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Speciation dependant antioxidative response in roots and leaves of sorghum (*Sorghum bicolor* (L.) Moench cv CO 27) under Cr(III) and Cr(VI) stress

Arun K. Shanker[1,2,3] & G. Pathmanabhan[1]

[1]*Department of Crop Physiology, Tamil Nadu Agricultural University, Lawly Road, Coimbatore, Tamil Nadu, India.* [2]*National Research Centre for Agroforestry (NRCAF), Pahuj Dam, Gwalior Road, Jhansi, Uttar Pradesh, India.* [3]*Corresponding author*

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Abstract

Growth, lipid peroxidation, H_2O_2 produciton and the response of the antioxidant enzymes and metabolites of the ascorbate glutathione pathway to oxidative stress caused by two concentrations (50 and 100 μM) of Cr(III) and Cr(VI) was studied in 15 day old seedlings of sorghum (*Sorghum bicolor* (L.) Moench CV CO 27) after 10 days of treatment. Cr accumulation in sorghum plants was concentration and organ dependant. There was no significant growth retardation of plants under 50 μM Cr(III) stress. 100 μM Cr(VI) was most toxic of all the treatments in terms of root and leaf growth and oxidative stress. 50 μM Cr(VI) treated roots exhibited high significant increase in superoxide dismutase (SOD), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) ($p < 0.01$) and significant increases in catalse (CAT), ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR) ($p < 0.05$). A high increase in ascorbic acid (AA) level was seen in roots of 50 μM Cr(VI) treated plants in comparison with control. Levels of reduced glutathione (GSH) showed a varied and complex response in all the treatments in both plant parts. GSH/GSSG ratio was not affected by Cr(III) treatment in leaves, in contrast, roots exhibited significant reduction in the ratio. Results indicate that GSH depletion increased sensitivity to oxidative stress (Cr(VI) roots and leaves and Cr(III) 100 μM roots) and AA in tandem with APX compensated for GSH depletion by acting directly on H_2O_2 and the mechanism of defensive response in roots as well as leaves varied in its degree and effectiveness due to the concentration dependant differences observed in translocation of the element itself, reactive oxygen species (*ROS*) generation and enzyme inhibition based on the oxidation state supplied to the plants.

Introduction

Chromium (Cr) finds its use in many different industries on a large scale, which include leather tanning, production of paints and pigments, wood preservation, pulp and paper production, Cr chemicals production, metallurgical and electroplating. Contamination of ground water and soil due to the use of chromium (Cr) in these anthropomorphic activities has become a serious source of concern to animal and plant scientist over the past decade. Cr in contrast to most trace metals like cadmium, lead, mercury and aluminum with no known function in plants has got relatively less attention from plant scientists. The physiological impact of Cr contamination in soil and water is dependant on the oxidation state and it has a direct impact on mobilisation of the metal, subsequent uptake and resultant toxicity in the plant system.

The toxic property of Cr(VI) originates from the action of this form itself as an oxidizing agent, as well as from the formation of free radicals during

Fax no: +91-517 273064. E-mail: arunshanker@mailcan.com)

the reduction of Cr(VI) to Cr(III) occurring inside the cell. Cr(III) on the other hand apart from generating reactive oxygen species (ROS), if present in high concentrations can cause toxic effects due to its ability to co-ordinate various organic compounds resulting in inhibition of some metallo-enzymes systems (Dixit et al., 2002; Von Burg and Liu, 1993; Mei et al., 2002). High concentrations of ROS such as singlet oxygen, hydroxyl ions and hydrogen peroxide cause oxidative stress and this explains most of the visual Cr toxicity symptoms observed at whole plant level (Zayed and Terry, 2003). However, the ROS that arise as a consequence of increased environmental stress are also important initiators of signalling pathways that lead to cellular, and ultimately, whole plant acclimation to adverse conditions. The main defence against the over-accumulation of ROS is an extensive network of enzymes and low molecular weight antioxidants inside the cell (Mitller, 2002). This antioxidant network constantly undergoes adjustment in response to both fluctuations in environmental conditions and intrinsic factors such as the developmental state of the plants. The synchronous action of antioxidant enzymes of the ascorbate glutathione pathway viz., catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) with the thiol regulated enzymes dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reducatse (GR) is a predominant mechanism of ROS quenching under heavy metal stress (Clijsters et al. 1999). Low molecular weight antioxidant metabolites like ascorbic acid (AA) and reduced glutathione (GSH) apart from being electron source to these enzymes the metabolites themselves play an important role in protecting plants from oxidative stress damage. The ascorbate glutathione pathway originally found in chloroplasts, has now since been identified in other subcellular compartments such as mitochondria and peroxisomes, in non-photosynthetic tissues, including roots (Jimenez et al. 1997; Paciolla et al. 2001). Every enzyme in the ascorbate glutathione pathway has been shown to be increased by Cu (Gupta et al., 1999). There are several reports of oxidation of various cellular thiols and ascorbic acid by Cr(VI) and also of formation of thiolate complexes (glutamate CrO_3^-) *in vitro* and in animal systems (Brauer and Wetterhahn, 1991; Brauer et al., 1996; Suzuki and Fukuda, 1990). There is dearth of literature with regard to the role of ascorbate glutathione pathway in cellular defense against chromium in general and chromium speciation in particular in plants parts. Roots accumulate several magnitudes higher Cr under both oxidation state as compared to shoots (Zayed et al. 1998) which suggest the possibility of different responses of this pathway in roots and leaves. The present study was taken up to investigate the effect of two different concentrations of Cr(III) and Cr(VI) in nutrient media on the enzymes and metabolites of ascorbate glutathione pathway in roots and leaves of sorghum (*Sorghum bicolor* (L.) Moench cv CO 27).

