Phytoaccumulation potential and enzymatic activities of *Oryza sativa* L. genotypes exposed to Cr (VI) in hydroponic culture

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Abstract
In this work, the phytoaccumulation efficiency and enzymatic activities of various rice (Oryza sativa L.) genotypes exposed to differential concentrations of Cr (VI) have been studied. The cultivars experimented were Pratikshya, Sankar, Annapurna, White swarna and Pimpudibasa. The seedlings were raised with ¼ strength Hoagland's nutrient solution prepared in deionised water supplemented with different concentrations (0, 100, 200, 300 and 400µg/L) of hexavalent Cr as potassium dichromate ($K_2Cr_2O_7$) under controlled environmental conditions. The seedlings were harvested at 7 days intervals and parameters like root and shoot elongation, enzymatic activities and tolerance to chromium ions were measured after different periods of exposure.

The highest concentration of Cr (VI) ions in shoots (9.92 µg/g dry weight) and roots (8.51µg/g dry weight) was found in Pratikshya exposed to 300 µg/L of Cr(VI) after 21 days of treatments. The growth parameters of five cultivars and tolerance to chromium were in the order of Pratikshya>Sankar>Annapurna>White swarna>Pimpudibasa. Cr(VI) toxicity was correlated with peroxidase and catalase enzyme activities of different rice cultivars. The interaction between chromium ions and rice cultivars biomass was studied using SEM-EDX and FTIR analysis.

Keywords: Rice cultivars, Hexavalent chromium, Accumulation, Catalase, Peroxidase.

Introduction

Phytoaccumulation techniques refer to the ability of green plants to take up contaminants into the roots and translocate them to the aboveground shoots and leaves. Researchers have developed a hydroponic cultures system which is eco-friendly, cost effective, easy operative and observation, making quick screening on the basis of plant relative growth rate and toxicity[1].

Heavy metals are generally found in soil and water. Some of them are beneficial and some are toxic to the living organisms. In the present time, toxic heavy metals are generated from different chemical industries and mining activities[4]. The presence of toxic heavy metals in the water bodies causes deterioration in the physical and chemical properties of the water and possibly results in the development of sever phytotoxic phenomena in the plant systems[7]. The different anthropogenic and natural activities increase the toxic levels of heavy metals in the water bodies[3]. The mechanism of toxicity of different metal ions in plant cells is mainly through their inhibitory effects on various enzymes involved in different key metabolic processes. These inhibitory effects may be on the catalytically active groups of enzymes or because of protein denaturation.[4,5].

Chromium is a naturally occurring element found in the rocks and minerals. Anthropogenic contribution of chromium ions contamination in water resources is different industrial processes. Hexavalent chromium ion is more toxic than the trivalent chromium ion, which is usually associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions[6]. The United States Environmental Protection Agency reported that the highest permissible limit for Cr(VI) in potable water is 0.05 ppm[8,12].

Application of different remediation techniques like physical absorption, chemical absorption and biological absorption has been reported by many workers for removal of chromium from contaminated water. But among all these methods, the use of aquatic plants to absorb toxic metals ions from contaminated water is extremely efficient[9]. The hydroponic culture techniques are useful for assessing the different plants' tolerance capacity to the toxic heavy metals along with their efficiency in mineral utilization[7,10].

Material and Methods

Plant materials and culture conditions: Viable seeds of some high yielding rice cultivars like Pratikshya, Sankar, Annapurna, White swarna and Pimpudibasa were collected from the Department of Plant Breeding and Genetics, Odisha University of Agriculture and Technology, Odisha. Seeds were washed with detergent solution and running tap water for 15 min. Seeds were then sterilized with 0.1% aqueous mercuric chloride solution for 20 min followed by repeated washings with distilled water for another 30 min and were spread on nylon nets stretched over plastic containers.

The containers were filled with around 250 ml of different concentrations (100, 200, 300 and 400 µg/L) of $K_2Cr_2O_7$ prepared in ¼ strength modified Hoagland nutrient solution. The Hoagland nutrient solution was prepared in deionised water.

