Efficacy of *Cicer arietinum* L. & *Vigna mungo* L. in remediation of Hexavalent Chromium

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**Abstract.** Hexavalent Chromium is a major soil pollutant; thus, its remediation from soil deserves due attention. Phytoremediation is an area of active current research which is eco-friendly and economic. Use of leguminous plants for phytoremediation will improve soil quality, fertility and nutrient balance and would help in restoration of natural soil ecosystem. The present study focuses on the use of two commonly growing legumes; *Cicer arietinum* (RP1) and *Vigna mungo* (RP2) to explore their remediation potential towards Cr(VI) with concentration ranging from 100-900 mg kg⁻¹ with the growth up to three weeks and were assessed for remediation potential and toxicity parameters. Higher percentage of decrease in root and shoot length was observed in RP2 as compared to RP1. Chlorophyll content was also found to be decreasing with increasing Cr stress in both the species. RP2 recorded higher BCF than RP1. Highest bioaccumulation factor 4.32 was observed in RP2 at 400 mg kg⁻¹ concentration. Translocation factor >1 was observed in both the plants with highest as 1.67 at 600 mg kg⁻¹ in RP2 and 1.93 at 400 mg kg⁻¹ in RP1. Remediation percentage of 72.25% in RP2 at 600 mg kg⁻¹ and 73.13% at 400 mg kg⁻¹ in RP1 was observed. Both the plants showed high tolerance and remediation potential towards Cr(VI) therefore has a great phytoremediation prospect, however, RP2 can be preferred over RP1.

1. **Introduction**

Extensive urbanisation, industrialization and development of technogenic progressions like mining, modern agricultural practices, automobile industries have
led to heavy pollution in the environment [1]. This has resulted in the substantial contamination of heavy metals in soil. Accumulation of heavy metals disturbs the overall biodiversity and soil quality thereby affecting the overall soil ecosystem [2]. Accumulation of toxic heavy metals like Arsenic(As), Mercury(Hg), Cadmium(Cd), Chromium(Cr), Copper(Cu) etc. in soil has caused great threat to soil biodiversity, plants and human health [3]. Due to extensive use in metallurgy, leather making, dyeing and stainless steel industries, Cr(VI) contamination has raised a great concern [4]. Chromium and its various forms alter the overall development of plants by altering enzymatic and non-enzymatic system, disturbing physiological equilibrium, and hindering overall biochemistry [5]. Two predominant forms Chromium exists in nature viz; trivalent Cr (III) and hexavalent Cr (VI). Cr(VI) is generated by the oxidation of trivalent state and is highly toxic; while Cr(III) is absorbed readily by the soil particles and utilised by the microbes for normal metabolic activities [5]. In both plant and animal cells, Cr(VI) diffuses easily within the cell and generates ROS. This causes DNA damage and inhibits RNA transcription [6]. Cr(VI) accumulation in human cells causes carcinogenicity, apoptosis, endocrine disruption and causes oxidative stress on vital organs (brain, lungs, kidney, etc.)[7]. Under natural conditions, heavy metal depletion can occur via process of leaching, erosion, deflation. But the process is very slow and has low efficiency. In order to cope up with this, various physico-chemical techniques are employed; oxidation/ reduction & chemical precipitation, reverse osmosis, ion exchange, electrochemical treatments etc.[8-9]. However, physico-chemical techniques are very expensive. Alternatively, a sustainable bioremediation method is promising for heavy metal removal through hyperaccumulation process [10-11]. Considering deleterious effects of Cr (VI) on all life forms, various methods have been used since decades to remediate, out of which phytoremediation tends to be a green approach. Phytoremediation efficiency largely depends on the plants to hyperaccumulate heavy metals in the plant parts, should have high biomass and fast growth rate. Family Fabaceae is the largest family of flowering plants and has about 20,000 species of 674 genera [12]. Use of leguminous plants for phytoremediation will also improve soil quality, fertility and nutrient balance and would help in restoration of natural soil ecosystem [13]. There are sufficient evidences that legumes such as; Anthyllis vulneraria, Coronilla varia, Lotus corniculatus, Lupinus albus, Trifolium repens, vicia faba are been effectively used in phytoremediation of heavy metal polluted sites [14][12]. Cicer arietinum (RP1) and Vigna mungo (RP2) are the two commonly growing leguminous plants in India. Therefore, remediation potential of these plants is been studied in hydroponic system in the present work. Apart from this, toxicity due to Cr(VI) accumulation have also been reported to further enhance the scope of the research on leguminous plants used for phytoremediation.
2. Materials & Methods

