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Exploring Phytoremediation Potential of *Vigna radiata* & *Vigna aconitifolia* Under Hexavalent Chromium Induced Stress in Hydroponics

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Abstract

A global concern regarding environmental pollution with chromium contamination of agricultural soil has set up a critical challenge as its accumulation is noxious. Toxicity of Chromium to plants depends on the valence state where hexavalent chromium; Cr(VI) is considered highly toxic. Therefore, present study investigates the phytoremediation potential of leguminous plants; *Vigna radiata* and *Vigna aconitifolia* for the reduction of Cr (VI) hydroponically. The study was designed by growing the two plants in varying concentration of chromium ranging from 100 mg kg^{-1} to 800 mg kg^{-1} and parameters were assessed for toxicity and remediation potential under hexavalent chromium stress. *V. radiata* as compared to *V. aconitifolia* showed a significant decrease in root and shoot lengths with increasing concentration with 17.31% & 78.85% and shoot length 26.39% & 72.22% in *V. radiata* at 100 mg kg^{-1} and 800 mg kg^{-1} respectively. Whereas; in *V. aconitifolia* it was found to be as 9.26% & 72.22% in root, 18.67% & 68% in shoot at 100 mg kg^{-1} and 800 mg kg^{-1} respectively at $p<0.05$. Translocation factor > 1 was observed in both the plants with maximum as 2 in *V. aconitifolia* at 600 mg kg^{-1} and maximum remediation percentage of 85.33% which makes it a better phytoremediator than *V. radiata*. Certain morphological aberrations such as necrosis of leaves and discoloration of roots and shoots were observed at 600 mg kg^{-1} and 800 mg kg^{-1} concentration of chromium. Therefore based on these observations *V. aconitifolia* can be considered as a potential phytoremediator and hyperaccumulator of Cr(VI) over *V. radiata*.

Keywords: Chromium stress, Phytoremediation, Leguminous plant, Hyperaccumulation, Phytotoxicity

Introduction

Increasing anthropogenic activities causes a widespread contamination of most ecosystems, with high dispersion rates of many contaminants including heavy metals. Countries are engaged into mining of ores working in collaborations with the industries has led to a widespread distribution and deposition of metal contaminants in soil. Wetlands and natural depressions get contaminated by subsequent deposition of heavy metals. Chromium (Cr) contamination is particularly increasing and posing a serious threat to the environment and all life forms. Soil pollution caused by noxious pollutants being released in to the environment by sources such as dyes-pigment manufacturing, chrome plating industries, leather industries, wood preservation etc. of which chromium is the most common pollutants, is of great concern (1)(2). Leather in India is extensively produced with about 80% involved in chrome tanning process releasing about 2000-32000 tonnes of elemental chromium in the environment (3) (4). International Agency for Research on Cancer has recognized Cr(VI) as a carcinogen based on sufficient evidences of carcinogenicity in humans and categorized it in Group I (5). Trace amounts of chromium is essential to all life forms and is considered as a major micronutrient involved in many physiological processes such as fat and glucose metabolism, proper functioning of insulin etc. Existence of chromium in environment is reported to be found in its valence state (-2 to +6); with Cr (III) and Cr (VI) are the most stable ones. Due to the binding of Cr(III) to other organic compound present in environment, it is less mobile and hence it is not readily taken up by the plants. On contrary, Cr (VI) is hundred folds more noxious than Cr (III) as it usually occurs associated with O_2 as $CrO4^{2-}$ or $Cr_2O_7^{2-}$ chromate & dichromate respectively. Due to its high solubility in water, are highly mobile and are considered as most noxious forms (6) (7). It is reported to cause oxidative DNA damage and causes teratogenesis, and carcinogenesis in humans (8). Due to expanding use of Cr and other heavy metals in the industries and their discharge in environment, it is posing critical impact on human health. One of the most common being oxidative damage due to the binding of Cr(VI) to nucleic acids and proteins (8)(9). This leads to the need for optimising heavy metal remediation methods viz; solidification, excavation and landfilling that abrupt soil fertility and are costly and non-eco-friendly (10). Plants, through the mechanism of phytoremediation tend to significantly contribute to heavy metal remediation by sequestering them in roots and shoots (11). Plants possess enhanced capacity to tolerate elevated concentrations of heavy metals due to the presence of strong antioxidant defence system. Hyper tolerance therefore is a significant plant characteristic that aids in hyperaccumulation (12) (13).