In another container, 250 ml of Hoagland solution without addition of $K_2Cr_2O_7$, was taken as control. The modified Hoagland nutrient solution consisted of 4.0 mM $Ca(NO_3)_2$, 2.0 mM $MgSO_4$, 4.0 mM KNO_3, 0.4 mM $(NH_4)_2SO_4$, 2 µM $MnSO_4$, 0.3 µM $CuSO_4$, 0.8 µM $ZnSO_4$, 30 µM NaCl, 0.1µM Na_2MoO_4, 1.43 µM KH_2PO_4, 10 µM H_3BO_3 and 20 µM Fe-Na-EDTA (All obtained from Merck Specialties) [11]. The pH of the nutrient solution was adjusted to 6.8 using 0.1 N HCl or 0.1N KOH as per requirement. The solution was changed regularly at 3 day intervals to maintain the desired pH. In each container, 30 surface sterilized seeds were spread. The containers were kept under cool, white fluorescent lamp (55 µmol $m^{-2}s^{-1}$) under 16 h photoperiod in a growth room maintained at 25 ± 2° C.

The experiment was laid in a completely randomized block design with three replicates. The experiments were repeated three times. The length of the primary root and the shoot were measured at 7 days intervals from the date of root emergence up to the 28 days. The rate of root length in each experiment was determined by subtracting the length of the root recorded on the day of germination noted on the 28th day. The significant differences of growth between the various cultivars were calculated and subjected to statistical analysis by using MINITAB 15 software [22]. Tolerance index (TI) for the tested plants was calculated using the formula:

$$TI\ (\%) = \frac{\text{Mean root or shoot length of seedlings exposed to potassium dichromate (300 µ g/L)}}{\text{Mean root or shoot length of control seedlings}} \times 100$$

Reagent preparation and analysis of Cr (VI): All the chemicals used in the studies were of analytical grade and used without further purification. In all experiments, double distilled water **(**Milli-Q Millipore 18.2 $M\Omega cm^{-1}$ conductivity**)** was used for standard preparation, dilution and analytical purposes of solutions. Stock hexavalent chromium solution of 1000 mg/L was prepared by dissolving 2.828 g of anhydrous potassium dichromate ($K_2Cr_2O_7$) and 1.5 mL of 1 M HNO_3 in 1000 mL of deionized water[2]. Subsequently, different working solutions of required concentrations were prepared by proper dilution. The pH was adjusted to the desired level with 0.1 M NaOH or 0.1 M HCl solution.

All the glassware were soaked in 30% HNO_3 overnight and washed in an ultrasonic bath for 15 min. Then the glassware was rinsed with distilled water and finally with deionized water and then dried by keeping in oven. Chromium ion accumulation in shoot biomass, root biomass and water samples was measured.

Digestion of samples in this study was performed according to the standard method[2]. Plant samples were dried by heating at 120° C for 24 h in hot air oven and the ash was digested with nitric acid and filtered into a volumetric flask with the help of Whatmann filter paper no. 42. The final volume was made up with deionized water and chromium analysis was carried out Spectrophotometrically at 540 nm following the diphenyl carbazide method.

Peroxidase analysis: Fresh shoot tissues were collected from the samples exposed to 300 µg/L of $K_2Cr_2O_7$ for 14 days along with the respective control samples of all cultivars. It was homogenized with mortar and pestle in 4 ml of cold 0.1M phosphate buffer (pH 6.1) containing 30 mg of insoluble polyvinyl pyrrolidone (PVP). The homogenate was filtered through four layers of miracloth and centrifuged at 12, 000 *g* for 10 min at -4° C. The supernatant was used as the enzyme extract for the peroxidase assay.

The assay mixture comprised of 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol, 3 mM H_2O_2 and 0.4 ml of crude enzyme extract. The total reaction volume was 1.2 ml. The rate of change in absorbance at 420 nm was measured using a spectrophotometer. The levels of enzyme activity were expressed as µmoles H_2O_2 destroyed $min^{-1}mg^{-1}$protein[5,15].

Catalase analysis: Fresh shoot tissues were collected from the samples exposed to 300 µg/L of $K_2Cr_2O_7$ for 14 days along with the respective control samples of all cultivars. The tissues were homogenized in 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 12,000 *g* for 10 min at -4 °C. The supernatant was used as the enzyme extract for assaying catalase activity. For the catalase assay, 1 ml of the enzyme extract was added to the reaction mixture containing 1 ml of 0.1 M H_2O_2 and 3 ml of 0.1 M sodium phosphate buffer (pH 7.0). The reaction was stopped by adding 10 ml of 2% H_2SO_4 after 1 min of incubation at 20 °C. The acidified reaction mixture with or without enzyme extract was titrated against 0.01N $KMnO_4$ to determine the quantity of H_2O_2 utilized by the enzyme. The catalase activity was expressed as µmoles H_2O_2 destroyed/min/mg protein[5,15].