Materials and methods

Plant material and hydroponic experiment

Sorghum (*Sorghum bicolor* (L.) Moench cv CO 27) seeds were cold treated (+4 °C) for 3 days to break dormancy and synchronize germination. Seeds were germinated in roll towels and germinating seedlings of similar size were placed in half strength Hoagland's solution containing (mM): 2.4 $Ca(NO_3)_2$, 1.0 KH_2PO_4, 3.0 KNO_3, 1.0 $MgSO_4$ and 0.5 NaCl and (μM) 23.1 H_3BO_3, 4.6 $MnCl_2$, 0.38 $ZnSO_4$, 0.16 $CuSO_4$, 0.052 H_2MoO_4 and 44.8 $FeSO_4$ (as ferric sodium ethylenediaminetetraacetate (EDTA) complex) on perforated polystyrene floats. The nutrient solution was bubbled with sterile air. The pH of the nutrient solution was adjusted to 6.8 using either 0.1 N HCl or 0.1 N KOH. The experiment was in completely randomized design with five replications. Growth chamber conditions were : photosynthetic photon flux density of 430 μmol m^{-2} s^{-1}, 16 hours of light, 8 hours of dark and a relative humidity of 60 per cent. After 15 days of seedling growth the plants were supplied with two forms of Cr: Cr(III) as chromium chloride and Cr(VI) as potassium chromate at a concentration of 50 and 100 μM each. Plants were harvested after 10 days of Cr treatment for estimation of enzyme activities, total Cr content, lipid peroxidation, H_2O_2, and metabolites of ascorbate glutathione pathway. Roots and shoots were weighed, parts of the root and shoot tissue were washed and dried for Cr estimation and the other part was divided into 1 gram fresh weight samples which were frozen in liquid nitrogen and stored at −70 °C.

Chromium content and plant growth

Measurement of chromium content (μg g^{-1}) was made on individual plants. Roots and shoots were separated and oven dried for three days at 80 °C. Samples

were then ground into fine powder using a grinding mill. The conditions used for digestion were according to Davies et al. (2002). Five millilitres of concentrated HNO_3 was added to 0.25 g of dried sample in a 50 ml digestion tube and allowed to stand overnight at room temperature. The digestion tubes were placed in a heating block for one hour at 150 °C, tubes were then removed allowed to cool and 2 ml of 30 per cent H_2O_2 was added. The tube contents were mixed by swirling, and then heated for 2 more hours at 150 °C. After cooling the solution was diluted to 50 mL total volume and the upper clear portion was used for chromium estimation. During dilution, NH_4Cl was added at 2 per cent and $CaCl_2$ was added at 0.5 per cent to each sample and standard to control interference caused by iron (Fe) and phosphorus (P) respectively during spectrophotometer analysis. Digested samples were analyzed for Cr in atomic absorption spectrometer (Varion Spectra AA-220) with air-acetylene flame at 358 nm with 0.2 mm spectral slit width. Plant growth was measured as root and shoot length and dry weights were measured after drying the samples in hot air oven at 60 °C till constant weight was reached.

Enzymes assays

For all enzymes activity estimations, frozen root tissue was homogenized in ice cold 0.1 M Tris-HCl buffer at pH 7.8 containing 1 mM EDTA, 1 mM dithiotreitol and 5 ml of 4 per cent polyvinyl pyrrolidone per gram fresh weight. The homogenate was filtered through a nylon mesh and centrifuged at 20,000 g at 4 °C. The supernatant was used for measuring enzyme activity. Protein was estimated according to Bradford (1976). *Catalase (CAT) (EC 1.11.1.6)* was estimated according to Samantaray (2002). One ml of the supernatant 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. The reaction was discontinued by adding 10 ml of 2 per cent H_2SO_4 after 1 min of incubation at 20 °C. The reaction mixture was then titrated against 0.01 M $KMnO_4$ to determine the quantity of H_2O_2 used by the enzyme. Enzyme activity was expressed as μmol H_2O_2 destroyed mg protein^{-1} min^{-1}.