Apart from the fact that legumes are rich in proteins, minerals and other nutrients they also aid in maintaining the overall nitrogen homeostasis in the soil. They contribute to increase the soil fertility by fixing nitrogen. In the present study, both the plants proved out be better translocators of chromium from root to shoot at varying concentrations (100-800mg kg^{-1}). Therefore, giving preference to these plants for phytoremediation and preparing them to counteract metal toxicities could serve the purpose better, which on one hand will improve soil fertility and on the other will remediate heavy metals. The present study shows the phytoremediation potential of *V. radiata* and *V. aconitifolia* against hexavalent chromium.

Materials and Methods

Plant growth experiment

Seeds of *Vigna radiata* and *Vigna aconitifolia* were surface sterilized with 70% ethanol and 0.1% mercuric chloride followed by germination and then grown hydroponically in Hoagland media in culture tubes (pH 6.5) with varying concentration of chromium (100ppm, 200ppm, 400ppm, 600ppm and 800ppm) using autoclaved sponge support (121°C, 15psi, 20 minutes) in triplicates. Negative controls with media and Cr(VI) and no seeds was used. Positive control was used to rule out any variation due to the absorption of nutrients from the media by the seeds which involved the growth of plant in the presence of media without Cr(VI). The seeds were grown for a period of 3 weeks and the culture tubes were covered and kept in plant growth room under controlled conditions for their growth with temperature between 25-30° C, with a temperature fluctuation of less than ±0.5°C and light intensity of 2000-2500 lux.

Determination of toxicity parameters:

Percentage decrease in root & shoot length

After 3 weeks of incubation, root and shoot lengths (cm) were observed and the percentage decrease was calculated with respect to the increasing chromium concentration.

Percentage decrease in Root & Shoot Length= (b-c/b) *100

b = Root/Shoot length of positive control

c= Root/Shoot length of plant with Cr(VI) stress

Determination of total chlorophyll content

The chlorophyll (chl) content was assessed inline by Arnon method (1949). To 1 gram of leaf (cut into small pieces), 10 ml 80% (V/V) acetone was added and homogenised to fine paste in a precooled mortar pestle. The extract was centrifuged at 3000 $rev.min^{-1}$ for 15 min and final volume made with up 80% (V/V) acetone to 25 ml. Optical density of the supernatant was measured at 645 nm and 663 nm, against an 80% acetone blank (Shimadzu 35 Double Beam spectrophotometer) and content (per gram fresh weight) was calculated from following equations (14);

chl 'a' (µ/g/ml) = (12.7 x O.D. at 663 nm) – (2.69 x O.D. at 645 nm)

chl 'b' (µ/g/ml) = (22.9 x O.D. at 645 nm) - (4.08 x O.D. at 663 nm)

Total chlorophyll (µ/g/ml) = (20.2 x O.D. at 645 nm) + (8.02 x O.D. at 663 nm)

Antioxidant enzyme estimation

In order to study the impact of hexavalent chromium on the antioxidant enzyme activity of the *V. radiata* & *V. aconitifolia*, the antioxidant enzyme activities were checked after 3 weeks of incubation against positive control. Enzyme extract was prepared by homogenizing 0.5 gm of fresh leaves in pre cooled mortar pestle in 5ml 100mM potassium phosphate buffer (pH 7.0) and centrifuged at 15000g/ 20 minutes and supernatant separated was further used for enzymatic assays.