Results and Discussion

Seed germination and tolerance index of shoots and roots: Seed germination and elongation of shoot and root are important parameters for assessing the chromium tolerance capability of various rice cultivars. In the present study, five rice cultivars were treated with four different concentrations of chromium and the seed germination percentage was recorded after 7 days of exposure.

The results are presented in table 1. It is found that the percentage of seed germination decreased significantly with increase in Cr (VI) concentration up to 300 µg/L of $K_2Cr_2O_7$, but at higher concentration (i.e. 400 µg/L), there was sharp decline in the germination percentage. A good degree of variation in seed germination and growth response was observed at 100 µg/L of chromium solution (Table 1).

The root and shoot elongation of the rice cultivars in the presence of 300 µg/L of $K_2Cr_2O_7$ solution as compared with their respective control samples were measured and the results are represented in table 2. The root elongation depends upon the inhibitory effect of metal ions[13].

In the present study, from the results shown in figure 1, it is found that Pratikshya and White swarna are tolerant to chromium having tolerance index values of 86.37% and 74.92 % for shoot and 79.82 % and 71.48% for root respectively. The cultivars with the lower tolerance index values were found in "Sankar, Annapurna and Pimpudibasa" and in the seedlings of these varieties, some disorders such as chlorosis and necrotic at the leaf tips and margin were noticed which might be due to chromium toxicity[20,21].

Accumulation and toxicity of Cr (VI) by shoot and root biomass: The accumulation of hexavalent chromium in root and shoot biomass varied in different cultivars of rice and the results are presented in figure 2. Accumulation of chromium ions in the shoot biomass was found to be more than in root biomass in all five cultivars. In the present study, the highest amount of chromium accumulation was found in the shoots of "Pratikshya" (i.e. 9.92 µg/g dry weight) followed by "Rudra" (i.e. 8.92 µg/g dry weigh) after 21 days of exposure (Figure 2).

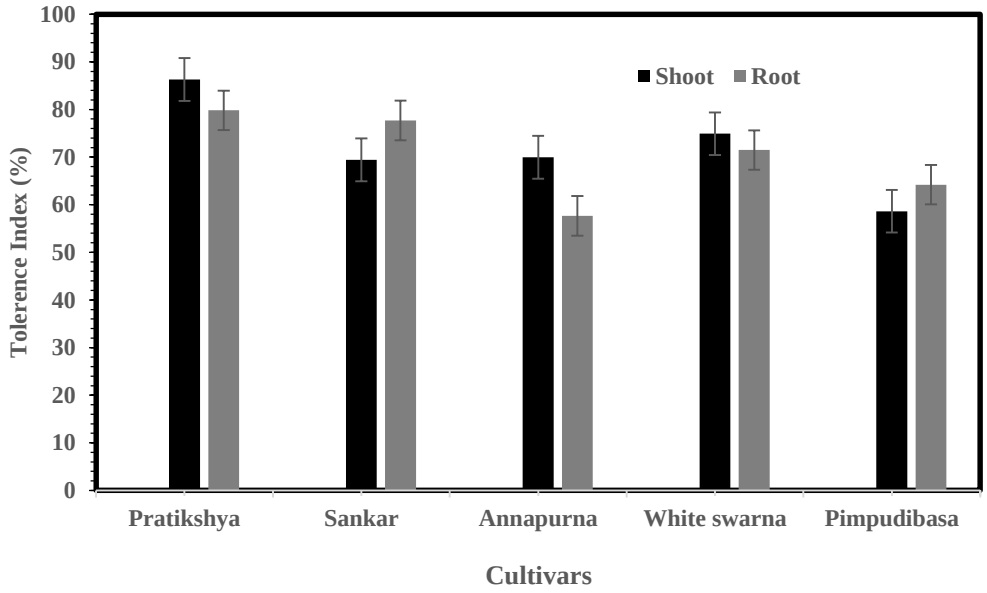

Figure 1: Tolerance Index of Shoot and Root of five rice (*Oryza sativa* L.) cultivars exposed to 300 µg/L of $K_2Cr_2O_7$. Values are means of 10 samples

Table 1
Effects of different concentrations of hexavalent chromium on the seed germination % of rice (*Oryza sativa* L) after 7 days of treatment with Cr (VI) solution.

