoxeroides* (Mart.) Griseb is a perennial stiloniferous herb, widely grown in rivers, lakes, ponds as well as many terrestrial habitats. Plants collected from highly contaminated area were grown under laboratory condition and was treated with different concentration of Cd and Cr (0.5, 0.8, 1.0, 1.2, 1.5 and 1.8 mM) individually and simultaneously for 10 days.

Digestion of plant sample for heavy metals:

Cadmium and Chromium concentration of the treated plant samples were estimated from Pollution Control Board, WB, Kolkata by utilizing Flame Atomic Absorption Spectrophotometer (Perkin Elmer) following digestion using the method described in the Perkin Elmer handbook for Atomic Absorption spectroscopy (APHA,1989). 0.8-1.0 gm of oven dried and crushed plant tissue (shoot and root separately) were digested with concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$).

From the result of metal analysis bioconcentration factor and translocation factor were determined. A plants ability to accumulate metals from soils can be estimated using the *bioconcentration factor*, which is defined as the ratio of metal concentration in the roots to that in soil (Yoon *et al*. 2006).

BCF (bioconcentration factor) : metal concentration in root / metal concentration in soil

TF (translocation factor) : metal concentration in shoot / metal concentration in root

Estimation of soil parameters

Organic carbon (OC): OC was measured according to the method of Walkley and Black (1934). 1g of oven dried and sieved soil was taken in 500ml conical flask. To it 10 ml 1N Potassium dichromate was added. Then 20 ml of conc. H_2SO_4 was added in each flask. After that the flask was incubated for at least 1hour. After 1hour, 200ml distilled water and 5ml phosphoric acid and 1ml DPA (used as indicator) were added. Then it was titrated against Mohr salt. The end point was determined by the appearance of green color.

Cation exchange capacity (CEC): CEC was measured according to Chapman (1965).

Estimation of proline:

500mg leaves were homogenized in 5ml 0.1M sulfosalicylic acid and centrifuged at 5000 rpm for 30 minutes. To the supernatant (2ml), 5ml glacial acetic acid and 5ml ninhydrin solution were added and heated at boiling water bath for 1 hour. The mixture was extracted with toluene in separating funnel and absorbance was taken at 520 nm. Free proline amount was determined (µg / gm tissue) from a previously prepared standard curve (Bates 1973).

Estimation of lipid peroxidation (MDA content):

The degree of lipid peroxidation was measured

in terms of malondialdehyde (MDA) content determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). 500 mg leaves were homogenized in 5 ml of 0.1% TCA and centrifuged at 10000 g for 5 min. For every 1ml of aliquot, 4 ml of 20% TCA containing 0.5% thiobarbituric acid was added and heated at 95°C for 30 min and quickly cooled on an ice bath. The resulting mixture was centrifuged at 10000 g for 15 min and the absorbance was taken at 532 nm and 600 nm. The non specific absorbance at 600 nm was subtracted from the absorbance at 532 nm. The concentration of MDA was calculated by using the extinction coefficient of 155/nm/cm.

Estimation of non protein thiol content (NP-SH):

0.5 g fresh samples were homogenized in 5 ml of 5% meta-phosphoric acid and centrifuged at 12000 rpm. Reaction mixture was prepared containing 0.5 ml plant extract, 2.5 ml of 150mM phosphate buffer (pH 7.4), 5 mM EDTA, 0.5ml 6mM 2- nitro benzoic acid. Following incubation at RT, the OD were measured at 412 nm. Calculation was done from the standard curve of reduced GSH (Cakmak and Marchner 1992).

Assay of Antioxidative isozymes:

Glutathione reductase: 200 mg leaf was homogenized in 50 mM Tris HCl buffer (pH 7.6). and centrifuged at 14000 rpm. The reaction mixture in total volume of 1 ml contained 50 mM Tris –HCl buffer pH 7.6, 0.15 mM NADPH, 1mM GSSG (oxidized glutathione), 3mM $MgCl_2$ and 200 µl enzyme extract. Reaction was monitored by decrease in absorbance of NADPH at 340 nm. The specific activity of enzyme was expressed as µmol NADPH oxidized /min/mg protein (Schaedle and Bassham 1977).

Superoxide dismutase: The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine,75 µM nitroblue tetrazolium, 2 µM riboflavin, 0.1 mM EDTA and a suitable aliquot of enzyme extract. The test tubes were shaken and placed 30 cm below light source consisting of 15 W fluorescent lamp for reading the absorbance at 560 nm. The activity of SOD was expressed as unit per milligram protein. One unit of activity is the amount of protein required to inhibit 50% initial reduction of nitroblue tetrazolium under light (Beauchamp and Fridovich 1971).

Zymogram Analysis of Guiacol Peroxidase (GPX) [EC No: 1.11.1.7]: In gel activity of Guiacol Peroxidase was done following the method of Reddy *et al*. (1971). The gel was incubated in 0.1 (M) Na-phosphate buffer for 30 mins at 4° C and stained with the staining solution till the appearance of brown bands. The reaction was stopped by washing with water after the colour has reached its desirable intensity.