Catalase activity assay: The enzyme activity was measured by the method of (Aebi, 1984). The reaction mixture (3 ml) contained 1.5 ml 50 mM potassium phosphate buffer at pH 7.0, 300µL enzyme extract and 1.2 ml H_2O_2. With three consecutive readings taken every minute, the consumption of H_2O_2 was monitored spectrophotometrically at 240 nm. And the activity was calculated from the following equation (15):

$$\text{Unit Activity (Units/min/g FW)} = \frac{\text{Change in abs./minute} * \text{Total volume (ml)}}{\text{Ext. coefficient} * \text{Volume of sample taken (ml)}}$$

Where, Extinction coefficient = $6.93 * 10^{-3}$ $mM^{-1}cm^{-1}$

Glutathione peroxidase activity assay: Reaction mixture (Tris buffer, sodium azide, EDTA, plant extracts; 2ml, 0.1 ml, 0.2 ml, 0.5 ml respectively) was assayed at 436 nm spectrophotometrically after addition of 0.2ml glutathione and 0.1 ml hydrogen peroxide (incubated 37°C for 10 minutes). The reaction was stopped by the adding of 0.5 ml of 10% trichloro acetic acid, centrifuged at 4000g and assayed for GPX activity; calculated by following equation (16).

$$\text{Glutathione peroxidase Unit activity} = \frac{\text{Change in absorbances } min^{-1} \times \text{total volume}}{*\text{Extinction coefficient} \times \text{weight of sample}}$$

Where, *Extinction coefficient = 25 $mM^{-1}cm^{-1}$

Assessing remediation potential of V. radiata and V. aconitifolia

Determination of metal accumulation in root and shoot

After 3 weeks of incubation, the samples were shade dried and ground to fine powder. Thereafter the samples were digested by the microwave assisted

digestion system (NuWav- Ultra); 1 gram of powdered sample in 5:1:1 ratio of 67% nitric acid, 30% hydrogen peroxide and MilliQ water & was subjected to microwave assisted digestion with max power 250 W, chromium content was determined by Inductively Coupled Plasma Mass Spectrometry (Agilent; 7700G).

Quantification of phytoremediation efficiency of plant

Bioconcentration Factor and Translocation Factor is the percentage decrease of pollutant Cr(VI)) in media and is calculated as;

BCF = Concentration of metal in plant/Concentration of metal in media (17)

Transfer of metals from roots to plant is Translocation Factor (TF)

TF = Concentration of metal in shoot/ Concentration of metal in root (17)

TF >1 is the indicator of the better phytoremediation potency of plant. Whereas, plants with TF value < 1 accumulate metals in the roots and die due to metal stress.

Calculation of percentage of remediation by respective species

After 3 weeks of germination, remediation percentage was calculated from the difference in initial concentration of the pollutant to the final concentration of the pollutant left in the media. For this, chromium content in media was determined spectrophotometrically (Shimadzu 1800, UV-Vis) by taking absorbance at 530 nm. A standard stock solution of 1000 mg kg^{-1} of K_2CrO_7 (Himedia, ≥99% purity) was prepared. A 0.2 N sulfuric acid (Thermo fisher scientific) was prepared. 1,5-diphenylcarbazide (DPC) solution by dissolving 250 mg of DPC (Sigma Aldrich, ≥99% purity) in 50 mL methanol was prepared. Working standard of Cr (VI) (100- 800 mg kg^{-1}) were prepared and calibration curve was plotted (18). The concentration of the chromium left after 3 weeks was calculated from the equation obtained from standard curve and remediation percentage was calculated w.r.t to the initial and final concentrations.

Data analysis

The experimental results were statistically assessed through analysis of variance (ANNOVA) at the significance level $p < 0.05$ by using R programming.