Rice cultivars	Percent of germination (%) (Mean ± S. E.)				
	$K_2Cr_2O_7$ concentration (µg/L)				
	0 µg/L (Control)	100 µg/L	200 µg/L	300 µg/L	400 µg/L
Pratikshya	95.3 ± 0.3	94.2 ± 0.4	91.9 ± 0.2	86.2 ± 0.2	31.5 ± 0.8
Sankar	94.3 ± 0.5	92.8 ± 0.4	89.2± 0.5	80.0 ± 0.3	30.2 ± 0.7
Annapurna	93.2 ± 0.6	90.2 ± 0.1	90.1 ± 0.8	88.1 ± 0.5	32.5 ± 0.2
White swarna	92.4 ± 0.7	90.1 ± 0.5	88.5 ± 0.9	85.6 ± 0.6	29.6 ± 0.1
Pimpudibasa	91.8 ± 0.8	88.6 ± 0.3	85.4 ± 0.2	84.5 ± 0.6	25.5 ± 0.2

Table 2
Root length and shoot length of five rice cultivars exposed to 300 µg/L $K_2Cr_2O_7$ solution for 14 days along with their respective control samples. *Values are means of ten samples ± S.E.

Rice cultivars	Root length (cm)		Shoot length (cm)	
	0 µg/L (Control)	300 µg/L	0 µg/L (Control)	300 µg/L
Pratikshya	8.08 ± 0.2	6.45± 0.21	7.01 ± 0.1	6.05 ± 0.31
Sankar	6.46 ± 0.1	5.02± 0.31	5.92 ± 0.4	4.11 ± 0.21
Annapurna	5.41 ± 0.3	3.12 ± 0.22	4.46 ± 0.3	3.12 ± 0.11
White swarna	5.89 ± 0.3	4.21 ± 0.11	4.76 ± 0.4	3.56 ± 0.12
Pimpudibasa	4.86 ± 0.4	3.12 ± 0.10	3.82 ± 0.5	2.24 ± 0.02

In "White swarna" and "Pimpudibasa" cultivars, the accumulation of chromium was less in comparison to other two cultivars after the same period of exposure (Figure 2). One of the basic strategies of metal tolerance is metal accumulation where there is no such restriction and metals accumulated in a detoxified form. Detoxification results from cell wall binding, active pumping of ions into vacuoles, complexing by organic acids and possibly by specific metal-binding proteins and alteration of membrane structure. The toxic effects of Cr are primarily dependent on the metal speciation which determines its uptake, translocation and accumulation mechanism. The oxidation state of chromium strongly influences the rate of chromium uptake[17].

Chromium (VI) can easily cross the cell membrane and the phosphate-sulphate carrier transports the chromate anions. The chromate ion has a large ionic potential and tetrahedral coordination and acts both as strong acid and an oxidizing agent. The toxic properties of Cr (VI) originate from the action of this form itself as an oxidizing agent as well as from the formation of free radicals during the reduction of Cr (VI) to Cr (III) occurring inside the cell. Induction and activation of antioxidative enzymes like superoxide dismutase (SOD) catalase are some of major metal detoxification mechanisms in plants[16].

The superoxide dismutase removes superoxide radical ($O_2^{\cdot-}$) which can undergo protonation to form a strong oxidizing agent, $HO_2^{\bullet}$ in negatively charged membrane surface that directly attacks the polyunsaturated fatty acids of membrane lipids. $O_2^{\cdot-}$ can also donate an electron to chromium (Cr^{6+}) to yield a reduced form of chromium (Cr^{3+}) which can reduce H_2O_2 produced as a result of SOD led dismutation of $O_2^{\cdot-}$ and $OH^{\bullet}$. Activation of superoxide dismutase and antioxidant catalase is a major metal detoxification mechanism reaction in plant cells[18] (Figure 3).

Enzyme activities of cultivars: The induction of enzymes is considered to play a significant role in the stress metabolism induced by metal phytotoxicity.