2.1 Selection & Plant Growth
Seeds of RP1 and RP2 were procured from the local market, sterilized and germinated for further growth in triplicates (5 seeds each tube) under various concentration range of \([\text{Cr(VI)}]\) (100 mg kg\(^{-1}\), 200 mg kg\(^{-1}\), 400 mg kg\(^{-1}\), 600 mg kg\(^{-1}\), and 800 mg kg\(^{-1}\) and 900 mg kg\(^{-1}\)) in Hoagland No. 2 basal salt solution (Himedia; pH 6.5) for 21 days with temperature (25-30°C) and light intensity (2000-2500 lux). Surface sterilization of seeds was done with ethanol and 0.1% mercuric chloride. Media + Stress + No Seed and Media + Seed + No stress were used as negative and positive controls respectively.

2.2 Analysis of growth parameters
Decrease in percentage of root and shoot individually was calculated 21 DAS as;
Root/Shoot length decrease (%) = \(\frac{(b-c)}{b}\) * 100. Where; \(b=\) Root/ Shoot length of positive control & \(C=\) Root/ Shoot length of treated plants.

2.3 Photosynthetic Pigment Content Assay
About 1 gram of fresh leaves was homogenized with 80% (V/V) acetone and 0.1 gram calcium carbonate. Total volume to 25 ml with 80% (V/V) acetone was made after centrifugation at 3000g-15 minutes. Absorbance at 645 nm and 663 nm was taken, against blank (Shimadzu 35 Double Beam Spectrophotometer). The content was expressed as mg chlorophyll per mililiter of sample and level was calculated as;
Chlorophyll ‘a’ (mg/ml) = (12.7 x O.D. at 663 nm) – (2.59 x O.D. at 645 nm),
Chlorophyll ‘b’ (mg/ml) = (22.9 x O.D. at 645 nm) - (4.7 x O.D. at 663 nm),
Total chlorophyll (mg/ml) = (20.2 x O.D. at 645 nm) + (8.2 x O.D. at 663 nm) [15].

2.4 Antioxidant Enzymes Activity
Enzyme extract was prepared in a pre-cooled mortar pestle by homogenizing 0.5 gm of fresh leaves in 5ml 100mM potassium phosphate buffer (pH 7.0), centrifuged at 15000g for 20 minutes. The supernatant separated was further used for enzymatic assays.

2.4.1 Catalase (CAT) Activity Assay
Catalase activity is measured in UV range. Hydrogen peroxide absorption increases as the wavelength decreases. The decomposition of \(\text{H}_2\text{O}_2\) is measured by the decrease in extinction/ unit time \(A_{240}\) nm which is a measure of CAT activity. The activity was determined by consumption of \(\text{H}_2\text{O}_2\) in the reaction mixture [16]. The reaction mixture (3 ml) contained 1.5 m 50 mM potassium phosphate buffer at pH 7.0, 1.2 ml \(\text{H}_2\text{O}_2\) and 300μL enzyme extract. The reaction was initiated by adding the \(\text{H}_2\text{O}_2\). One unit of the CAT activity is calculated as the amount of enzyme required to liberate 1/2 the peroxide oxygen from \(\text{H}_2\text{O}_2\); CAT Unit Activity
(Units/min/g FW ) = Change in abs. per minute x total volume(ml)/0.00693 (mM⁻¹ cm⁻¹) x volume of sample (ml) [17].

2.4.2 Glutathione Peroxidase (GPX) Activity Assay
To the reaction mixture (0.5ml plant extract, 2ml tris buffer, 0.1ml sodium azide, 0.2ml EDTA); 0.1ml H₂O₂ & 0.2ml glutathione were added. The mixture was incubated at 37°C for 10 minutes. 500 μl 10% trichloroacetic acid was added to stop the reaction. Supernatant was assayed for GPX activity after centrifugation. The activities are expressed as μg GSH consumed/minute/mg protein at 436 nm spectrophotometrically and the activity by using 0.00622 as extinction coefficient [18].

