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Antioxidant Response of Indian Wild Rice Exposed to Different Concentration of Chromium for Phytoremediation

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ABSTRACT

The present study was conducted to evaluate the phytotoxic effects induced by hexavalent chromium (Cr) on germination and antioxidant response in Indian wild rice (*Oryza nivara* and *Oryza rufipogon*). After exposure to varying concentration of Cr, two varieties of wild rice *O. nivara* and *O. rufipogon* showed significant changes in growth and physiological parameters. Higher concentration of chromium resulted in significant reduction of germination, fresh weight, dry weight and dry matter accumulation in both the wild rice seedlings compared to the control plants. In low concentration of Cr (25; 50 µM) the activities of antioxidative enzymes were significantly increased in both the varieties over control and the activities were significantly decreased under high concentration of Cr (75; 100 µM). The results from the present experiments suggest that high concentrations of Cr caused oxidative damage as evidenced by increased anti-oxidative enzymes and the induction of antioxidative enzymes could be the reason for tolerating higher levels of metals by wild rice. The findings suggest that phytoremediation of Cr from soil by application of weeds like Indian wild rice.

1. Introduction

Chromium (Cr) is one of the most toxic heavy metals found abundantly in the earth's crust, which attenuates the environment and is discharged into the environment through mining activities and various human activities [1]. Heavy metal toxicity, especially due to Cr (VI) has been considered as a threat to the agriculture as well as surrounding flora and fauna. In many reports chromium is phytotoxic and has been demonstrated to induce the formation of reactive oxygen species (ROS) such as singlet oxygen, hydroxyl radicals and hydrogen peroxide, which cause oxidative damage of plant biomolecules (proteins, nucleic acids, lipids) and alter essential biological processes (seed germination, photosynthesis, enzyme activity, cell division, growth) [2-4]. To avoid this kind of cellular damage, plants possess a complex system of antioxidative enzymes like superoxide dismutase, catalase, peroxidase and ascorbate peroxidase or by the action of non-enzymatic mechanisms in order to scavenge ROS [3]. Unlike other heavy metals, relatively few information is published on Cr-induced oxidative stress in higher plants [2].

Keeping in view of serious phytotoxic impacts of Cr, it becomes the need to remediate such heavy metals from the cultivated land in the contaminated areas. Present investigation is a phytoremediation approach through testing the potentiality of a weed species *i.e.* Indian wild rice. Wild rice plants are annual plants is a wild progenitor of the cultivated rice. It is an annual short (usually <2 m) seasonal grass found growing in swampy areas, at edge of pond and tanks, beside streams and in ditches [5]. As the wild relatives and ancestor of cultivated rice, it carries various characteristics resistant to biotic and abiotic stresses and abundant genetic diversity [6]; therefore it can be used as a convenient plant material for heavy metal toxicity investigations [5].

The present study aims to evaluate the phytotoxic effects induced by Cr^{6+} on germination and seedling vigour characteristics along with antioxidant response in wild rice. It will give an overview of the impact of varying concentration of Cr on two cultivars of Indian wild rice and removal of these toxic contaminants from soil by potent application of weeds like Indian wild rice.

2. Experimental Methods

2.1 Plant Material and Growth Condition

The study was conducted by taking two varieties of Indian wild rice *Oryza nivara (*Accession No.100367) and *Oryza rufipogon (*Accession No. 100377). The dry healthy seeds were collected from Central Rice Research Institute (CRRI), Cuttack, India. Uniform sized seeds were selected and disinfected with 0.1% mercuric chloride ($HgCl_2$) for 5 minutes and then thoroughly washed with distilled water 3 times. The seeds of both the genotypes were placed in sterilized petriplates over saturated tissue paper and regularly irrigated under varying concentrations of Cr^{6+} (0 as control, 25 µM, 50 µM, 75 µM and 100 µM) [source: $K_2Cr_2O_7$] at 25 °C in the laboratory condition. The experiments were carried out in three replications in a randomize block design. The rate of seed germination was recorded daily. On the 9th day after sowing various seed germination characteristics, like rate of germination and seedling growth parameters such as fresh weight and dry weight and dry matter accumulation were recorded.