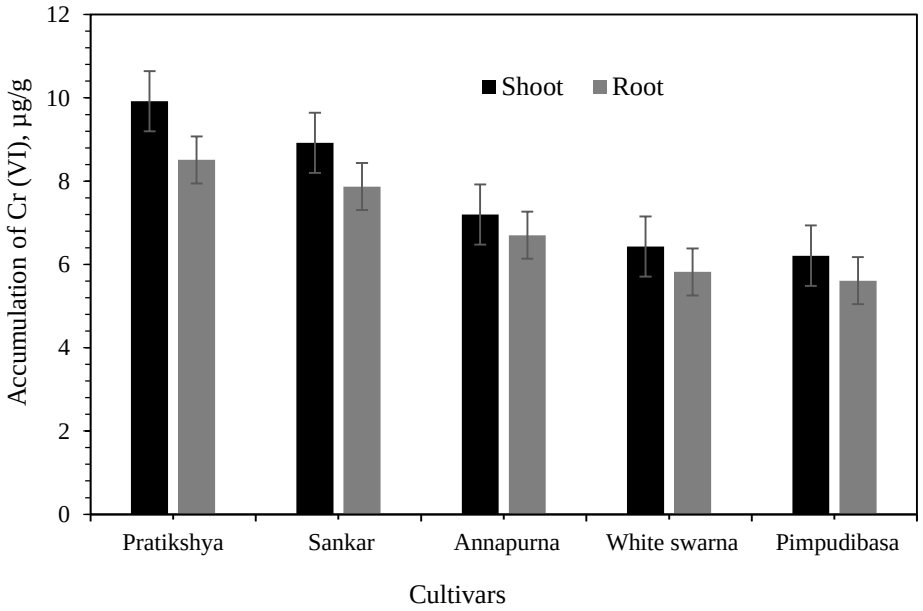

Figure 2: Accumulation of chromium (µg/g) in five rice (*Oryza sativa*) cultivars in presence of 300 µg/L of $K_2Cr_2O_7$ after 21 days of treatment. The values are means of 10 replicates repeated three times

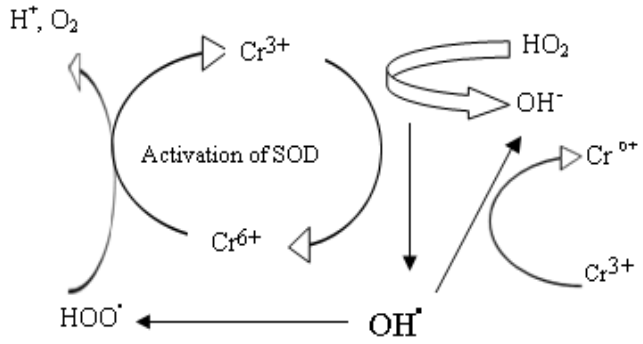

Figure 3: Metal detoxification mechanism in plant cells in presence of superoxide dismutase (SOD)

Table 3
Enzyme activities in the shoot tissues of different rice cultivars exposed to 300 µg/L of $K_2Cr_2O_7$ solution for 14 days. Values given are the average of three assays replicated of 10 plants. Standard deviations are indicated.

Rice cultivars	Enzyme activity (µmoles H_2O_2 destroyed $min^{-1}mg^{-1}$protein)			
	Catalase		Peroxidase	
	Control (µg/L)	$K_2Cr_2O_7$ (300)	Control (µg/L)	$K_2Cr_2O_7$ (300)
Pratikshya	21.2 ± 0.11	24.8 ± 0.03	28.9 ± 0.6	30.11 ± 1.6
Sankar	6.1 ± 0.12	4.21 ± 0.01	11.04 ± 0.5	10.11 ± 0.1
Annapurna	10.6 ± 0.14	14.21 ± 0.21	16.8 ± 0.4	13.05 ± 0.12
White swarna	16.7 ± 0.11	18.22 ± 0.23	19.2 ± 1.2	20.12 ± 0.04
Pimpudibasa	24.1 ± 0.17	22.71 ± 0.12	27.8 ± 0.5	30. 12 ± 0.12

From the results presented in Table 3, it is found that both catalase and peroxidase activities varied in different cultivars in the presence of 300 µg/L of chromium in comparison to their respective control samples. The catalase activity is higher in the case of tolerant cultivars than the non-tolerant cultivars. The activity varied from 9.10% to 34.05% in the case of catalase and 4.18% to 8.34% in the case of peroxidase respectively as compared to control[15].