2.5 Accumulation of Cr(VI) in Root and Shoot and determination of percentage remediation
21 days after treatment, dried powder of root and shoot was subject to microwave assisted digestion (NuWav-Ultra) with, 5:1:1 ratio of 67% HNO₃, 30% H₂O₂ (Thermo Fisher Scientific) and MilQ water; max power 450 W, and programmed as (90°C-10 minutes, 150°C-10 minutes, 165°C-5 minutes), with final volume 10 ml v/v. Cr(VI) in filtered sample was determined by Inductively Coupled Plasma Mass Spectrometry (Agilent; 7700G). Phytoextraction potential is estimated by calculating Bioconcentration factor (BCF) and Translocation factor (TF). BCF = Cr(VI) concentration in plant/ Cr(VI) concentration in media, TF = Concentration of Cr(VI) in shoot/ Concentration of Cr(VI) in root [19]. Cr(VI) content in media was determined spectrophotometrically at 530 nm (Shimadzu 35 Double Beam Spectrophotometer) by Diphenyl Carbazide Method. A standard curve was obtained from the reaction mixture; working standards (50ppm – 950 ppm) of potassium dichromate (Himedia, >99% purity), 0.2 N sulphuric acid (Thermo Fisher Scientific), and 1,5 diphenyl carbazide (250mg/50 ml methanol). Remediation percentage was calculated by the difference in initial concentration of Cr(VI) to final concentration left in media [20].

2.6 Data Analysis
The data were analysed as mean ± standard error of n=3. The experimental results were statistically assessed through regression model and analysis of variance (ANOVA) and were considered to be significant at \( p < .05 \). Statistical analyses were performed using R studio (version; R 4.1.0). Graphs are made using Origin 2021b.

3. Results and Discussion
Root and shoot lengths (cm) showed a gradual decrease upon increasing stress. The effects of Cr(VI) on percentage decrease of root and shoot lengths were observed in the morphological parameter between treated plants and control. A significant
increase in root and shoot length percentage decrease with 30.69% & 85.58% and 23.83% & 80.44% at 100 mg kg\(^{-1}\) and 900 mg kg\(^{-1}\) in RP1 and RP2 root respectively; whereas as 17.04% & 75.06% and 15.16% & 68.27% in RP1 and RP2 shoot at 100 mg kg\(^{-1}\) and 900 mg kg\(^{-1}\) respectively were observed with increasing Cr(VI) concentrations in all the treated plants (Figure 1). A single line regression model was formulated; equations (Table 2) to predict the effect of increasing Cr(VI) stress on root and shoot length (Figure 2 A,B,C,D). The model shows increase in percentage decrease in root and shoot lengths with increasing stress. By using ANOVA on regression line, the value of \(F_{(1,4)} = 120.51\), \(F_{(1,4)} = 84.28\), \(F_{(1,4)} = 58.84\) & \(F_{(1,4)} = 151.27\) (RP1 root, RP2 root, RP1 shoot and RP2 shoot respectively) and \(p<.05\) shows a significant impact of Cr(VI) on morphological change in both the plants. Similar to our findings, a gradual decrease in root length was observed in *Cicer arietinum* with increasing Cr(VI) concentration [21]. Cr(VI) induced toxicity decreased root and shoot length in *Oryza sativa* L. [22], *Brassica napus* L. [23] and *Vigna mungo* L. [24]. The reduced plant growth may be due to the transport of chromium to the ariel plant parts with water and nutrients which had a direct impact on the cellular metabolism of cell. A complex formation of chromium with cellular proteins and ultimate inhibition of protein synthesis could be the reason for reduced plant growth [25].

