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Uptake and Translocation of Tri- and Hexa-Valent Chromium and Their Effects on Black Gram (*Vigna mungo* L. Hepper cv. Co4) Roots

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An equal concentration (100 μM) of Cr(III)- and Cr(VI)-induced changes in activities of antioxidative enzymes and metabolites of ascorbate-glutathione cycle was studied in 7-d-old black gram (*Vigna mungo* L. Hepper cv. Co4) seedlings for 5-d after infliction of Cr stress. Seeds were germinated and grown in the presence or absence of Cr under controlled environmental conditions. Uptake and translocation of Cr rate was relatively higher during first 12 h of treatment with both speciation of Cr, Cr(III)- and Cr(VI)-treated black gram roots retained 15 times more Cr than the shoots. Significantly increased lipid peroxidation was observed in the form of accumulation of malondialdehyde (MDA) and production of hydrogen peroxide (H_2O_2) molecule and superoxide ($O_2^{\cdot -}$) radical after 6 h of infliction with Cr(VI) and after 12 h in Cr(III)-treated black gram roots. Superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities were significantly increased under Cr(VI)-treatment after 12 and 6 h, respectively. However, catalase (CAT) and monodehydroascorbate reductase (MDHAR) activities were not significantly increased under Cr(III)-treatment. There was a steep increase of 2.71 μmol g^{-1} FW in ascorbic acid (AA) content was observed between 6 and 24 h of Cr(VI)-treatment. Oxidized glutathione (GSSG) content was steadily increased through the course of Cr(III)- and Cr(VI)-treatments, where as reduced glutathione (GSH) level was decreased after 24 h of treatment. GSH/GSSG ratio was rapidly decreased in treatment with Cr(III) than the Cr(VI). There was significant increase of 99 nmol g^{-1} FW in non-protein thiol (NPT) content was recorded between 6 and 24 h of Cr(VI)-treatment. The present results showed differential response to AA and H_2O_2 signaling by Cr(III) and Cr(VI). AA in combination with APX was more effective in mitigating oxidative stress as against the role of GSH as an antioxidant.

Keywords: antioxidative enzymes, black gram, Cr phytotoxicity, lipid peroxidation, metabolites, reactive oxygen species

The extensive use of chromium (Cr) compounds in steel, alloys, cast iron, chrome plating, dyes and pigments, textile, leather tanning, wood preserving, and chemical production makes these industries have become large contributors of Cr contamination in the environment (Zayed and Terry, 2003). In nature, Cr exists in toxic Cr(III) and Cr(IV) forms, Cr(III) is readily oxidized to Cr(VI) in soil in the presence of oxidized Mn, which serves as the electron acceptor in this oxidation reaction. The accepted view that Cr(VI) is more toxic to plants than the Cr(III), derived from the experiments in which nutrient solution culture techniques were used to compare the uptake, translocation and toxicities of nominally equal amounts of the two Cr speciation (Peterson and Girling, 1981). In plants, Cr(VI) ion is known to affect several metabolic, physiological, and biochemical processes (Howe et al., 2003). In plants, only limited studies have been carried out the bioaccumulation of Cr, resulted in inhibition of seed germination or of early seedling development, reduction in plant growth, root injury, leaf chlorosis, wilting of tops, and depressed biomass (Howe et al., 2003; Karuppanapandian et al., 2006c, 2008). Cr significantly affects the metabolism of plants, such as, cauliflower (*Brassica oleracea*) (Chatterjee and Chatterjee, 2000), green gram (*Vigna radiata*) (Karuppanapandian et al., 2006a,c, 2008), and vegetable crops (Zayed et al., 1998).

Cr-induced to inactivate the mitochondrial electron transport chain and hydrogen peroxide (H_2O_2) molecule and superoxide ($O_2^{\cdot -}$) radical production has been well reported in higher plants (Chatterjee and Chatterjee, 2000; Han et al., 2004; Karuppanapandian et al., 2006a). High concentrations of reactive oxygen species (ROS), such as, $O_2^{\cdot -}$, H_2O_2, singlet oxygen ($^1O_2$), and hydroxyl radical ($OH^{\cdot}$) at cellular level cause oxidative damage and this explains most of the visual Cr toxicity symptoms observed at whole plant level under biotic and abiotic stresses (Dat et al., 2000; Karuppanapandian et al., 2006c). ROS may play two different roles: aggravating oxidative damage and/or activation of defense genes against oxidative stresses. Such a dual role has been recently reported in various plant systems under various heavy metal stresses (Dat et al., 2000; Liszkay et al., 2004). The synchronous action of various antioxidative enzymes, such as, catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and superoxide dismutase (SOD) with the thiol regulated enzymes (dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR) of the ascorbate-glutathione cycle is a predominant mechanism of ROS scavenging under various heavy metal stress (Ali et al., 2000a,b;

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Abbreviations: AA, ascorbic acid; APX, ascorbate peroxidase; CAT, catalase; Cr, chromium; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DW, dry weight; FW, fresh weight; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; NPT, non-protein thiol; ROS, reactive oxygen species; SOD, superoxide dismutase