Zymogram Analysis of Superoxide Dismutase (SOD) [EC No:1.15.11]: Zymogram analysis of super oxide dismutase was done according to the method of Beauchamp and Freidovich (1971). The gel was first soaked in Solution A containing (MTT) for 25 mins in dark (30° C) and then the solution incubated almost for one hour under intense light with Solution B containing (TEMED and riboflavin) till the achromatic zones appeared against blue background.

Zymogram Analysis of Ascorbate Peroxidase (APX)[EC No: 1.11.1.11]: In gel activity of APX was done according to the method of Chat Field and Dalton (1993). The gel was pre run for 30 minutes in electrophoresis buffer containing 2mM ascorbate. The gel was soaked with 50mM Na-PO_4 buffer (pH 7.0) containing 2mM ascorbate for 30 minutes and incubated in 50mM Na- PO_4 buffer (pH 7.0)

containing 4mM ascorbate and 2 mM H_2O_2 for 20 minutes. Gel was washed with 50 mM Na-PO_4 buffer (pH 7.0) for 1 minute. After this the gel was stained with a solution of 50 mM Na-PO_4 buffer (pH 7.8) having 28mM TEMED and 2.45 mM NBT with gentle agitation for 10 min. APX activity was observed by the formation of achromatic bands on a purple background.

Zymogram Analysis of Glutathione Reductase (GR) [EC: 1.8.1.7]: Zymogram analysis of Glutathione Reductase was done according to the method of Hou Wen-Chi *et al*. (2004). Leaf samples were homogenized in 50 mM Tris-HCl buffer (pH 7.9). The gel was dipped in substrate solution containing 4 mM oxidized glutathione, 1.5 mM NADPH, 2mM nitrobenzoic acid for 20 min. Then the gel was negatively stained by incubating the gel with staining solution (1.2 mM MTT, 1.6 mM PMS) for 10 min in dark. The contrast between the clear zone of GR activity and the purple background was found immediately.

Statistical analysis: All measurement was made on samples drawn in triplicate. Variance analysis (ANOVA) was performed on experimental data. For mean seperations, Duncan's multiple test (DMRT) was used at $p \leq 0.05$.

RESULTS

The metal accumulating capacity of this plant grown *ex situ* varied specifically (Table 1). For Cr it was observed that metal concentration in roots increased with soil metal concentration (28.38 mg/kg to 73.77 mg/kg root Cr corresponded to 25.6 mg/kg to 93.7 mg/kg soil Cr). But accumulation by the shoots were not directly proportional to soil Cr concentration, here; maximum accumulation (53.13 mg/ kg) was observed in 1.2 mM (62.0 mg/kg soil) Cr treated plants, then, with increasing concentration accumulated Cr concentration in shoots decreased. At 1 mM and 1.2 mM concentration the accumulation by the shoots were more than the roots (TF> 1), a characteristic property of hyperaccumulators (Baker 1981)

For Cd, the accumulation by the roots and shoots did not increase linearly along with the external Cd concentration. Here, accumulation by the roots increased maximally at 1 mM concentration (98.11 mg/kg root Cd at 112.5 mg/kg soil Cd), after that at 1.2 mM concentration root Cd content decreased, then increased marginally at 1.8 mM concentration of Cd (93.58 mg/ kg root Cd at 202.34 mg/kg soil Cd). Cd, accumulation by the shoots also varied with soil Cd concentration; maximum accumulation was observed at 1 mM concentration (117.3 mg/kg shoot Cd), then it decreased gradually. In 0.8 mM and 1 mM concentration of Cd, accumulation by the shoots was more than the roots, showing TF > 1. So it can be said that in regulated condition this plant can act as hyperaccumulator. Another important observation of this study was that *A. philoxeroides* can tolerate very high concentration of Cd in soil (phytotoxic level - Cd: 5-30 mg/kg, Cr: 5-30 mg/kg; Kabata- Pendias 2001) under laboratory condition, but tolerance towards Cr was observed maximum in field condition (1833.3 mg/kg soil Cr). Soil pH, CEC, OC also have an important role for this differential absorption capacity of metals.

Soil Parameters:

As metal bioavailability depends on the edaphic factors; soil pH, electron conductivity (EC), organic carbon (OC) and cation exchange capacity (CEC) of Cd treated soils used in pot for growing *A. philoxeroides* was measured (Fig 1). Different parameters indicate how these factors control Cd availability to the plant. pH of all treatment was uniform and more or less acidic to neutral. Lowest

EC (0.82 ms) was observed at 1.2 mM Cd concentration. OC content varies from 1.25% -1.9% having highest (1.9 %) in 1.0 mM treatment. Highest cation exchange capacity (29.8 $Cmol_c/kg$) was observed at 1 mM Cd concentration which was 3.8 folds in comparison to control set (7.8 $Cmol_c/kg$).

Our result indicates (Fig 2) how Cr availability depends different soil properties. pH of Cr treated soil was mildly acidic to neutral. But acute acidic pH (5.56) was recorded in 1.2 mM Cr treated soil. Electron conductivity (EC) varied from 1.02 – 5.04 ms. OC (organic carbon) content was maximum (1.8%) in the soil of 0.8 mM Cr concentration. CEC showed huge variation among Cr treated soils. CEC increased initially and reached the highest peak (22.7 $Cmol_c/kg$) at 0.8 mM Cr treated soil then declined gradually.