![Figure 1. Percentage Decrease Root & Shoot Length in RP1 & RP2](image-url)
Figure 2. Regression model for percentage decrease in length in RP1 & RP2

Total chlorophyll content, in general, gradually decreased as concentration of Cr(VI) increased as compared to its control in both the treated plants, from 13.43 mg/ml & 14.70 mg/ml in control to 1.70 mg/ml & 3.18 mg/ml at 900 ppm concentration in RP1 & RP2 respectively. Total Chlorophyll content was found to be lower with increasing Cr(VI) concentration in RP1 than RP2 (Figure 3). From the single line regression model formulated and the equations obtained (Table 2), effect of increasing chromium concentration on decreasing chlorophyll content in both the plants is evident where it decreased by 1.4% and 1.3% in RP1 & RP2 respectively (Figure 4 A,B). There is a significant effect of increasing Cr(VI) stress on total chlorophyll (mg/ml) content at 5% level significance with $F_{(1,5)} = 431.91$, $F_{(1,5)} = 360.11$ and $p < 0.05$. The results are in line with the results on *Brassica napus* and *Dolichos biflorus* L. where decline in photosynthetic pigment was observed upon Cr toxicity [26-27]. *Sesbania sesban*, grown on chromite mine soil also experienced decline in Chlorophyll content [28]. Chlorophyll is the key molecule to prepare photosynthesize which makes it the important component of plant cell. Due to Cr(VI) toxicity, chlorophyll synthesis reduces due to disruption in electron transport chain [29]. It was also studied that chromium toxicity inhibited the activities of δ-aminolaevulinic acid dehydratase, and essential enzyme
in chlorophyll biosynthesis [30]. Destabilization and degradation of peripheral proteins maybe the reason for decrease in chlorophyll content. Hexavalent chromium also facilitates enzyme inactivation by substituting Mg at the active sites. Toxic effects of Cr(VI) on PSI and PSII activities are well reported [26, 31].

![Chlorophyll content in RP1 & RP2](image1)

**Figure 3.** Chlorophyll content in RP1 & RP2

![Regression model for Total Chlorophyll in RP1 & RP2](image2)

**Figure 4.** Regression model for Total Chlorophyll in RP1 & RP2

Catalase activity showed an increase with increasing Cr(VI) concentration till 600 mg kg\(^{-1}\) in RP1 and till 800 mg kg\(^{-1}\) in RP2. Maximum unit activity recorded was; 37.52 U min\(^{-1}\) g\(^{-1}\) & 44.25 U min\(^{-1}\) g\(^{-1}\) observed at 600 mg kg\(^{-1}\) for RP1 & at 800 mg kg\(^{-1}\) for RP2.
kg$^{-1}$ in RP2 respectively, after which it was found to decrease in both the plants; CAT activity of control being 12.51 U min$^{-1}$g$^{-1}$ and 9.62 U min$^{-1}$g$^{-1}$ in RP1 & RP2 respectively (Table 1). Similar to our findings, decreased CAT activity was observed at high chromium concentration in *Kandelia candel* [32]. Similarly, CAT activity significantly increased in *Capsicum annum* L. [29], *Platanus orientalis* [33] upon stress. Increased activity was also observed with increasing chromium stress in both the varieties; rosea and alba of *Catharanthus roseus* (L.) [34]. Catalase are the important antioxidant enzymes involved in quenching ROS and dismutation of H$_2$O$_2$ into O$_2$ and H$_2$O from the cells [35]. Decreased CAT activity at high concentration of Cr (VI) maybe due inactivation of enzyme protein by ROS. Increment in antioxidant enzyme activity is the indication of mitigation of oxidative stress generated by Cr(VI) toxicity [36].

Similar to this GPX activity also increased with increasing Cr (VI) stress till 800 mg kg$^{-1}$ in both the plants after which a decrease was observed with unit activity as, 10.03 U min$^{-1}$g$^{-1}$ & 16.32 U min$^{-1}$g$^{-1}$ in control and 18.91 U min$^{-1}$g$^{-1}$ & 32.41 U min$^{-1}$g$^{-1}$ at 800 mgkg$^{-1}$ in RP1 and RP2 respectively (Table 1). Higher GPX activity was observed in RP2 as compared to RP1. Similar results for GPX activity were recorded in *Vigna radiata* plants under Cr(VI) stress [37]. Redox state of the plant’s cell is perturbated by the heavy metals stress due to ROS production. GPX is a Se-containing antioxidant enzyme which is involved in the reduction process of hydrogen peroxide and lipid peroxides to water and lipid alcohols and in turn oxidises glutathione to glutathione disulphide. This process helps in regulating the intracellular redox state of cell thereby maintaining many biochemical pathways by providing reducing equivalents. This entire process is responsible for combating the antioxidative stress caused by heavy metal in plants [38]. From the single linear regression model presented in (Figure 5 A,B,C,D), it is evident that Cr (VI) had a significant impact on CAT and GPX activities and as concentration increases CAT activity increases by 2.4% and 2.8% and GPX activity increases by 0.9% and 1.5% in RP1 in RP2 respectively (Table 2). From ANOVA, the value of $F(1,5) = 12.17$, $F(1,5) = 9.56$ for CAT and $F(1,5) = 9.51$, $F(1,5) = 19.09$ for GPX activity of RP1 and RP2 respectively at 0.05 levels of significance and $p<0.05$ shows a significant impact of Cr(VI) on antioxidant defence system in both the plants.