Superoxide dismutase (SOD)(EC 1.15.1.1) was determined by nitroblue tetrazolium (NBT) method (Beyer and Fridovich, 1987) by measuring the photoreduction of NBT at 560 nm. One unit of SOD activity equaled to the amount required to inhibit photreduction of NBT by 50 per cent.

Ascorbate peroxidase (APX) (EC 1.11.1.11) enzyme activity was measured according to Gerbling et al. (1984) following the oxidation of ascorbate to dehydroascorbate spectrophotometrically. The enzyme activity was calculated from the observed rates of absorbance decrease and the milli molar extinction coefficient for ascorbate which is 0.80 and 0.055 mmol^{-1} cm^{-1} at 298 and 310, respectively. One unit of enzyme activity was that which catalysed the oxidation of 1 μmol ascorbate min^{-1} mg protein^{-1}.

Monodehydroascorbate reductase (MDHAR) (EC 1.6.5.4) enzyme activity was measured in the supernatant at 25 °C as described by Hussain and Asada (1984). MDHAR was assayed spectrophotometrically by following the decrease in absorbance at 340 nm due to NADPH oxidation using an absorbance coefficient of 6.2 mmol^{-1} cm^{-1}. Monodehyroascorbate formed by ascorbate oxidase was used as standard. One enzyme unit equaled to 1 nM NADPH oxidised min^{-1} mg protein^{-1}.

Dehydroascorbate reductase (DHAR) (EC 1.8.5.1) enzyme activity was measured similar to MDHAR by measuring the reduction of dehydroascorbate at 265 nm according to Foyer et al. (1989). One enzyme unit equaled to 1 nmol ascorbate produced g^{-1} min^{-1} mg protein^{-1}.

Glutathione reductase (GR) (EC 1.6.4.2) estimation method was based on the increase in absorbance at 412 nm when 5.5′- dithiobis (2-nitrobenzoic acid) (DTNB) is reduced by reduced glutathione (GSH) (Smith et al., 1988). The reaction mixture contained 1 ml of 0.2 M potassium phosphate buffer at pH 7.5 containing 1 mM EDTA, 0.5 ml 3 mM DTNB in 0.01 M phosphate buffer, 0.25 ml water, 0.1 ml of 2 mM NADPH, 0.05 ml of GR (1 Uml^{-1}) and 0.1 ml of 20 mM GSSG. The components of the reaction mixture were added in the order stated in 4.5 ml cuvette and the reaction was initiated by the addition of GSSG. The temperature was maintained at 24 °C. The increase in absorbance at 412 nm was monitored. The activity of the enzyme was expressed in mg protein^{-1} wherein unit activity is the amount of enzyme which reduces 1 μmol of GSSG in 1 hour at 24 °C.

Metabolite estimation

One gram of tissue was ground with inert sand in 5 ml of 6.5 percent m-phosphoric acid containg 1 mM NaEDTA with a mortar and pestle. Total ascorbate (AA) and reduced ascorbate was estimated according to Hodges et al. (1996). Dehydroascorbate (DHA) was

Table 1. Growth and chromium content (±SEM) of sorghum after 10 days under different Cr concentration and speciation. * significant at 0.05 and ** significant at 0.01. Nd (Not detected)

Seedling growth	Control	50 μM Cr(III)	100 μM Cr(III)	50 μM Cr(VI)	100 μM Cr(VI)
Shoot length (cm)	16.8 ± (2.1)	15.6 ± (2.3)	14.2 ± (2.6)	10.2 ± (1.8)*	8.2 ± (1.4)**
Root length (cm)	14.3 ± (1.6)	12.4 ± (1.5)	10.2 ± (0.9)*	9.1 ± (1.0)**	6.1 ± (1.8)**
Shoot dry weight (g)	0.121 ± (0.017)	0.117 ± (0.020)	0.114 ± (0.018)	0.103 ± (0.023)**	0.089 ± (0.026)**
Root dry weight (g)	0.036 ± (0.003)	0.032 ± (0.003)	0.028 ± (0.005)*	0.021 ± (0.007)**	0.013 ± (0.009)**
Leaf Area cm^2	24.6 ± (3.8)	22.8 ± (3.6)	18.2 ± (3.3)	13.2 ± (2.3)*	9.4 ± (2.6)**
Chromium content ($\mu g\ g^{-1}$)					
Root	Nd	207.4 ± (12.6)	301.7 ± (11.4)	324.5 ± (19.1)	432.2 ± (21.9)
Leaf	Nd	3.2 ± (0.87)	4.1 ± (1.0)	5.7 ± (1.3)	7.9 ± (1.8)
Stem	Nd	0.9 ± (0.06)	1.2 ± (0.42)	1.3 ± (0.39)	1.8 ± (0.26)

calculated from the difference between the total and reduced ascorbate. Reduced glutathione (GSH), oxidised glutathione (GSSG) and total glutathione were assayed by the enzymatic GSSG recycling method advocated by Bergmeyer et al. (1974) The reaction was monitored by stoichometric conversion of NADPH spectrophotometrically at baseline level of NADPH absorbance at 340 nm. Standard calibration was done using 100 μl GSSG instead of sample. The final concentration of GR amounted to 0.5 unit ml^{-1} in the total glutathione assay (GSH+GSSG), and 1 unit ml^{-1} in the assay of GSSG. GSH content was calculated by subtracting GSSG content from total glutathione. All the metabolites were expressed in nmol g^{-1} fresh weight.