Free Proline Content

Alternanthera philoxeroides grown over a period of 10 days with increasing concentration of Cd and Cr, revealed that there was a significant increase in free proline content in comparison to the control plant (Fig 3). Irrespective of the type of metals proline accumulation was found to be linear dose dependent with respect to control plant (40.61 µg/ g tissue FW), reaching a peak at 1.2 mM (271.68 µg/ g tissue FW) of simultaneous Cd, Cr treatment and then showed abrupt decline.

MDA content:

MDA (malonyldialdehyde) is the ultimate product of oxidative damage of membrane lipids. In the present study, MDA content increased significantly when plants were subjected to Cd and Cr compared to control (Fig 4). In each case, synergistic effect of both Cd and Cr was much more pronounced than their individual effect. Maximum MDA content (3.2 µM / g leaf tissue FW) was observed in 1.8 mM Cd and Cr co- treated plants which was almost 11.42 times higher than the MDA content (0.28 3.2 µM / g leaf tissue FW) of plants grown under control condition. A noticeable difference was found in the alteration of MDA content among two metals. Cr was found to be the better inducer of MDA content in alligator weed.

NP-SH content:

With the increase in the metal concentration, NP-SH content showed sharp and linear increase, reached the peak at 1.2 mM concentration and then declined (Fig 5). In comparison to the control plant (4.55 µg SH group / g leaf tissue FW) the highest non protein thiol content (37.8 µg SH group / g leaf tissue FW) of 1.2 mM combined Cd, Cr treated plant was almost 8.3 folds higher. Though combined treatment of Cd and Cr caused much enhancement in NP-SH accumulation than individual treatment, it is evident from our result that Cd is better inducer of NP-SH production.

Glutathione Reductase activity:

Under low concentration of Cd, Cr treatment (0.5 mM and 0.8 mM) GR activity enhanced not too significantly but at combined treatment of 1.2 mM Cd and Cr, GR activity increased 27 folds (0.35 µmol NADPH oxidized /min/mg protein) in comparison to control plant (0.013 µmol NADPH oxidized /min/mg protein) (Fig 6). After reaching the highest activity at 1.2 mM concentration, GR activity showed sharp decline. It is noticeable that Cd induced much more elevation of GR activity than single Cr, and simultaneous Cd Cr treatment.

Superoxide Dismutase activity:

At low metal concentration SOD activity increased linearly and became highest at 1 mM concentration (28.5 Unit SOD/ mg protein), which

was 3.5 times higher than the SOD activity shown by the control plant (8 Unit SOD/ mg protein) (Fig 7). But further increase in metal concentration caused reduction of SOD activity. Lowest activity (7.64 Unit SOD/ mg protein) was observed in plants treated simultaneously with 1.8 mM Cd and Cr which was comparable to control plant.

Zymogram analysis of *A. philoxeroides* treated with Cd and Cr:

Elevated expression of GPX under synergistic effect of both Cd and Cr treatment (O, P,Q and R lane) was evident from our result by the appearance of a new band (R.f. 43.75) in comparison to control plants (Fig 8).

The plants treated separately with 0.5 mM, 0.8 mM, 1mM Cd and Cr of same concentrations had a single band (R.f 28.57) like control plants. But the increased activity of SOD (Fig 9) under higher concentration of separately Cd (1.2 mM, 1.5 mM), Cr treated (1.2 mM, 1.5 mM and 1.8 mM) plants and in all concentrations of combined treatment was evident by appearance of one new band (R.f 24.34) in comparison to a single band (R.f 28.57) of control plants. The zymogram analysis of SOD supports our previous observation where enhanced SOD activities of *A. philoxeroides* under combined Cd, Cr stress was documented.

Table 1 : Metal concentration of different parts of treated plants

Conc. (mM)	Cr					Cd				
	Soil mg/kg	Root mg/kg	Shoot mg/kg	TF	BCF	Soil mg/kg	Root mg/kg	Shoot mg/kg	TF	BCF
Cont	0.033± 0.015	BDL	-----	-------	------	-------	-----	------	-----	-----
0.5	25.6 ± 3.2	28.38 ± 2.54	26.38 ± 1.27	0.93	1.11	56.3 ± 1.8	29.98 ± 1.31	22.63 ± 2.51	0.75	0.54
0.8	41.6 ± 2.8	39.53 ± 1.33	35.53 ± 0.63	0.898	0.95	90.0 ± 2.3	86.71 ±0.55	107.8 ±0.789	1.24	0.97
1.0	52.0 ± 1.1	46.13 ± 1.2	47.31 ±0.91	1.025	0.89	112.5 ± 1.67	98.11 ± 1.2	117.3 ±0.916	1.2	0.88
1.2	62.0 ± 2.2	51.81 ± 0.8	53.13 ± 1.0	1.02	0.84	135 ± 3.3	73 ± 2.17	67.91 ± 2.15	0.93	0.54
1.5	80.0 ± 1.98	69.85 ± 1.73	46.48 ± 1.3	0.665	0.88	168.7 ± 2.0	87.162 ± 1.56	59.51 ± 1.46	0.68	0.52
1.8	93.7 ± 1.2	73.77 ± 0.82	32.31 ± 1.0	0.505	0.79	202.34 ± 1.5	93.58 ± 0.97	36.38 ± 1.04	0.38	0.47