Results and Discussion

A significant decrease in percentage of root and shoot lengths were observed, with maximum decrease in *V. radiata* as compared to *V. aconitifolia*. Root and shoot lengths were recorded with decrease of 17.31% & 78.85% in root length and 26.39% & 72.22% of shoot length in *V. radiata* at 100 mg kg^{-1} and 800 mg kg^{-1} respectively. Whereas; in *V. aconitifolia* it was found to be 9.26% & 72.22% in root as well as 18.67% & 68% in shoot at 100 mg kg^{-1} and 800 mg kg^{-1} respectively (Figure 1-2). Percentage decrease in root and shoot lengths was higher in *V. radiata* as compared to *V. aconitifolia.* With increasing chromium stress, both the species showed significant decrease in root and shoot

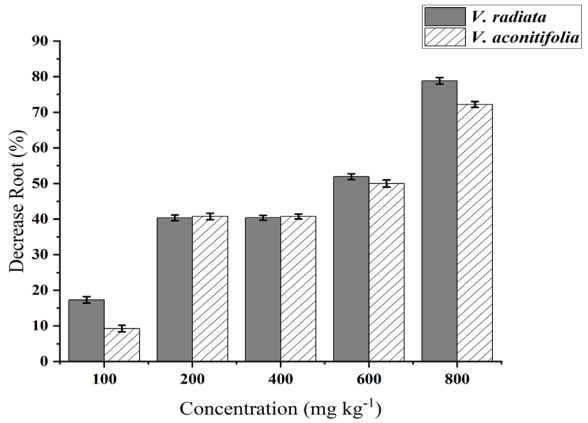

Figure1. Percentage decrease of root in V. radiata & V. aconitifolia *($p<0.05$)

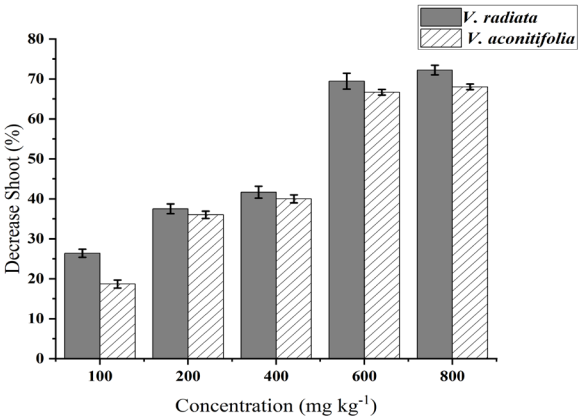

Figure 2. Percentage decrease of shoot in V. radiata & V. aconitifolia *($p<0.05$)

lengths after 400 mg kg^{-1}. Chromium as a pollutant showed a significant impact on root and shoot length of both the plants with $p<0.05$ where it was found to be decreasing with the increasing concentration. Drying of older leaves, chlorosis, and necrosis of young leaves, reduction in length of lateral roots, and browning of roots was observed. Both the species, *V. radiata* and *V. aconitifolia* showed a decline in growth with increasing concentrations of chromium. Decrease in root and shoot by heavy metals in various crops like *Salix viminalis, Caesalpinia pulcherrima,* rice, sorghum, Oats, *Curcuma sativa, Lactuca sativa, Panicum miliaceum, Sinapsis alba* (400 mg kg^{-1}) is well studied (19)(20). Root primordia of *Salix viminalis* showed heavy metal toxicity in order of Cadmium>Chromium>Lead whereas root length was greatly affected by chromium than cadmium and lead (21). In a study on mung bean cultivated on chromite mine soil showed significant reduction in root and shoot length after 28 days of root emergence (22). This could

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be due to development of Cr speciation causing inability of roots to absorb nutrients (23). Upon increase in Cr concentration in medium, oat plants showed decrease in shoot growth by 41% (24). The reduction in shoot length may be due to reduced root growth which disturbs translocation of nutrients (23).