2.2 Measurement of Antioxidant Enzyme Activity: Extraction

Each 0.5 g of germinated seed of wild rice was homogenized in 10 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM ascorbate and 10% (w/v) sorbital and 0.1% tritonX -100. The homogenate was centrifuged at 0-4 °C at 12,000 rpm for 15 minutes and the supernatant was used for enzyme analysis. All the operation was performed at 0-4 °C. An aliquot of the extract was used to determine protein content following Lowry et al [7].

2.3 Superoxide Dismutase (SOD: EC. 1.15.1.1)

Superoxide dismutase was measured by the photochemical method described by Gianopolitis and Ries [8] with modification suggested by Choudhury and Choudhury [9]. Two mL reaction mixture contained 1.3 mL (50 mM) Na_2PO_4 buffer (pH 7.8) containing 0.1 mM EDTA, 63 µM nitro blue tetrazolium chloride (NBT) and 13 µL L-methionine, 0.2 mL of enzyme extract and 0.5 mL of riboflavin (1.3 µM). Riboflavin was added last. The reaction was monitored in the presence of two 40V fluorescent lamp for 20 min. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction at 560 nm and was expressed unit g^{-1} fresh weight.

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2.4 Ascorbate Peroxidase (APX: EC. 1.11.1.11)

Ascorbate peroxidase was measured following Nakano and Asada [10] by monitoring the rate of ascorbate oxidation at 290 nm (E=2.8 mM cm^{-1}). The reaction mixture (2 mL) containing 50 mM Na_2PO_4 buffer of pH 7.0 (1.2 mL), 100 mM H_2O_2 (0.3 mL) and 0.5 mM ascorbic acid (0.3 mL) and 0.2 mL of enzyme aliquot. The reaction was monitored for 3 min and activity was expressed as mM ascorbate oxidized min^{-1} g^{-1} fresh weight.

2.5 Catalase (CAT: EC.1.11.1.6)

Catalase activity was measured, following Cakmak and Marschner [11], in a reaction mixture (2 mL) containing 50 mM Na_2PO_4 buffer of pH 7.0 (1.5 mL), 100 mM H_2O_2 (0.3 mL) and 0.2 mL enzyme extract. The decomposition of H_2O_2 was monitored at 240 nm for 3 min and the activity was expressed as change in OD min^{-1} g^{-1} fresh weight.

2.6 Guaiacol Peroxidase (GPX: EC. 1.11.1.7)

The activity of guaiacol peroxidase was assayed following the method of Rao et al [12]. The reaction mixture (2 mL) contained 0.1 mM guaiacol (1.5 mL), 0.1 mM H_2O_2 (0.3 mL) and 0.2 mL enzyme extract. Enzyme activity was measured by increased in absorbance at 470 nm caused by guaiacol oxidation (E = 26.6 mM cm^{-1}) and the activity was expressed in mM guaiacol oxidized min^{-1} g^{-1} fresh weight.

2.7 Statistical Analysis

Differences between various growth parameters and antioxidant enzyme activities were compared by ANOVA using CROPSTAT (International Rice Research Institute, Philippines) software's least significant difference (LSD*p<0.05), as this is a good test for determining whether means were significantly different. Correlation coefficients and regression analysis were done following the standard procedure.

3. Results and Discussion

The extent of visible injury caused by chromium stress was used as an indicator of the sensitivity of a plant to metals and the plants grown in high levels of metals show visible symptoms of injury reflected in terms of germination of seed, reduction in plant growth, and finally death [13]. In these experiment two varieties of wild rice *O. nivara* and *O. rufipogon* showed same growth response in relation to different concentration of Cr as significant varietal difference was not observed in different treatments in terms of germination and growth parameters. The test of significance of different growth parameters and antioxidant enzymes activities are presented in Table 1 and 2. The differences were non-significant in the case of replication, whereas in most of the cases the differences in the case of variety, treatment and variety x treatment interactions were highly significant.

Table 1 Changes of Antioxidant enzymes of wild rice seedlings exposed to different concentration of chromium. SOD: Superoxide dismutase, APX: Ascorbate peroxidase, CAT: Catalase, GPX: Guaiacol peroxidase. Data are the mean of 3 replications.