The data represents means ± SD of three independent replicas. BDL: Below detectable limits

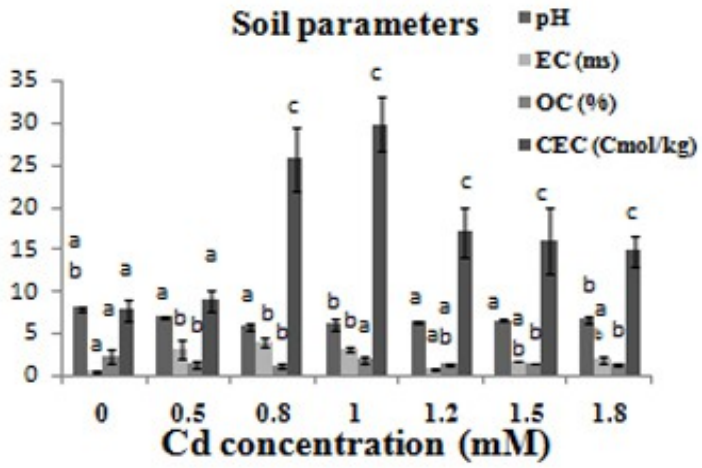

Figure 1 : Comparative account of soil parameters of Cd treated experimental pots

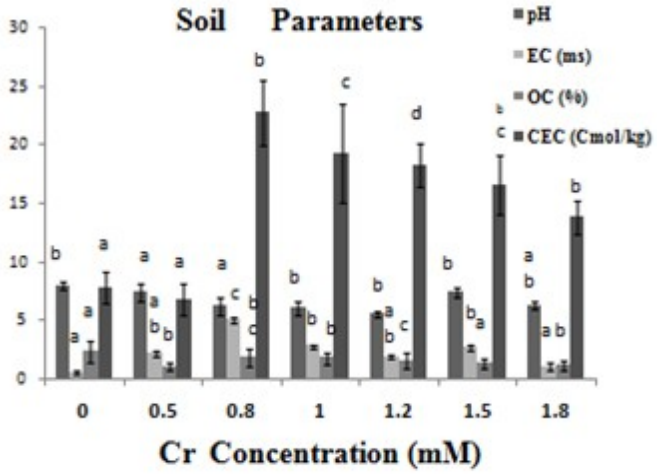

Figure 2 : Comparative account of soil parameters of Cr treated experimental pots

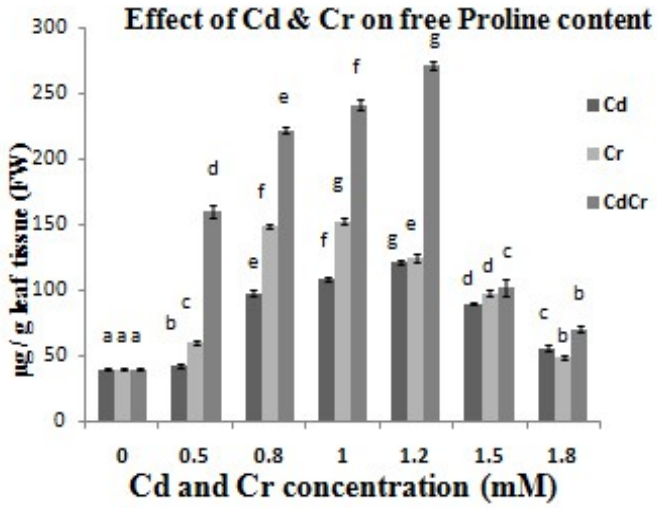

Figure 3 : Individual and synergistic effect of Cd and Cr on free proline content of *A. philoxeroides*

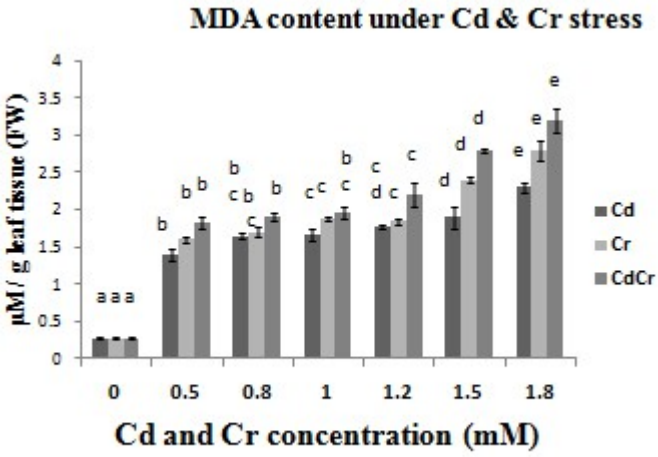

Figure 4 : Individual and synergistic effect of Cd and Cr on MDA content of *A. philoxeroides*