Lipid peroxidation and ROS assay

The lipid peroxidation was determined by malondialdehyde (MDA) content produced by thiobarbituric acid (TBA) reaction at low pH as described by Behra et al. (1999). The pink chromogen was measured at 532 nm and 600 nm for the correction of blank. Lipid peroxidation was expressed as melondialdehyde content in μmol g^{-1}. The H_2O_2 level was colorimetrically measured as described by Okuda et al. (1991) H_2O_2 was extracted by homogenising 0.5 g of tissue with 4 ml of perchloric acid (200 nM). The homogenate was centrifuged at 12,000 g for 10 min. Oxidation of ferrous ions to ferric ions by H_2O_2 in acidic pH was monitored and stable complex of ferric ions with Xylenol orange dye at 560 nm was measured. H_2O_2 was expressed in nmol g^{-1} DW.

Statistical analysis

Three replicates were taken for all the metabolite, ROS, enzyme assays and Cr estimations from each set of experiments ($n = 15$). The mean values ± SE are given in all the tables and figures. The data was analyzed statistically using a general linear model for analysis of variance (repeated measures Anova) (Wilkinson et al., 1996). Significance between control and treatments were compared at 0.05 and 0.01 probability levels

Results

Cr accumulation and growth

Cr accumulation in sorghum plants was concentration, speciation and organ dependant. Plants exposed

to higher concentration (100 μM) of both the speciation accumulated more Cr than lower concentration (50 μM). Under equal concentration of Cr(III) and Cr(VI): Cr(VI) treated plants exhibited higher Cr accumulation in all the plant parts analyzed than Cr(III) treated plants (Table 1). With regard to the plant parts analyzed, highest accumulation was seen in the roots followed by leaves and stem irrespective of the concentration or speciation of Cr. The difference between Cr accumulated by all plant parts treated with 100 μM Cr(III) and 50 μM Cr(VI) was least (22.8, 1.6 and 0.1 μg g^{-1} root, leaf and stem respectively). Roots accumulated 64.6 and 73.4 times higher Cr than leaf in 50 and 100 μM Cr(III) treated plants as against 56.8 and 54.7 times under 50 and 100 μM Cr(VI) treated plants. The least presence of Cr was seen in the stems of all plants in all concentration and speciation treatments. There was no significant growth retardation of plants under 50 μM Cr(III) stress although all the growth parameters exhibited slightly lower values than control. 100 μM Cr(III) treated plants exhibited significant growth retardation in terms of root length and dry weight whereas shoot growth was not significantly affected (Table 1). On the other hand both concentrations of Cr(VI) affected all the parameters studied with root length, shoot dry weight and root dry weight severely affected in plants under 100 μM Cr(VI) stress.

ROS generation

H_2O_2 levels significantly increased over control in all the treatments and both plant parts studied excepting leaves of 50 μM Cr(III) treated plants (Figure 1). Cr(VI) at 100 μM induced higher H_2O_2 in both leaf and root tissue (significant at 0.01 probability level), Lipid peroxidation in terms of MDA accumulation followed a similar trend as observed in the case of H_2O_2 accumulation with the only difference being higher degree of significance as compared to control in all the treatments.

Antioxdative enzyme activities

Significant increase in SOD, DHAR and GR activities was observed in roots of 50 μM Cr(III) treated plants, CAT, APX and MDHAR did not exhibit any significant increase although there was numerical increase in the activity of these enzymes over control (Table 2). However, in the leaves of the plants studied under the same treatments none of the enzymes assayed exhibited any significant increase or decrease over control.

Table 2. Antioxidant enzyme activity (± SEM) in roots and leaves of sorghum after 10 days under different Cr concentration and speciation. * significant at 0.05 and ** significant at 0.01