Treatment	SOD (Units g^{-1} Fwt)		APX(mM Ascorbate oxidised min^{-1} g^{-1} Fwt)		GPX (µM guaiacol oxidised min^{-1} g^{-1} Fwt)		CAT(Change in OD. min^{-1} g^{-1} Fwt)	
	O. nivara	*O. rufipogon*	*O. nivara*	*O. rufipogon*	*O. nivara*	*O. rufipogon*	*O. nivara*	*O. rufipogan*
Control	97.81	97.16	2.59	1.65	578	575	1.44	2.31
25 µM	109.0	103.9	2.52	1.91	588	581	1.63	2.45
50 µM	127.3	150.8	2.95	2.79	681	631	1.86	2.75
75 µM	54.04	49.05	0.45	1.34	190	234	1.25	1.56
100 µM	21.86	47.51	0.67	0.76	186	246	1.05	1.31
LSD *P<0.05	16.27		0.85		104		0.45	

Table 2 F value of different parameters of Indian wild rice under different concentration of chromium as studied by ANOVA. *:significance at P<0.05, **: significance at P<0.001, ns: not significant.

Source of Variation	SOD	APX	GPX	CAT	Protein	Fwt	Dwt	DMA
Variety (V)	16.54*	3.32 ns	0.06 ns	5.05*	1.65 ns	5.43*	6.70*	5.12*
Treatment(T)	58.69**	9.82*	78.17**	31.84**	27.76**	4.44*	530.13**	14.90**
Replication (R)	0.92ns	0.37 ns	2.48 ns	1.10 ns	0.08 ns	0.76 ns	0.01 ns	0.01 ns
VXT	3.29 ns	5.84*	0.47 ns	10.72*	0.77 ns	75.32**	16.33**	0.49 ns

3.1 Chromium Effect on Germination and Growth Parameters

Germination is a prime plant-growth process, which play a major role in deciding subsequent growth and yield. Seed germination and early seedling growth are vital for continuation of life of seeds and seedlings are extremely vulnerable to environmental stresses due to presence of polluting agents in the environment. In the present study impact of Cr on germination of wild rice seedlings revealed that germination of wild rice starts after 4 days in each treatment and gradually increased up to 9 days. Varietal difference was not observed among the two varieties of wild rice *Oryza nivara* and *Oryza rufipogon* but germination percentage was varied from 21% to 98% in different treatments and it decreased with the increase of chromium concentration. In high concentration of Cr remarkably reduce the germination and the percentage of reduction was 78% and 76 % in *Oryza nivara* and *Oryza rufipogon* respectively under 100 µM of chromium compared to the control (Fig. 1). Reduction in germination in wild rice by the higher concentration of Cr in the present study may be due to osmotic inhibition of water absorption and or heavy metal toxicity inhibiting the functions of essential enzymes during germination [14, 15].

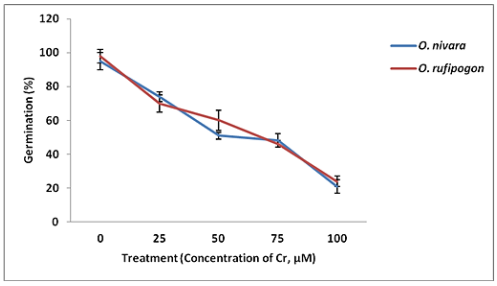

Fig. 1 Germination percentage of wild rice under different concentration of Chromium. Data are the mean of three replications and Bar represents the standard deviation.

High concentrations of heavy metals can negatively affect crop growth, as these metals interfere with metabolic functions in plants [16, 17]. The present study evident that the early seedling characteristics of wild rice, such as fresh weight, dry weight and dry matter accumulation showed higher in control condition and significantly decreased under different concentration of Cr (Fig. 2 and 3). Fresh weight and dry weight of *O. nivara* and *O. rufipogon* seedlings were significantly reduced with increase in the Cr concentrations (Fig. 2). The more reduction of dry matter accumulation in both the variety was observed under 100 µM Cr. Adverse effects of higher concentration of Cr on growth and yield of various crops were evaluated by different workers [5].