### Table 1. CAT and GPX Activity in RP1 and RP2

| Metal Concentration (ppm) | RP1 CAT activity (U min$^{-1}$ g$^{-1}$) | RP2 CAT activity (U min$^{-1}$ g$^{-1}$) | RP1 GPX activity (U min$^{-1}$ g$^{-1}$) | RP2 GPX activity (U min$^{-1}$ g$^{-1}$) |
|---------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| Blank                     | 12.51 ± 0.83*                          | 9.62 ± 1.67*                          | 10.03 ± 0.85*                          | 16.32 ± 0.79*                          |
| 100                       | 15.87 ± 0.68*                          | 18.76 ± 0.97*                          | 13.12 ± 1.04*                          | 21.22 ± 0.98*                          |
| 200                       | 26.46 ± 0.76*                          | 28.86 ± 0.94*                          | 13.89 ± 0.98*                          | 23.54 ± 1.02*                          |
| 400                       | 32.23 ± 0.96*                          | 36.56 ± 0.89*                          | 17.36 ± 0.80*                          | 28.17 ± 1.21*                          |
| 600                       | 37.52 ± 0.83*                          | 39.92 ± 1.11*                          | 20.84 ± 1.03*                          | 32.03 ± 0.87*                          |
| 800                       | 36.56 ± 0.56*                          | 44.25 ± 0.94*                          | 18.91 ± 0.89*                          | 32.41 ± 0.66*                          |
| 900                       | 31.75 ± 0.64*                          | 32.71 ± 0.84*                          | 16.98 ± 0.91*                          | 29.32 ± 0.94*                          |
± denotes standard error, *indicates significant difference at 5% level of significance

Figure 5. Regression model for CAT & GPX activity in RP1 & RP2

There was an increasing trend seen in the percentage remediation up to the concentration of 400 mg kg$^{-1}$ as 73.13 in RP1 and 80.71% in RP2 and thereafter, a decline in percentage remediation was observed (Figure 6) which could be the result of Cr(VI) induced high toxicity and subsequent growth reduction which resulted in the decline in remediation. Single line regression model formulated
showed an initial increase and a subsequent decrease in remediation percentage with increasing stress (Table 2). The results obtained are statistically significant at p < 0.05. Hexavalent chromate remediation by various plant species has been studied. High potential Salix viminalis is been studied to remove Cr(VI) from soil [39].

![Percentage Remediation in RP1 & RP2](image)