Total chlorophyll content, in general, showed gradual decrease with increase in chromium concentration as compared to its control from 15.95 mg/ml & 15.77 mg/ml in control to 4.16 mg/ml & 3.87 mg/ml at 800 mg kg^{-1} concentration in *V. radiata* & *V. aconitifolia* respectively. Chlorophyll content as compared with positive control, was found to be low with increasing Cr concentration in *V. aconitifolia* than *V. radiata* (Figure 3). This could be attributed to the fact that Chlorophyll biosynthesis is inhibited by heavy metals, particularly by lowering down the activity of amino levulinic acid dehydrogenase and protochlorophyllide reductase (25). This fact is also supported statistically using single factor ANNOVA where value of $p<0.05$ shows that there is significant impact of increasing chromium concentration on total chlorophyll content of plant. A similar trend has been reported in *Oryza sativa* L in which total chlorophyll content decreased significantly with increasing chromium concentration (26). A decrease in the chlorophyll content with increasing chromium concentration (from 0.35 mg/gm of tissue at 1 ppm to 0.24 mg/gm of tissue at 10 ppm) was also observed in the seedlings of *Vigna radiata* (27). Chromium negatively impacts enzyme aminolevulinic acid dehydratase which impacts ALA resulting in decrease in Chlorophyll levels (28). Electron inhibition during photosynthesis could also be a reason to lower levels of Chlorophyll (29).

Catalase activity was found to increase with increasing heavy metal concentration with maximum activity; 52 Umin^{-1}g^{-1} & 62.53 Umin^{-1}g^{-1} observed at 400 ppm and 600 ppm for *V. radiata* and *V. aconitifolia* respectively (Table 1). After which it was found to decrease which may be due inactivation of enzyme protein by ROS. Catalase is an antioxidant enzyme, the synthesis of which increases with stress. Chromium had a significant impact on catalase activity in both the plants at $p<0.05$. A significant increase in the catalase activity was seen in *Amaranthus viridis* and *Parthenium hysterophorous* under hexavalent chromium stress (30). Free radicals within the cells generate ROS which causes oxidative stress, lipid peroxidation, membrane damage and inactivation of enzymes (31). Hydrogen peroxide if not eliminated from the cells, could convert into a hydroxyl radical which damages plant cell (32). Catalase breaks down H_2O_2 and restricts the formation of reactive hydroxyl radicals inside the cell. Catalase activity is greatly enhanced by zinc and chromium, which suggest that catalase provides defence mechanism under metal stress to plants (30).

There was a consistent increase observed in glutathione peroxidase activity with increasing heavy metal concentration in both *V. radiata* & *V. aconitifolia* and was found to be significant at $p<0.05$ with highest at 800 mg kg^{-1} as 57.6 Umin^{-1} g^{-1} & 56.64 Umin^{-1} g^{-1} respectively with blank to be observed as 14.4 Unit min^{-1} g^{-1} and 16.32 Unit min^{-1} g^{-1} (Table 2). GPXs are present in the various subcellular organelles in plants and are known to catalyse reduction of hydrogen peroxide and organic peroxides etc.(33). A increase in significant GPX activity was found in *Allium cepa* with various heavy metals including chromium (34), in the leaves and roots of *Kandelia candel*, in the leaves and stems of *Bruguiera gymnorrhiza*. (35). A consistent increase in glutathione peroxidase activity was seen with increasing heavy metal concentration in both *V. radiata* and *V. aconitifolia*. Though in some plants, GPX activity is reported to decrease with increase in HM stress, therefore according to literature it can be said that GPX activity in response to heavy metal exposure is diverse, and depends on

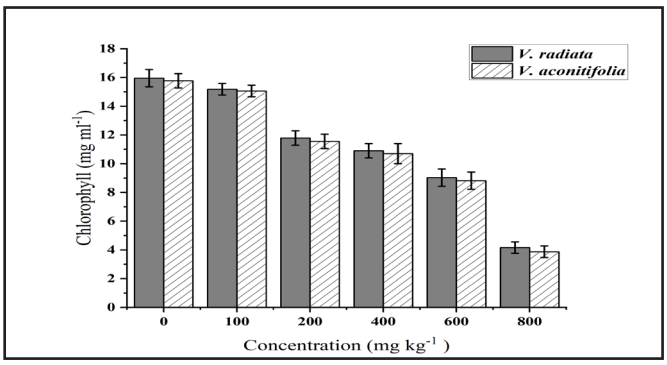

Figure 3. Chlorophyll content in *V. radiata and V. aconitifolia* *($p<0.05$)