Enzyme	Control		50 μM Cr (III)		100 μM Cr (III)		50 μM Cr (VI)		100 μM Cr (VI)	
Unit mg protein^{-1}	Roots	Leaves	Roots	leaves	roots	leaves	Roots	leaves	roots	Leaves
SOD	34.9 ± 2.3	27.4 ± 2.1	38.9 ± 3.2*	29.1 ± 3.4	39.7 ± 2.7*	32.1 ± 2.9*	41.0 ± 3.1**	35.8 ± 3.0**	22.7 ± 2.9*	34.2 ± 2.1*
CAT	24.7 ± 1.8	28.8 ± 1.7	27.9 ± 1.9	29.7 ± 2.0	29.6 ± 2.1*	30.4 ± 2.4	29.8 ± 1.7*	31.6 ± 2.7	27.8 ± 2.4	32.6 ± 2.3*
APX	0.53 ± 0.08	0.28 ± 0.03	1.12 ± 0.09	0.31 ± 0.03	1.19 ± 0.07*	0.36 ± 0.02*	0.62 ± 0.11*	0.78 ± 0.09**	0.51 ± 0.14	0.69 ± 0.10**
MDHAR	1.72 ± 0.15	1.83 ± 0.15	1.97 ± 0.15	1.87 ± 0.19	1.74 ± 0.18	1.85 ± 0.20	2.07 ± 0.26*	1.92 ± 0.20	1.80 ± 0.23	1.88 ± 0.22
DHAR	0.68 ± 0.07	0.77 ± 0.07	1.01 ± 0.10*	0.79 ± 0.09	1.28 ± 0.12**	0.82 ± 0.12*	1.47 ± 0.17**	0.97 ± 0.09*	0.75 ± 0.16	0.82 ± 0.09*
GR	0.05 ± 0.006	0.06 ± 0.009	0.09 ± 0.012*	0.07 ± 0.012	0.15 ± 0.013**	0.09 ± 0.008*	0.21 ± 0.019**	0.12 ± 0.008**	0.07 ± 0.005	0.09 ± 0.009*

superoxide dismutase (SOD), catalse (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR)

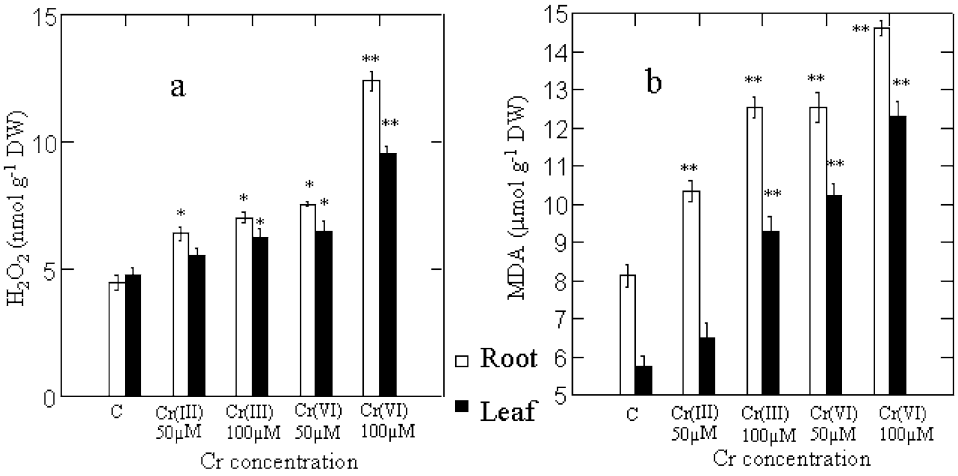

Figure 1. Levels of H_2O_2 (a) and lipid peroxidation expressed as malondialdehyde (MDA) (b) in roots and leaves of sorghum treated with different concentrations of Cr (III) and Cr (VI). Data represents mean ± SE of five separate experiments ∗ significant at $P < 0.05$ ∗∗ significant at $P < 0.01$.

In roots of Cr(III) 100 μM treated plants a significant increase in all the enzymes except MDHAR was observed. 50 μM Cr(VI) treated roots exhibited high significant increase in SOD, DHAR and GR ($P < 0.01$) and significant increases in CAT, APX and MDHAR ($P < 0.05$). Leaves of the plants under the same concentration showed a similar trend as observed in 100 μM Cr(III) treated plants and the only difference being the degree of significance of SOD, GR and APX activities which was higher in 50 μM Cr(VI) treated leaves. The activity of CAT, APX, MDHAR, DHAR and GR came down to control levels in roots of 100 μM Cr(VI) treated plants. SOD activity was significantly lower than control levels in this treatment. On the contrary, leaves of the plant exposed to the same treatment showed high significant increase in APX activities and significant increase in SOD, CAT, DHAR and GR. Changes in MDHAR were not significant.

Metabolites

DHA levels exhibited a high degree of significant increase irrespective of speciation or concentration of Cr in the medium. AA concentration in leaves of Cr(III) treated plants under both concentration fluctuated around control levels in contrast to roots wherein a significant increase in the level of the metabolite was observed (Figure 2). A very high increase (of about 2.5 folds) in AA level was seen in roots of 50 μM Cr(VI) treated plants in comparison with control. This was contrary to the very small increase seen in the 100 μM Cr(VI) treated roots. In contrast to leaves of Cr(III) (both concentration) treated plants AA in Cr(VI) (both concentration) treated plants showed significantly higher levels. Levels of GSH showed a varied and complex response in all the treatments in both plant parts (Figure 3). Significant increase was only observed in Cr(III) 50 μM treatment in roots as well as leaves. In the Cr(III) 100 μM treatment (roots) and both concentration of Cr(VI) (leaves and roots) there was a drastic reduction in the GSH levels. Whereas GSH in Cr(III) 100 μM treated leaves varied around control levels.