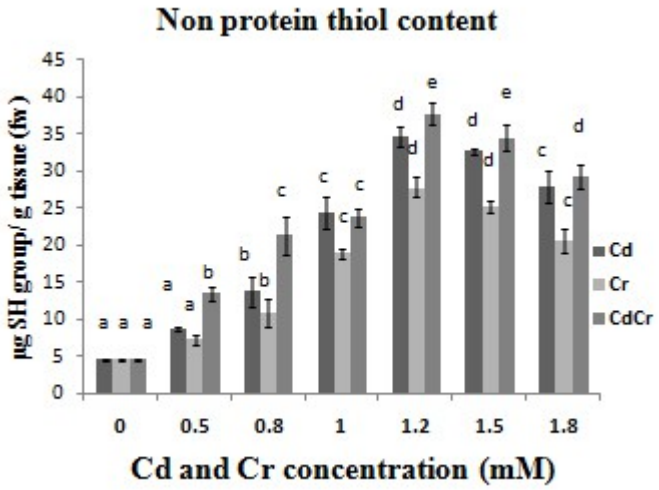

Figure 5 : Individual and synergistic effect of Cd and Cr on non protein thiol content of *A. philoxeroides*

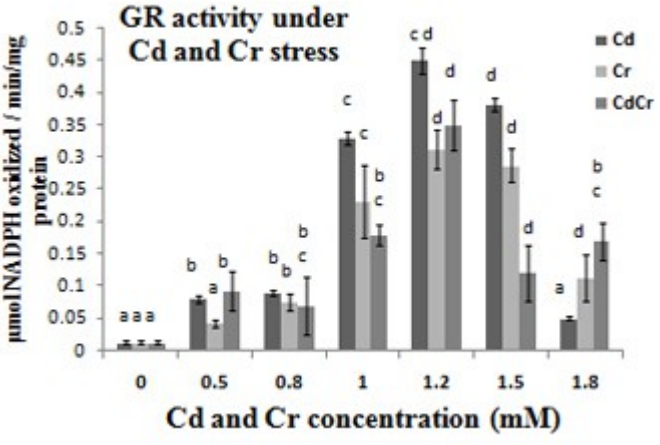

Figure 6 : Individual and synergistic effect of Cd and Cr on Glutathione reductase activity of *A. philoxeroides*

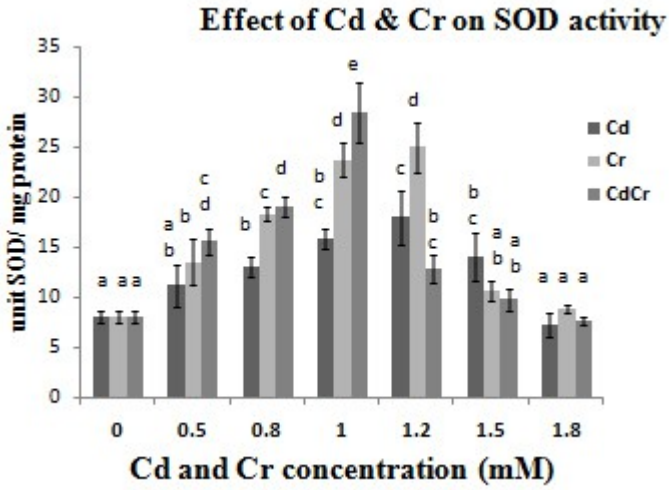

Figure 7 : Individual and synergistic effect of Cd and Cr on Superoxide dismutase activity of *A. philoxeroides*

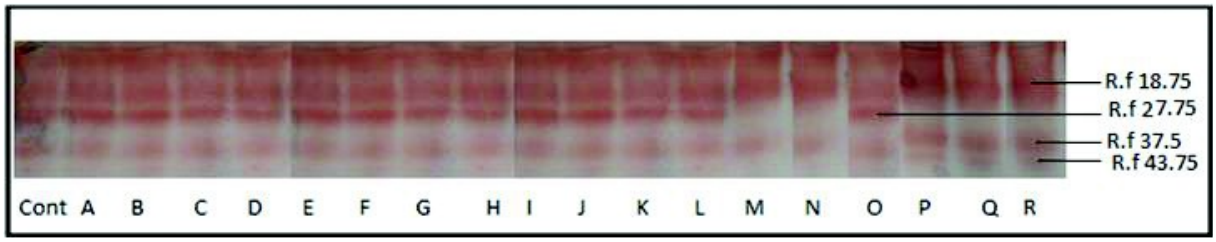

Figure 8 : Zymogram analysis of Guiacol peroxidase (GPX) of *A. philoxeroides* treated with Cd and Cr

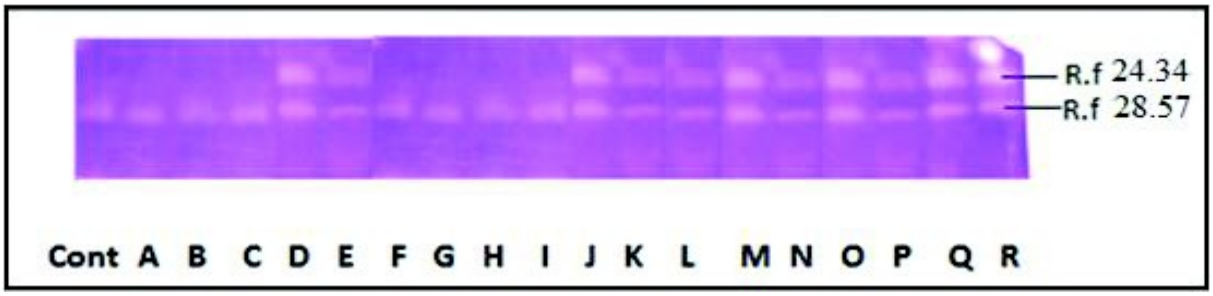

Figure 9 : Zymogram analysis of Superoxide dismutase (SOD) of *A. philoxeroides* treated with Cd and Cr