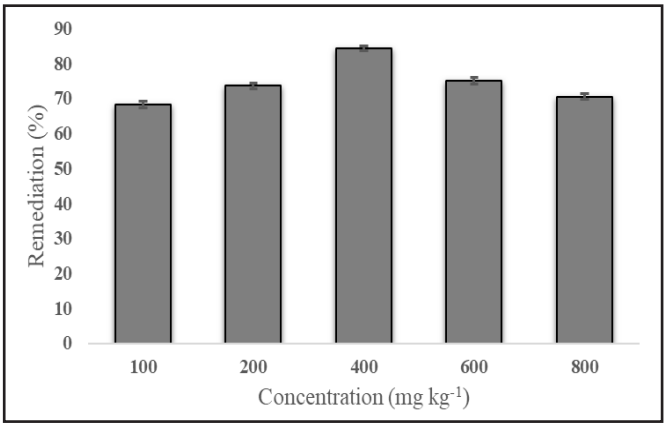

Figure 4. Percentage Remediation in *V. radiata*

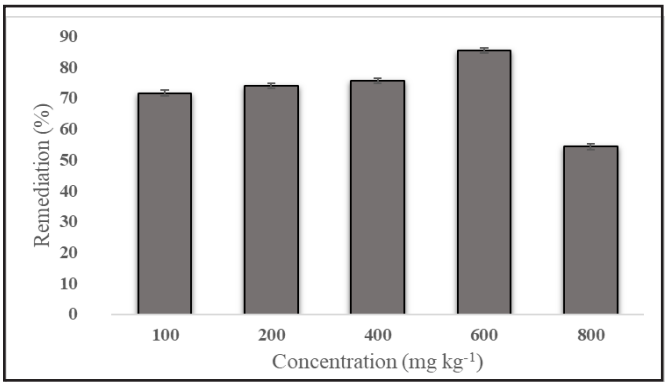

Figure 5. Percentage Remediation in *V. aconitifolia*

from which metal plant is impacted (36).

There was an increasing trend seen in the percentage remediation up to the concentration of 400 mg kg^{-1} as 84.22% in *V. radiata* and 600 mg kg^{-1} as 85.33% in *V. aconitifolia* and thereafter, a decline in percentage remediation was observed (Figure 4 and 5). It could be attributed to the fact that at a concentration greater than this, higher toxicity was induced in the plants therefore, reduced plant growth and remediation were observed. Phytoremediation of various species of Vigna family viz., *V. radiata* and *V. mungo* is largely been studied. According to a study it was clear that *V. mungo* showed remediation percentage of 51.58% of Cr(VI) which was higher as compared to *V. radiata* (37). In a study on Phyto-stabilisation of plants against lead stress,

Table 1. Catalase Activity in *V. radiata and V. aconitifolia*

Metal Concentration (ppm)	*V. radiata* Catalase activity (Umin^{-1} g^{-1})	*V. aconitifolia* Catalase activity (Umin^{-1} g^{-1})
0	18.27±0.23	19.24±0.33
100	33.7±0.21	48.1±0.22
200	48.1±0.34	52.91±0.34
400	52±0.24	58.68±0.42
600	48.1±0.32	62.53±0.23
800	24.05±0.25	33.67±0.31

*Significant at p<0.05

Table 2. Glutathione Peroxidase Activity in *V. radiata and V. aconitifolia*

Metal Concentration (ppm)	*V. radiata* Glutathione Peroxidase activity (Umin^{-1} g^{-1})	*V. aconitifolia* Glutathione Peroxidase activity (Umin^{-1} g^{-1})
0	14.4±0.32	16.32±0.36
100	24.96±0.24	25.92±0.32
200	25.92±0.26	26.88±0.35
400	27.84±0.23	36.48±0.33
600	38.4±0.31	37.44±0.32
800	57.6±0.44	56.64±0.33