GSSG levels of leaves treated with Cr(III) in both concentration fluctuated around control levels which was not the case in roots wherein increase was observed in 50 μM treatment as against a decrease in 100 μM treatment. Both concentration of Cr(VI) increased levels of GSSG in both plant parts studied by almost more than 1.5 folds observed in control. Total glutathione was not affected in Cr(VI) treated roots under both concentrations. The only significant decrease in total glutathione was in Cr(III) 100 μM treatment (Figure 4). With regard to leaves, increase in total glutathione was seen in Cr(III) treatment and a decrease was observed in Cr(VI) treatment. GSH/GSSG ratio was not affected by Cr(III) treatment in leaves, in contrast, roots exhibited significant reduction in the ratio. The ratio was severely affected as evidenced by a high decrease in both plant parts under both concentration of Cr(VI).

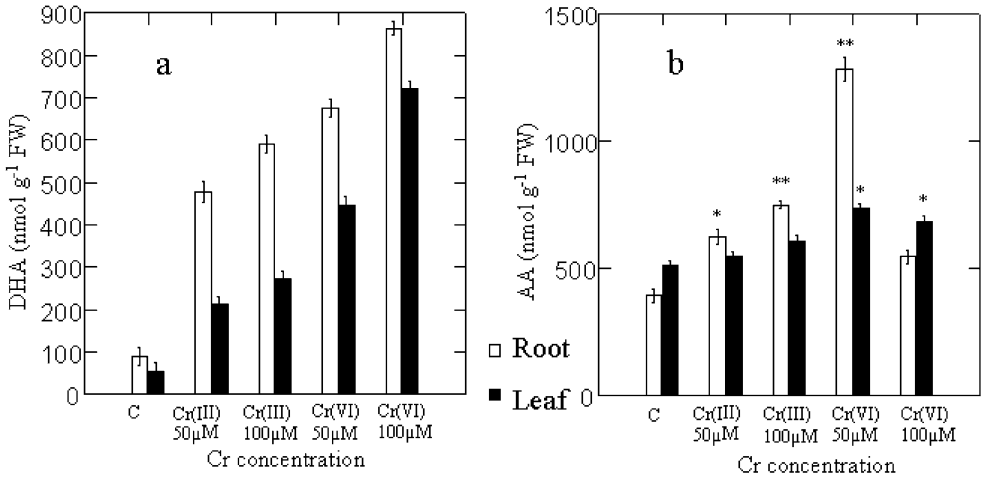

Figure 2. Levels of dehydroascorbate (DHA) (a) (significant in all treatments at 0.01) and total ascorbate (AA) (b) concentration in roots and leaves of sorghum treated with different concentrations of Cr (III) and Cr (VI). Data represents mean ± SE of three separate experiments ∗ significant at $P < 0.05$ ∗∗ significant at $P < 0.01$.

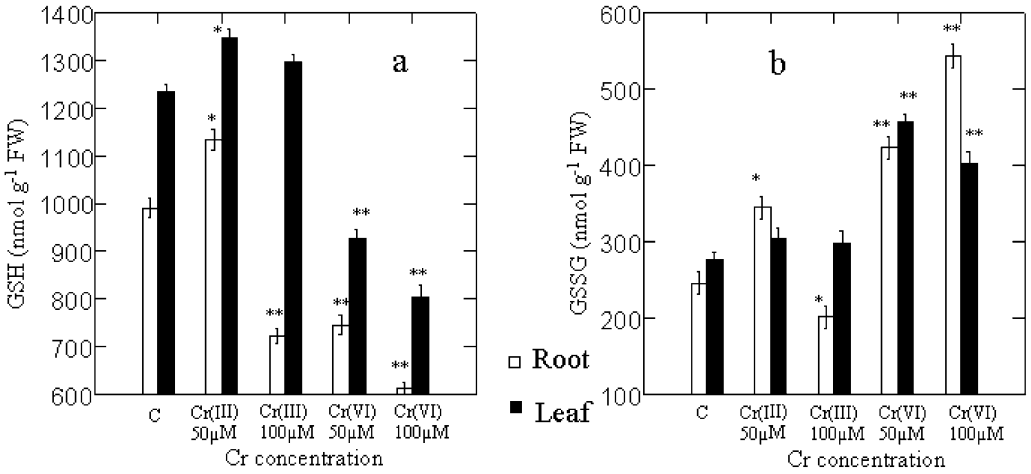

Figure 3. Levels of reduced glutathione (GSH) (a) and oxidised glutathione (GSSG) (b) in roots and leaves of sorghum treated with different concentrations of Cr (III) and Cr (VI). Data represents mean ± SE of three separate experiments ∗ significant at $P < 0.05$ ∗∗ significant at $P < 0.01$.