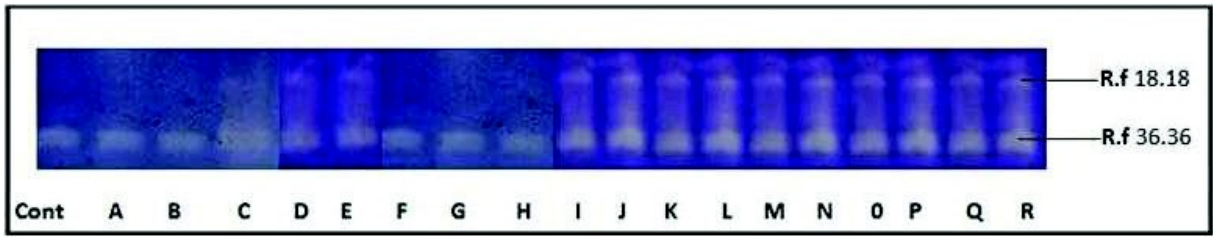

Figure 10 : Zymogram analysis of Glutathione reductase (GR) of *A. philoxeroides* treated with Cd and Cr

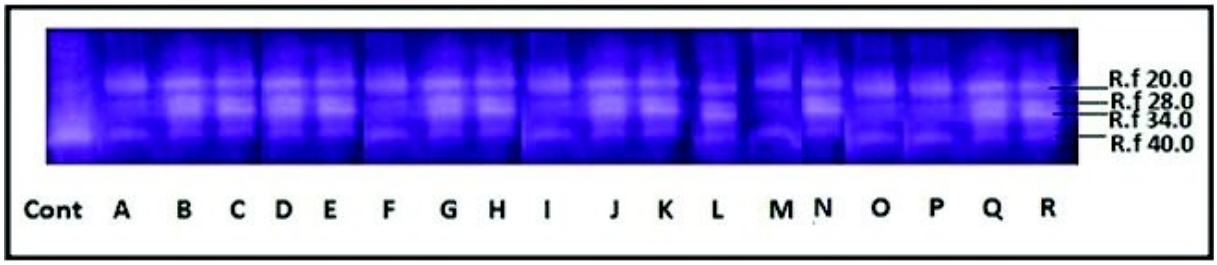

Figure 11 : Zymogram analysis of Ascorbate peroxidase (APX) of *A. philoxeroides* treated with Cd and Cr

A: 0.5 mM CdCl2 (56.3 mg Cd/kg soil)
B: 0.8mM CdCl2 (90 mg Cd/kg soil)
C: 1 mM CdCl2 (112.5 mg Cd/kg soil)
D: 1.2 mM CdCl2 (135 mg Cd/kg soil)
E: 1.5 mM CdCl2 (168.7 mg/kg soil)
F: 1.8 mM CdCl2 (202.34 mg Cd/kg soil)
G: 0.5 mM K2Cr2O7 (25.6 mg Cr/kg soil)
H: 0.8 mM K2Cr2O7 (41.6 mg Cr/kg soil)
I: 1 mM K2Cr2O7 (52 mg Cr/kg soil)
J: 1.2 mM K2Cr2O7(62 mg Cr/kg soil)
K: 1.5 mM K2Cr2O7(78 mg Cr/kg soil)
L: 1.8 mM K2Cr2O7(93.7 mg Cr/kg soil)
M: (28 mg Cd, 13 mg Cr / kg soil)
N: (45 mg Cd, 21 mg Cr/ kg soil)
O: (56.3 mg Cd, 26 mg Cr /kg soil)
P: (67.5 mg Cd, 31.3 mg Cr/kg soil)
Q: (84.4 mg Cd, 39 mg Cr/ kg soil)
R: (97.3 mg Cd, 47 mg Cr/ kg soil)

Like control plant the normal activity of GR was found in plants treated with 0.5mM, 0.8mM, 1mM Cd and 0.5mM, 0.8 mM Cr separately, evident by the presence of a single band (R.f 36.36) (Fig 10). Higher concentration of Cd/Cr and synergistic application of Cd and Cr induced elevated expression of GR, which was proved by the appearance of another extra band (R.f 18.18) in comparison to control plants.

Enhanced ascorbate peroxidase (APX) activity of

Cd, Cr treated *A. philoxeroides* provides metal detoxification capacity to this plant is well documented in our result (Fig 11). In comparison to a single band (R.f 40.40) of the plants grown under control condition four isoforms were observed almost all in individually (Cd/ Cr) and synergistically (Cd+ Cr) treated plants.