*Significant at p<0.05

Table 3. Translocation Factor and Bioconcentration Factor of *V. radiata and V. aconitifolia*

Concentration of chromium (ppm)	*Vigna radiata*		*Vigna aconitifolia*	
	TF	BCF	TF	BCF
0	0.72±0.76	0.01±0.56	0.8±0.64	0.01±0.33
100	1.43±0.43	2.15±0.53	1.7±0.34	2.54±0.25
200	1.67±0.26	3.05±0.34	1.9±0.35	3.56±0.43
400	1.37±0.33	3.71±0.42	1.7±0.32	3.75±0.54
600	1.28±0.37	2.49±0.55	2.0±0.54	4.05±0.60
800	1.60±0.21	2.44±0.39	1.8±0.35	3.13±0.79

Vigna radiata showed higher tolerance as compared to *Raphnus sativus* and *Cicer areitinum* (38). Study on *V. radiata* is largely been done for heavy metal remediation, although study on *V. aconitifolia* is not being reported so far. Also, *V. aconitifolia* showed better remediation than *V. radiata* at 600 mg kg^{-1}

TF was found to be greater than 1 at all the concentrations in both *V. radiata* and *V. aconitifolia*. On comparing the two plants, higher value of TF was seen in *V. aconitifolia* with maximum TF i.e., 2 at 600 ppm. Plants with TF value less than 1 accumulate metals in the roots and TF values > 1 indicate that the metals are accumulated in the stems and leaves. BCF was found to be increasing with increasing chromium concentrations in both the species. Maximum BCF (4.29) was seen in *V. aconitifolia* at 600 ppm (Table 3). The trend for bioaccumulation factor was observed increasing with increased concentration with highest accumulation at 600 mg kg^{-1} after which it declined significantly. The trend observed was, 100 mg kg^{-1} <200 mg kg^{-1}<400 mg kg^{-1}>600 mg kg^{-1}>800 mg kg^{-1} in *V. radiata* and 100 mg kg^{-1} <200 mg kg^{-1}<400 mg kg^{-1}<600 mg kg^{-1}>800 mg kg^{-1}

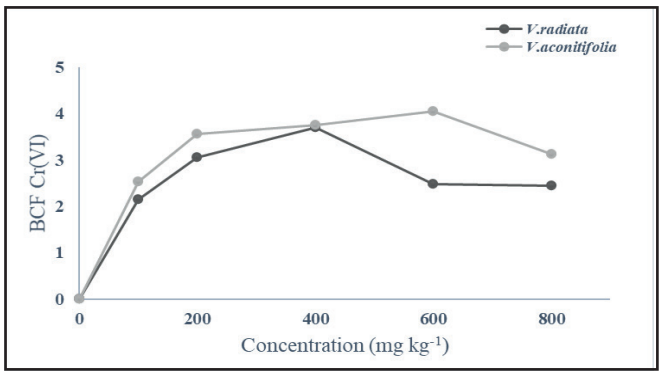

Figure 6. Bioaccumulation of Chromium by *V. radiata & V. aconitifolia*

in *V. aconitifolia* (Figure 6).

Conclusion:

Phytoremediation proves to be an economical and greener solution for the removal of excess heavy metals from soil. Several plants including legumes have been reported for their phytoremediation potential. Effects of chromium and its phytoremediation at high concentrations up to 800 mg kg^{-1} with *Vigna aconitifolia* is very less studied and not reported. The study also explained the toxic effects of Cr(VI) on plants. At the specific metal concentration upon root and shoot lengths in both the plants. A significant percentage of remediation 85.33% at 600 mg kg^{-1} shows excellent phytoremediation potential of *Vigna aconitifolia* towards hexavalent chromium in hydroponics. This study shows the interesting view points for the use of the leguminous plant, *Vigna aconitifolia* for removal of hexavalent chromium from various contaminated sources.

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