Disscusion

Cr accumulation, translocation and growth

Cr has a complex chemistry and hence the detailed mechanism of toxicity of Cr is yet to be clearly explained in higher vascular plants. There is evidence that no conversion occurs for Cr oxidation state in the nutrient solution before absorption by plant roots. McGrath (1982) measured the valence of Cr remaining in solution that originally contained either Cr(III) or Cr(VI) after culturing oat plants for 4 weeks. The results showed that both Cr ions remained unchanged in the nutrient solution for the duration of the experiment. In our experiment Cr(VI) was readily taken up by the plants in comparison to Cr(III) but there was a distinct restriction of translocation of both speciation to shoots irrespective of the concentration in nutrient solution. This is explained by the fact that Cr(III) is known to penetrate plant cells only very slowly by diffusion and retained by cation exchange sites of the cell wall (Shewry and Peterson, 1974) thus restricting its further translocation to shoots. The lower toxicity of Cr(III) to the above ground parts can be attributed to this specific reason. However, Cr(VI), is taken up by the plants by active mechanisms involving metabolic energy (Skeffington et al., 1976), thus the reason for its higher uptake could be that it is taken up with the es-

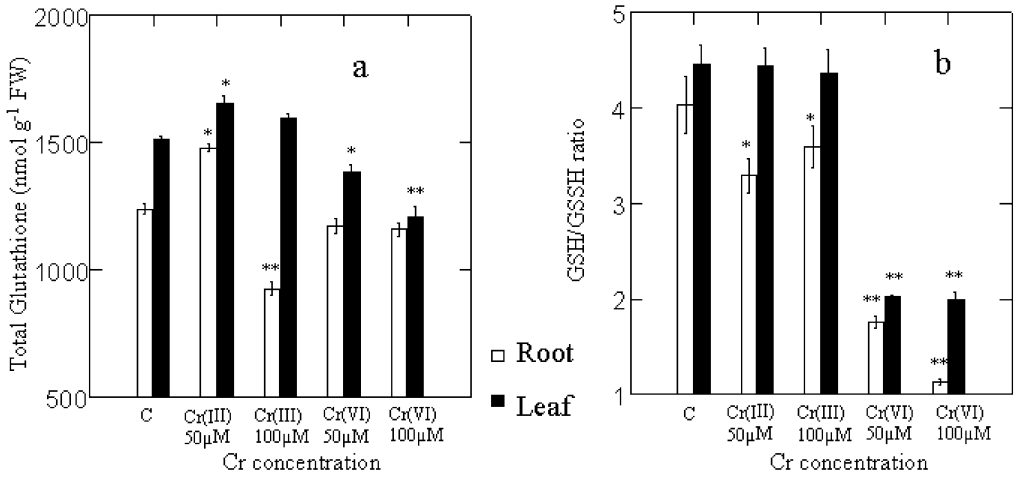

Figure 4. Levels of total glutathione (a) and GSH/GSSG ratio (b) in roots and leaves of sorghum treated with different concentrations of Cr (III) and Cr (VI). Data represents mean ± SE of three separate experiments ∗ significant at $P < 0.05$ ∗∗ significant at $P < 0.01$.

sential elements with similar molecular structure like Fe and S. In addition it is known that P and Cr are competitive for surface sites, Mn is also known to compete with Cr for transport binding (Wallace et al., 1976). Hence it is possible that Cr competed effectively with these elements to gain rapid entry into the plant roots. Poor translocation of Cr to the shoots could be due to vacuolar sequesterization of most of the Cr in the root cells to render it non-toxic, which may be a natural toxicity response of the plant. It must be noted that Cr is a toxic and nonessential element to plants and hence the plants may not possess any specific mechanism of transport of Cr. Both oxidation states of Cr have the potential of generating ROS (Figure 1) although this potential is concentration dependant. Cr(VI) is a strong oxidant with a high redox potential in range of 1.33 and 1.38 eV (Kotas and Stasicka, 2000). Higher H_2O_2 production and lipid peroxidation observed in the present study indicates that extensive oxidative damage could have occurred to the outer root cells under Cr(VI) thus preventing uptake of mineral nutrients and water leading to deficiency in the shoot and culminating in poor growth. On the other hand, Cr(III) apart from its lower potential to produce ROS, is kinetically inert to ligand substitution and therefore can form substitution inert metaloprotein complexes *in vivo* thus greatly reducing its role in causing toxic symptoms. In contrast, under higher concentrations of Cr(III), H_2O_2 can act as an oxidizing agent and may oxidize Cr(III) to Cr(VI) (Rock et al., 2001) hence a possibility of such an endogenous oxidation of Cr(III) to Cr(V) and Cr(IV) (both unstable) cannot be ruled out (Micera and Dessi, 1988). On the other hand, Cr(III) can be endogenously reduced to C(II) by biological reductants L-Cysteine and NADPH. In turn the newly formed Cr(II) reacts with hydrogen peroxide producing hydroxyl radicals causing tissue damage in roots thus accounting for its reduced growth (Stohs and Bagachi, 1995).