**Figure 6.** Percentage Remediation in RP1 & RP2

| Regression Equation                                      | R²   |
|---------------------------------------------------------|------|
| Decrease Root (%) RP1 = 27.260+0.061 (Cr (VI))          | 0.968|
| Decrease Root (%) RP2 = 25.261+0.057 (Cr (VI))          | 0.967|
| Decrease Shoot (%) RP1 = 13.754+0.061 (Cr (VI))         | 0.936|
| Decrease Shoot (%) RP2 = 11.430+0.061 (Cr (VI))         | 0.974|
| Total Chlorophyll RP1 = 13.517-0.014 (Cr (VI))          | 0.989|
| Total Chlorophyll RP2 = 14.545-0.013 (Cr (VI))          | 0.986|
| CAT RP1 = 17.379+0.024 (Cr (VI))                        | 0.709|
| CAT RP2 = 17.973+0.028 (Cr (VI))                        | 0.657|
| GPX RP1 = 12.188+0.009 (Cr (VI))                        | 0.655|
| GPX RP2 = 19.606+0.015 (Cr (VI))                        | 0.792|
| Remediation RP1 = 72.177-0.022 (Cr (VI))                | 0.529|
| Remediation RP2 = 76.678-0.017 (Cr (VI))                | 0.978|
Bioconcentration and Translocation Factors are the important parameters used to determine the phytoremediation potential of plants [40]. However, translocation and distribution of chromium within plant cells is diverse and depends upon species, oxidation state and concentration of chromium used [41]. In the present study, translocation factor was found to be greater than 1 at the concentrations ranging from 100 mg kg\(^{-1}\) to 400 mg kg\(^{-1}\) in RP1 and 100 mg kg\(^{-1}\) to 600 mg kg\(^{-1}\) in RP2 after which a decline (less than 1) was observed. However, TF near 1 was observed at higher concentrations. Bio Concentration Factor increased with increasing Cr (VI) concentration till 400 mg kg\(^{-1}\) in RP1 and 600 mg kg\(^{-1}\) in RP2, after which a decline was observed with highest value of 4.32 at 400 mg kg\(^{-1}\) in RP2 (Table 3). The trend observed was; 100 mg kg\(^{-1}\) < 200 mg kg\(^{-1}\) < 400 mg kg\(^{-1}\) < 600 mg kg\(^{-1}\) < 800 mg kg\(^{-1}\) < 900 mg kg\(^{-1}\) in RP1 and 100 mg kg\(^{-1}\) < 200 mg kg\(^{-1}\) < 400 mg kg\(^{-1}\) < 600 mg kg\(^{-1}\) < 800 mg kg\(^{-1}\) < 900 mg kg\(^{-1}\) in RP2. Data of BCF and TF is presented in Table 3. Accumulation of Cr(VI) in root and shoot of respective species is represented in Fig. 7 & 8. The higher accumulation of Cr(VI) in roots at high concentrations maybe due to the sequestration of metal ions in the root vacuoles [42]. This tends develop a natural protective mechanism against chromium thus reducing further damage [43]. Although, results in soil experiments may vary; the findings suggest the use *Cicer arietinum* and *Vigna mungo* for phytoextraction of chromium at 400 mg kg\(^{-1}\) and 600 mg kg\(^{-1}\) respectively.

![Figure 7. Accumulation of Cr (VI) in Shoot & Root of RP1](image-url)
Table 3. BCF and TF of RP1 and RP2

| Concentration of Chromium (mg kg⁻¹) | RP1       |            | RP2       |            |
|-----------------------------------|-----------|------------|-----------|------------|
|                                   | TF        | BCF        | TF        | BCF        |
| 100                               | 1.07±0.09 | 1.64±0.09  | 1.50±0.05 | 2.11±0.06  |
| 200                               | 1.21±0.08 | 2.32±0.06  | 1.46±0.04 | 2.60±0.07  |
| 400                               | 1.93±0.09 | 2.78±0.08  | 1.57±0.08 | 4.32±0.08  |
| 600                               | 0.94±0.08 | 1.60±0.06  | 1.67±0.09 | 2.67±0.08  |
| 800                               | 0.95±0.07 | 1.28±0.09  | 0.96±0.09 | 1.91±0.09  |
| 900                               | 0.81±0.08 | 0.86±0.05  | 0.92±0.06 | 1.13±0.07  |

± denotes standard error
Results significant at 5% level of significance

4. Conclusions
Hexavalent chromium is widely distributed in the environment and is highly toxic. Screening of phytoremediator plants and using them as a sustainable approach for Cr (VI) remediation will aid in restoring the soil natural nutrient balance in chromium contaminated soil. Although upon treating both the plants in different Cr (VI) concentrations, root and shoot lengths and photosynthetic pigment were significantly impacted, both the plants showed a high potential to remediate chromium at 400 mg kg⁻¹. The plants also showed high potency to translocate Cr(VI) from root to shoot up to 400 mg kg⁻¹ (RP1) and 600 mg kg⁻¹ (RP2). In addition, both the plants are leguminous and aids in maintaining the nitrogen balance and soil fertility. Therefore, *Cicer arietinum* and *Vigna mungo* can be used...
to remediate Cr (VI) and has great phytoremediation prospects. A further need to assess the results in soil is necessary along with molecular studies to unlock more viewpoints.

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