DISCUSSION

The present study indicated that *A. philoxeroides* can accumulate considerable amount of Cd and Cr under laboratory condition and it was also well established from *in situ* study also (Pal and Kundu 2011). But, the amount of metal accumulated by the root and shoots varied greatly for these two metals. It was observed that the root metal content increased upto 1.8 mM and 1 mM respectively for Cr and Cd treated plants, showing a linear relationship. On the other hand shoot metal content also increased with increasing soil metal concentration upto 1.2 mM and 1 mM for Cr and Cd. Metal bioavailability and bioaccumulation is governed by several environmental factors, such as chemical speciation of the metal, pH, organic chelators, humic substances, presence of other metals and anions, ionic strength, temperature, salinity, O_2 level and other prevailing electrochemical functions (Greger 1999). In this study the accumulation pattern was also governed by these factors. At 0.8 mM soil Cr concentration almost 95% of soil Cr was accumulated, this enhanced uptake was facilitated by low pH value (6.18). Along with pH, CEC of soil also play an important role in metal accumulation which is evident from our study. Plants treated with 1.2 mM Cr showed lesser (83.5%) metal uptake due to low CEC value (18.27 $Cmol_c$/kg; pH-5.25). This indicates that an elevated CEC and acidic pH of the soil might help the plant to uptake greater metal content, while the reverse might have a role in limiting the metal availability to them. Similar observation obtained from Cd treated plants also supported this. In case of Cd at 0.8 and 1 mM soil Cd concentration plants satisfied both the criteria of being a hyperaccumulator for Cd, regarding metal accumulation (> 100 mg/kg in aerial part) and translocation capacity (TF>1). Here low soil pH (pH 5.8) along with high CEC value (25.7 $Cmol_c$/kg) made Cd more bioavailable to the plants. So, it can be said that the same plant can behave as a hyperaccumulator and excluder, under variable soil condition. At higher concentration of soil Cd and Cr, maximum metal accumulation was observed in the underground parts. This result is in accordance with the observation of Gupta and his coworkers (1994), where he has reported that roots *Bacopa monnieri* and *Scirpus lacustris* accumulated 1600 mg/kg and 739 mg/kg Cr respectively after 7 days exposure. Roots act as a barrier against heavy metal translocation and this is one of the tolerance mechanisms present within the root system. There are ample evidences which showed significant amount of metals in the roots of aquatic plants exposed in externally supplied metal solution (Singh *et al*. 2006; Majid *et al*. 2011). Heavy metal tolerance with high TF and BCF value was suggested for phytoaccumulation of contaminated soils (Yoon 2006).

Plant cell membranes are considered as the primary sites of metal injury. Membrane distillation is frequently attributed to lipid peroxidation, which can be initiated by AOS or by the action of lipoxygenase (Halliwell and Gutteridge 1984). Measurement of malondialdehyde (cytotoxic product of lipid peroxidation) is routinely used as a sensitive index of oxidative stress due to heavy metal toxicity. Our result is in pretty good

accordance with the findings of various workers who showed progressive increase in MDA content with increasing Cd and Cr concentration (Srivastava 2011; Bah *et al*. 2011; Tantrey and Agnihotri 2010; Pandey and Singh 2012, Pal and Kundu 2011). In each case Cd induced higher membrane damage than Cr, but the synergistic effect was much more devastating, resulting extremely high lipid peroxidation. If free proline accumulation and lipid peroxidation are considered, their levels were found to be inversely proportional. At low concentration of treatment (0.5mM – 1.2 mM) the enhanced accumulation of proline resulted moderate amount of MDA formation. While at higher concentration (1.5 mM, 1.8 mM), sharp decline of proline content failed to give enough antioxidative protection, resulting maximum lipid peroxidation (3.2 µM/ g FW). Proline mediated reduction of lipid peroxidation is in agreement with previous and recent findings (Mehta and Gaur 1999; Singh *et al*. 2010).

Plants exhibit both enzymatic and nonenzymatic mechanisms for ROS scavenging. Proline and non protein thiol (NPSH) are two important non enzymatic ROS scavenging mechanism of plants. One of the important mechanisms by which plants respond and apparently detoxify toxic heavy metals is the production and accumulation of free proline which acts as an osmoprotectant (Delauney and Verma 1993; Taylor 1996), protein stabilizer, metal chelator (Farago and Mullen 1979), inhibitor of lipid peroxidation (Mehta and Gaur 1999) and OH^- scavenger (Alia *et al*. 2001). The metal tolerance capacity of *A. philoxeroides* was reflected by the enhanced production of free proline both in *in situ* (Pal and Kundu 2011) and *ex situ* study. In comparison to control plants, simultaneously treated plants with both Cd and Cr (1.2 mM) showed 6.7 folds higher proline (271.68 µg/ g FW) production which was also 1.8-2.2 times higher than the proline content of individual Cd, Cr treated plant. Though it was previously reported that Cd is the strongest inducer of proline among Pb, Zn, Co, Hg (Arora and Saradhi 1995; Handique 2009), our findings suggested that Cr acted as better inducer than Cd in *A. philoxeroides*. Elevated proline accumulation, might have ameliorated the oxidative damage due to the generation of ROS under Cd and Cr. Our result is in conformity with the findings of previous workers (Rai *et al*. 2004; Vernay *et al*. 2008), where they have concluded that; being more toxic than Cr (III), Cr (VI) could be better inducer of proline than Cd.