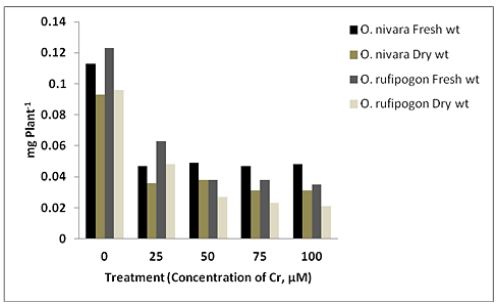

Fig. 2 Changes of growth parameters like fresh weight and dry weight of wild rice seedlings exposed to different concentration of chromium. Data are the mean of 3 replications.

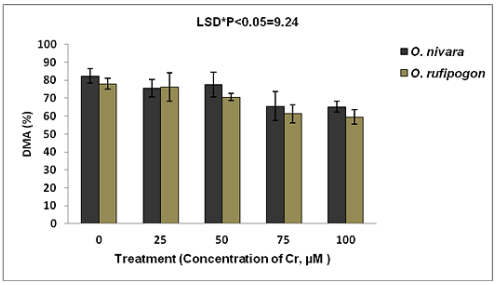

Fig. 3 Changes of dry matter accumulation (DMA) of wild rice seedlings exposed to different concentration of chromium. Data are the mean of 3 replications and bar represents the standard deviation.

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3.2 Chromium Effect on Soluble Protein

The soluble protein content of wild rice seedlings was found to be significantly decreased over control under different concentration of Cr in both the varieties (Fig. 4). The main cause of variance was proved to be the treatment 89% of total variance (Table 3). Reduction in protein content may cause due to decreased protease activities seems to be a common feature involved in metal toxicity in plants and it may be affecting total protein and growth of plants [18].

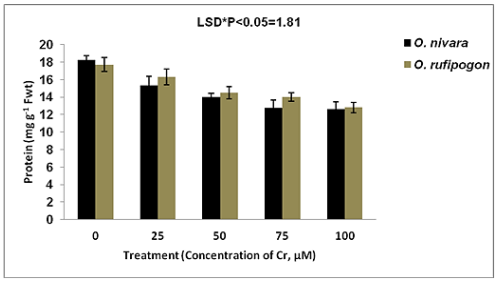

Fig. 4 Changes of soluble protein in wild rice seedling exposed to different concentration of Chromium. Data are the mean of three replications and Bar represents the standard deviation

Table 3 Sum square as absolute value and percentage of total (in bracket) of main effect resulting from analysis of variance of studied parameters in wild rice subjected to different concentration of chromium.

Parameters	Source of Variation		
	Variety (df=1)	Treatment(df=5)	Variety X Treatment (df=5)
SOD	855.6*(6.02)	12142.3**(85.6)	680.5^{ns}(4.8)
APX	0.434^{ns}(4.41)	5.13*(52.12)	3.05*(31)
GPX	133.6^{ns}(0.02)	661708**(95.9)	4012.5^{ns}(0.58)
CAT	0.176*(2.7)	4.43**(68.7)	1.49*(23.1)
Protein	1.06^{ns}(1.32)	71.22**(89)	1.97^{ns}(2.5)
Fresh weight	0.000013*(0.07)	0.017**(94.4)	0.0007**(3.9)
Dry weight	0.00004*(0.29)	0.013**(94.20)	0.0004**(2.9)
Dry Matter Accumulation	85.6*(6.8)	995.14**(78.7)	32.9^{ns}(2.6)

*df, Degrees of freedom; Total df=19; The P of overall ANOVA for variety, treatment and variety X treatment interaction for each parameters *P<0.05 ,**P<0.01,ns not significant.*