Antioxidative response of ascorbate glutathione pathway

All the scavenging enzymes studied were unaffected by lower concentration of Cr(III) in leaves as there was no significant ROS production in leaves. The combined action of SOD and CAT is critical in mitigating the effects of oxidative stress, since the former merely acts on the superoxide anion converting it to another reactive intermediate (H_2O_2) and the latter acts on H_2O_2 converting it to water and oxygen.

SOD in contrast to CAT was more active in scavenging under both the concentration and speciation studied in both plant parts with the exception of roots under 100 μM Cr(VI) stress. Under abiotic stress the differential response of CAT and SOD has been recently reported (Jung, 2003). APX which essentiality catalyses the same reaction as CAT compensated for the reduced CAT activity. This could be because unlike CAT, which is present only in the peroxisome and has low substrate affinities since it requires simultaneous access of two molecules of H_2O_2, APX is present through out the cell and has higher substrate affinity in the presence of AA as a reductant (Willekens et al., 1995). This was evident by the fact that AA turn

over was high as was APX activty in all the stressed treatment again with the exception of 100 μM Cr(VI) treated roots. Higher DHAR activity is reported to enhance efficient AA recycling (Chen et al., 2003). It is possible that ROS generation acted as a signal for enhancing DHAR synthesis and consequently increased effective scavenging. DHA was high in all the treatments inspite of no significant increases observed in MDHAR activities indicating that the short life of MDA and/or inhibition of MDHAR by Cr ions routed a non-enzymatic dispropotionation of MDHA to DHA (Noctor and Foyer, 1998). The failure of this system to operate in 100 μM Cr(VI) treated roots could be because, Cr(VI) apart from generating ROS which consequently sets off a signaling response for active scavenging, could actually inhibit the enzyme activities at acute concentrations. The high content of DHA in combination with an absence of active scavenging and the recently ascribed role of blockage of normal cell cycle progression by DHA (Potters et al., 2002) explains the poor growth and high toxic effect at this concentration of Cr(VI). Glutathione pool dynamics was complex in both the plant parts studied and under both speciation and concentrations studied. The depletion of GSH and total glutathione in spite of higher GR activities observed especially in the roots indicates that mechanism of antioxidant defense was by enhanced oxidation of GSH to GSSG by DHAR yielding AA. This AA in addition to the AA produced by non enzymatic dispropotionation of MDHA was used by APX to directly detoxify H_2O_2. Role of GSH as a signal intermediate in increasing APX expression under metal stress has been reported (Pekker et al., 2002).It is also known that certain other enzymes (thioltranferase, protein disulphide isomerases and kunitz type trypsin inhibiotors) can participate in the GSH – DHA reaction (Morell et al., 1997). In view of the amounts of metabolites present in the stressed plants the role of GSH could have been more of a signal intermediate rather than direct participation in detoxification of ROS. This mechanism is more likely because of the reported absence of phytochelatin production under Cr stress (Toppi et al., 2002) and that glutathione serves as a precursor for phytochelatin production (Grill et al., 1989). The cellular redox status is maintained by the ratio of GSH/GSSG. The GSH-GSSG redox pair can function effectively only when there is an adequate supply of NADPH and that GSH itself can serve as a cellular sensor to maintain the NADPH pool (Potter et al., 2002). Cr(VI) can function as a hill reagent (Desmet et al., 1975) and can inhibit electron transport both in the photosynthetic and mitochondrial apparatus thus accounting for reduced NADPH pool. This in part explains the high reduction in the ratio observed in Cr(VI) treated plants. GR uses NADPH to reduce GSSG to GSH whereas free radicals are able to oxidize GSH to GSSG (Noctor et al., 1998) hence the critical balance between the available NADPH pool and ROS production by Cr would decide the redox status of the cell. Our results indicate that this balance was favorably tilted in the Cr(III) stressed plants especially in the leaves. In addition it is possible that GSH can prevent enzyme inactivation by protecting succeptible protein thiol groups (Alscher, 1989) thus accounting for reduced toxicity in leaves seen under Cr(III) 100 μM treatment. GSH depletion increased sensitivity to oxidative stress (Cr(VI) roots and leaves and Cr(III) 100 μM roots) and AA in tandem with APX compensated for GSH depletion. There is considerable evidence that AA can effectively substitute GSH depletion under stressful condition (May et al., 1998). In conclusion it can be said that the mechanism of defensive response in roots as well as leaves varied in its degree and effectiveness due to the concentration dependent differences observed in translocation of the element itself, ROS generation and enzyme inhibition based on the oxidation state of Cr supplied to the plants.

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