Glutathione is a major NPSH and possess strong antioxidative properties and directly involved in the synthesis of Cd binding peptide phytochelatins. Highest NPSH content of 1.2 mM treated plant showed 8.3 (37.8 µg/ g FW) times increase under synergistic effect of both Cd and Cr , while individual application of Cd alone showed 7.6 folds (34.6 µg/ g FW) increase. Our result is in accordance with reports of other workers (Wang *et al*. 2010; Pandey and Singh 2012) that Cd is a better inducer of NP-SH. *A. philoxeroides* exhibited higher amount of NPSH synthesis during Cd exposure indicated its ability to tolerate cellular metal load, which might be due to the promotion of PC biosynthesis. The sharp decline of NPSH content after the optimum metal concentration might be due to the imbalance between NPSH production capacity and its consumption for GSH and PC synthesis under unbearable metal toxicity.

Peroxidase, SOD and GR are the main components of enzymatic defense of the plant. Superoxide dismutase plays a pivotal role in the first line of defense against ROS, reducing the oxidative

stress by the dismutation of two superoxide radical to H_2O_2 and O_2. H_2O_2 is further converted to H_2O and O_2 by peroxidases. Elevated level of APX and GPX activity was also recorded in the Cd treated *B. monnieri* roots and leaves (Weckx and Clijsters 1996) suggesting its role in the detoxification of H_2O_2.

Enzyme assay: Being a key enzyme of ascorbate glutathione cycle, that protects cell against oxidative damage, GR is responsible for the continuous maintenance of GSH pool for uninterrupted supply of phytochelatin, that explains the dose dependent increase of GR activity upto a optimum concentration in our result. The highest GR activity of 1.2 mM Cd treated plants is corroborated with the highest NPSH content of the same. Increased GR activity maintains high GSH/GSSG ratio and thus play a bridging role between primary and secondary responses of the plants against metal toxicity. It allows ascorbate-glutathione cycle to operate at high rate to detoxify ROS and provide substrate for PC biosynthesis. The decline of GR activity after optimum metal concentration could be due to the sensitivity of thiol pool to Cd.

Treated plant showed concomitant increase in SOD activity under low concentration of metals and became highest at 1mM treated plant. Our result corroborated with the concentration and time dependent increase in SOD activity of Indian mustard (Wang 2008), suggesting that metal accumulators are equipped with higher level of SOD activity conferring ROS scavenging activity. Under oxidative damage induced by Cd and Cr these antioxidative isozymes (SOD, GR) harmonize to ameliorate the membrane damage evident by low MDA content in the particular treatment with higher SOD, GR activity.

Zymogram analysis of isozymes: With respect to the plant grown on control condition, treated plants showed distinct variation evident by the appearances of new bands of POX in combined treatments of Cd and Cr. This is because enhanced expression of this H_2O_2 scavenging enzyme is required for the dissipation of excess production of H_2O_2 under dual activity of Cd and Cr. Induction of peroxidase activity under Cd and Cr stress was reported by various workers (Radotic *et al*. 2000; Singh *et al*. 2006; Wang *et al*. 2008; Pandey *et al*. 2009).

The most important antioxidative isozyme SOD catalyzes the conversion of highly reactive superoxide radical (O_2^-) into H_2O_2, followed by the action of GPX and CAT to convert H_2O_2 into O_2 and H_2O. Appearance of one extra isoform confirmed the upregulation of SOD in plants exposed to combined treatment of Cr and Cd. The result of SOD activity of treated plants showed a good reflection in zymogram analysis. Higher concentration of single application of Cd (1.2 mM, 1.5 mM), Cr (1.2, 1.5, 1.8 mM) and all combined treatment of Cd, Cr showed enhanced SOD production.

Enhanced GR activitiy was observed in higher concentration of Cd and Cr treated plants, evident by similar type of banding pattern. Synergistic treatment had more acute and devastating effects which were reflected by the formation of new isoform at all concentration of combined treatment and also showed resemblance with the result of GR assay. Appearance of new isoforms was also reported by Yannarelli (2007) in Cd treated wheat.

Treated plant showed newly appeared bands of APX in higher concentration of Cd , Cr and almost all combined treatment of Cd and Cr both. Enhanced accumulation of cellular antioxidant such as ascorbate, glutathione under heavy metal stress

was pretty well reported and the resultant increase in APX activity is also well documented (Gajewaska and Sklodowsha 2007). If in gel activity of GR and APX are taken under consideration, it would be noticeable that with concomitant increase in single and combined doses of Cd, Cr the appearance of new isoforms followed the same pattern in both zymograms. The increase in APX and GR (evident by extra bands) under Cd and Cr exposure was also established by early worker (Pandey *et al*. 2009 in spinach), and it suggests an induction of ascorbate-glutathione pathway and this could have a relevant role in protecting cell against heavy metals.

Our study was mainly focused on the deadly effects of Cd, Cr on the physiology of *A.philoxeroides* and the estimation of certain relevant physicochemical parameters for the understanding of its metal tolerance and detoxification ability. Inspite of lipid peroxidation, the enzymatic (SOD, GR, APX, POX) and nonenzymatic (proline, NPSH) defense systems provide enough protection against oxidative damage upto a threshold level of Cd, Cr. It is evident from the study that plants have different mechanism to combat different metal toxicity. Alligator weed (*A. philoxeroides*) plants can cope the Cd burden of the soil by increasing its NPSH content, while for mitigating Cr toxicity increase in SOD activity was observed. Still much more information is needed at the sub-cellular and molecular levels in order to get a deeper insight into the reason of metal tolerance capacity of this plant.

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