The increase in catalase and peroxidase activities is indicator of heavy metal toxicity and stress situation in plants. The mechanism of metal tolerance in plants was proposed which includes production of intercellular metal binding compounds, cellular metabolism and membrane structure. Finally, absorbed toxic metal in green plants was not completely inert, but stimulated the activity of certain enzymes[17,19].

Characterization of *Oryza sativa* L. shoot biomass after absorption of chromium ions: The leaf surface morphology of Pratikshya cultivar shoot biomass exposed to 300 µg/L of $K_2Cr_2O_7$ along with that of the respective control sample was measured with the help of SEM-EDX after 21 days of exposure and the results are presented in figure 4(a) and figure 4(b). Figure 4(a) clearly shows the surface structure and texture in the materials. It is evident that the carbon particles are in the form of spheres with a wide range of sizes.

Figure 4(b) shows the morphological changes with respect to shape and size of the biomass after absorption of chromium ion treatment. It is clearly observed that the surface of leaf has been changed into new bulky particles and small whitish patch like structures after chromium absorption. The EDX spectra of chromium unloaded and loaded biomass is shown in figure 4(a) and figure 4 (b), respectively. So, it is concluded that chromium ions are absorbed on the surface of the biomass[10].

These results are further confirmed with the results of FTIR spectra analysis. FTIR spectra (presented in figure 5) of the Pratikshya rice cultivar shoot biomass exposed to different concentration of chromium ions display a number of absorption peaks which indicate the complex nature of the biomass. The broad absorption spectra at 3,484.93 cm^{-1} due to bonded - OH stretching vibration may be possibly due to complexation of –OH groups with chromium ions. The peak at 2927.45 cm^{-1} indicates -CH stretching vibration of alkyl chains with chromium ions.

The absorption peak appearing at 1655.12 cm^{-1} may be due to C=O stretching vibration of carboxylic with chromium ions. The absorbance peaks at 1,170.77 cm^{-1} and 1003.15 cm^{-1} may be attributed to N-H stretching vibration complexation of nitrogen from amino group with chromium ions. The appearance of the spectra may be attributed to the interaction of chromium ions with the hydroxyl, carboxyl and amino groups present in the surface of the cultivar biomass[3,10,14].

Conclusion

Aqueous solution treatment is an efficient method for selection of chromium tolerant plants of rice cultivars for breeding programme. Rice (*Oryza sativa* L) cultivars were tested for their tolerance, absorption, toxicity and enzymatic activities after exposure to different concentrations of chromium (VI) ions in nutrient solution at pH 7. Based on the parameters studied, the chromium tolerance in five cultivars of rice is in the order of Pratikshya >Sankar > Annapurna >White swarna > Pimpudibasa respectively.

The maximum absorption efficiency of Cr (VI) ions is 9.92 µg/g dry weights in shoot and 8.51 µg/g dry weight in root biomass of Pratikshya cultivar after 21 days of treatment. FTIR analysis shows that the hexavalent chromium ions may be coordinated with the hydroxyl, carboxyl and amino groups present in the biomass. SEM-EDX analysis also confirms the absorption of hexavalent chromium ions in the rice cultivars.