3.3 Chromium Effect on Levels of Antioxidant Enzyme Activity

To explain the difference observed between the two varieties and to understand how the protective mechanism might be functioning in *O. nivara* and *O. rufipogon*, we measured the levels of the activities of some antioxidant enzymes. The activities of all the antioxidant enzymes exhibited almost significant changes in different concentration of Cr as compared to the control. Analysis of variance (ANOVA) showed that the treatment describe the main cause of variance in all the antioxidant enzyme activities (Table 3). Superoxide dismutase (SOD) activity was significantly increased over the control in both the variety under lower concentrations of Cr (25, 50 µM) but the activity was remarkable decreased under higher concentration of Cr (Table 1). The reduction of SOD activity was 78% and 52% in *O. nivara and O. rufipogon*, respectively under 100 µM Cr compared to the control. The main cause of variance proved to be the treatment 86% of total variance followed by interactions of variety × treatment (5%) and the variety (6%) (Table 3). The APX activity of wild rice seedlings was found to be significantly more in 50 µM Cr over control conditions but considerably decreased in 100 µM concentration of Cr (Table 1). The reduction of APX activity was 75% and 56% in *O. nivara and O. rufipogon*, respectively under 100 µM Cr compared to control seedlings. The APX activity was greatly affected by treatment which accounted for 52% of total variance followed by variety × treatment (31%) and the variety (4%) (Table 3). The GPX activity of wild rice seedlings was found to be significantly more in 50 µM Cr over control conditions but considerably decreased in 100 µM Cr (Table 1). The significant reduction of GPX activity was observed in *O. rufipogon* (68%) *and O. nivara* (58%) under 100 µM Cr over control seedlings. The GPX activity was greatly affected by treatment which accounted for 96% of total variance (Table 3). CAT activity was increased over the control condition in both the variety under 25 and 50 µM Cr (Table 1) but higher concentration of Cr, CAT activity are significantly reduced. The percentage of reduction was 38% and 44% in *O. nivara and O. rufipogon* respectively under 100 µM Cr over control seedlings. The CAT activity was greatly affected by treatment, which accounted for 69% of total variance followed by variety × treatment (23%) and the variety (3%) (Table 3). Correlation of antioxidant enzymes, protein and growth parameters were presented in Table 4. There was highly significant positive association of antioxidant enzyme activities with protein, and dry matter accumulation. The levels of APX, GPX and CAT also showed highly significant positive correlation with different growth parameters.

Table 4 Correlation coefficient (r value) of different parameters. *P<0.05, **P<0.01,ns not significant.

	SOD	CAT	APX	GPX	Protein	Fwt	Dwt	DMA
SOD	1							
CAT	0.619*	1						
APX	0.783**	0.372ns	1					
GPX	0.828**	0.798**	0.728**	1				
Protein	0.877**	0.665**	0.752**	0.909**	1			
Fwt	0.815**	0.764**	0.571*	0.774**	0.826**	1		
Dwt	0.827**	0.761**	0.591*	0.801**	0.859**	0.993**	1	
DMA	0.606*	0.654*	0.464*	0.787**	0.784**	0.659**	0.732**	1

Metal stress can induce oxidative stress, causing an increased production of reactive oxygen species (ROS) [19]. Plants possess a number of antioxidant molecules and enzymes that protect them against the oxidative damage caused by ROS. To combat the metal toxicity of several free radicals, there is a mobilization of the antioxidant reserves in the plant, which react both enzymatically and non-enzymatically with these toxic molecular species, making them less harmful [20, 21]. In low concentration of Cr (25; 50 µM) the activities of antioxidative enzymes were significantly increased in both the varieties over control. This result agreed with the reports on the other crops [19]. This early rise of enzymes was considered to be the response to active oxygen activities caused by metal. Possibly, increased levels of active oxygen stimulate the cellular protective mechanism to mitigate damages, but with the high concentration of metal *i.e.* (75; 100 µM) the significant decreased activities of antioxidative enzymes like SOD, APX, GPX and CAT indicating the low activity of wild rice seedlings to decompose to H_2O_2 and O_2 as has been reported for other crops [19]. Another possible cause of the reduction of these enzymes is the decrease of the production and/ or activity of ROS. This finding is consistent with the results obtained from previous studies with Cr tolerance in green gram conducted by Mohanty and Patra [22].The results from the present experiments suggests that high concentrations of Cr caused oxidative damage as evidenced by increased anti-oxidative enzymes and the induction of antioxidative enzymes could the reason for tolerating higher levels of metals by wild rice.

4. Conclusion

The findings suggest that removal of these toxic Cr by potent application of weeds like Indian wild rice and can be used as phytoremediation. Further research on screening the cultivars for Cr tolerance potentiality is needed and the ability of different cultivars of this plant for increasing phytoaccumulation potential needs to be tried along with various biosorption models.

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