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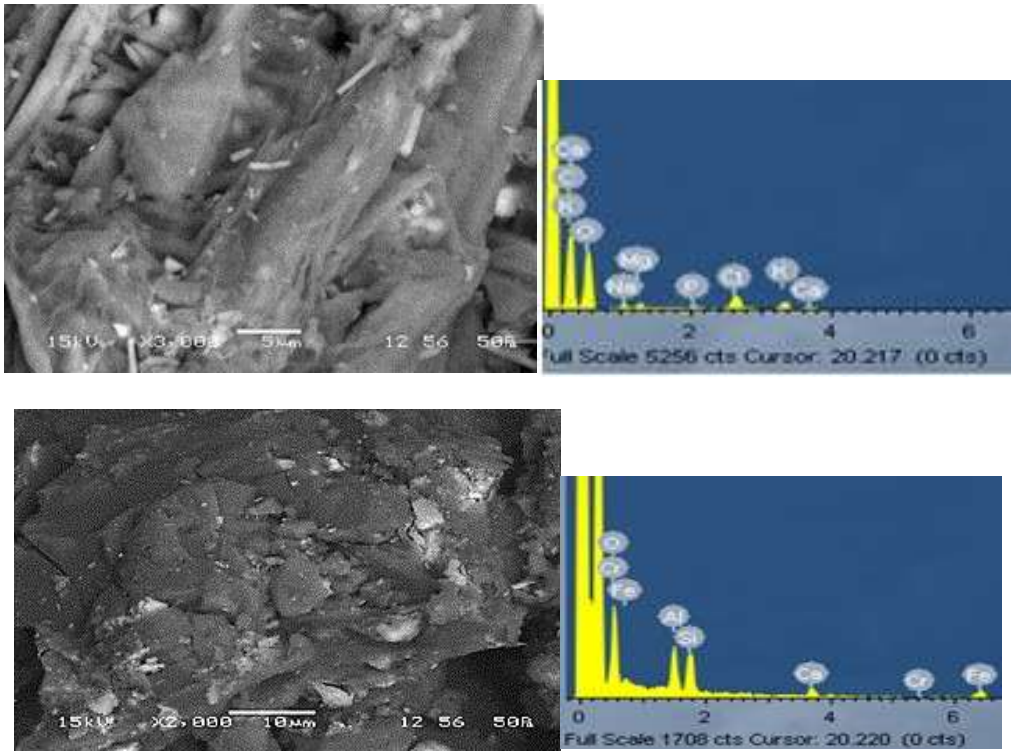

Figure 4: (a) SEM-EDX of Pratikshya rice cultivar biomass control at 2000 x (b) SEM-EDX of Pratikshya rice cultivar biomass (exposed to 300 µg/L of $K_2Cr_2O_7$ solution) at 2000 x

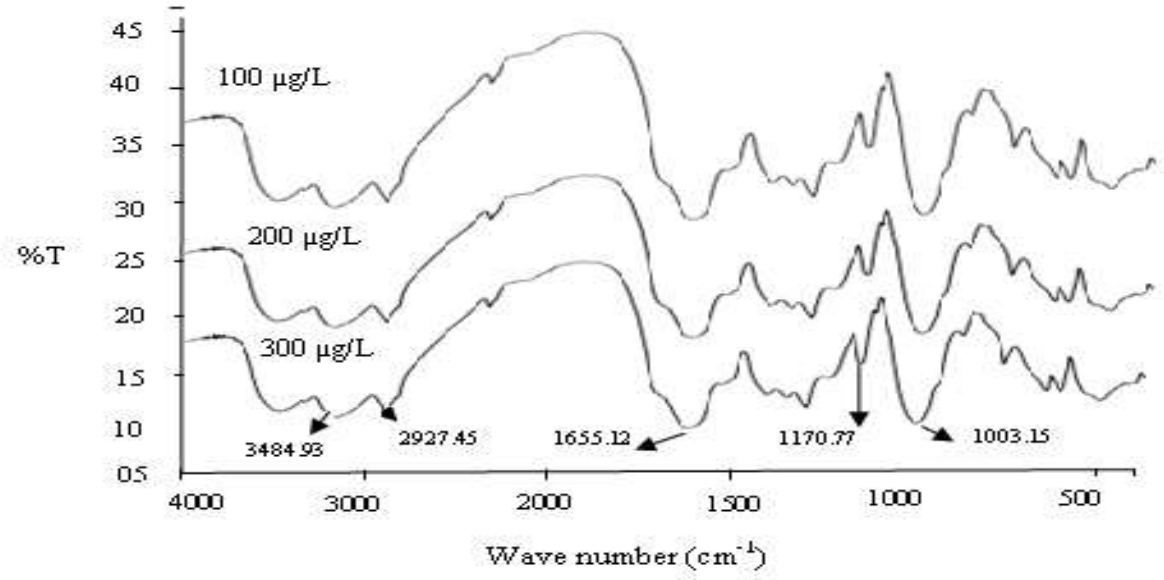

Figure 5: FTIR spectra of the Pratikshya rice cultivar biomass treated with different concentration of chromium at pH 7.0

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