Panda and Choudhry, 2005; Karuppanapandian et al., 2006a,b, 2008). Apart from these enzymes, low molecular weight antioxidants, such as, ascorbic acid (AA) and reduced glutathione (GSH) play an important role to protecting plant cells from oxidative damage due to heavy metal stress. Every enzymes in the ascorbate-glutathione cycle have been shown to be affected by copper, Cr, and aluminium were already reported in mustard (*Brassica juncea*) roots and green gram leaves and roots, respectively (Wang et al., 2004; Karuppanapandian et al., 2006a,b,c, 2008). In plants, roots accumulate several fold higher Cr then the shoots (Zayed et al., 1998), although there are several reports on oxidation by Cr(VI) of different cellular thiols in solutions, i.e. GSH, there is a distinct dearth of literature with regard to the role of ascorbate-glutathione cycle in cellular defense against in various forms of Cr in plants.

The inhibition of plant growth and crop production by excess of various heavy metals in contaminated soil is a global agricultural problem. Using metal-accumulating plants to remove these excessive heavy metals from soil has been proposed as a solution (Ahmad et al., 2005). In addition, the knowledge of uptake, translocation or compartmentation of heavy metals in plants is also biotechnologically important (Han et al., 2004; Lee and Kang, 2005). In the present study, we have used black gram as test plant belong to the family Leguminaceae, because it is characterized by rapid growth, high biomass, fast growing and an appreciable capacity to absorb Cr as well as other toxic metals from soil. However, no information has been reported on Cr(III)- and Cr(VI)-induced oxidative damage in this species. Therefore, the present study was focused on the effect of equal concentration (100 µM) of Cr(III)- and Cr(VI)-induced changes in contents of malondialdehyde (MDA) and non-protein thiol (NPT), production of ROS including H_2O_2 and $O_2^{\cdot -}$, and activities of ascorbate-glutathione cycle related antioxidative enzymes and metabolites on black gram roots in Hoagland nutrient medium.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Healthy and uniform black gram (*Vigna mungo* L. Hepper cv. Co4; obtained from Tamil Nadu Agricultural University, Coimbatore, India) seeds were germinated aseptically after surface sterilization with 70% ethanol, followed by a treatment with 0.1% $HgCl_2$ for 5 min. Subsequently, seeds were thoroughly washed 5 times in sterile double distilled water and germinated in Petri dishes in darkness containing Whatman No. 1 filter paper moistened with Hoagland nutrient medium (Hoagland and Arnon, 1950). After 48 h of germination, seedlings were transferred to plastic glasses containing Hoagland nutrient medium. The pH of the nutrient medium was adjusted to 5.8 using either 0.1 N NaOH or 0.1 N HCl and kept in growth chamber. Growth chamber was maintained at 25±1°C under cool, white fluorescent lamps (150 µmoL s^{-1} m^{-2}) with 16 h photoperiod. Relative humidity was maintained at 35% during the day time and 60% at night. Two different forms of Cr was added to the Hoagland nutrient medium: Cr(III) as chromium chloride ($CrCl_3$) and Cr(VI) as potassium dichromate ($K_2Cr_2O_7$). For the control, seedlings were placed on the filter paper moistened with just double distilled water.

Determination of Morphometric Parameters

Morphometric parameters were measured after 120 h of Cr-treatment, at end of treatment, the seedlings were divided into separate parts (shoot and root). The root and shoot length were measured and the rate of elongation was calculated. The dry weight (DW) of the shoot and root (adventitious roots plus primary root) were measured. DW estimation of shoot and root were recorded after washing the shoot and root with distilled water and blotting with absorbent paper and left in hot air oven at 70°C till constant weight was reached. The leaf area was measured using Li-Cor 3100 leaf area meter (Li-Cor, USA). Seedlings were harvested after 0, 3, 6, 12, 24, 48, 72, and 120 h of Cr-treatment for determination of enzyme activities, and 6, 12, 24, and 120 h of Cr-treatment for estimation of contents of Cr, MDA, H_2O_2, $O_2^{\cdot -}$, NPT and metabolites of ascorbate-glutathione cycle.

Estimation of Cr Content

Cr content was estimated according to Davies et al. (2002). Root and shoot were separated and oven dried for 72 h at 80°C and ground into fine powder. Five mL concentrated HNO_3 was added to 250 mg of dried sample in a 50 mL digestion tube and allowed to stand for overnight at room temperature. The digestion tubes were placed in a heating block for 1 h at 150°C, then tubes were removed and allowed to cool and 2 mL of 30% H_2O_2 was added. The contents in the tubes were mixed by swirling and then heated for 2 h at 150°C. After cooling to room temperature the solution was diluted to 50 mL in total volume, the upper clear portion used for Cr estimation. During dilution, 2% of NH_4Cl or 0.5% of $CaCl_2$ was added to each sample and standard to eliminate interference caused by iron (Fe) or phosphorus (P), respectively, during spectrophotometeric analysis. Atomic absorption spectrometer (Spectra AA-220; Varion, USA) with air-acetylene flame at 358 nm and 0.2 mm spectral slit width was used for Cr estimation. Cr content was expressed as mg kg^{-1} DW.

Determination of MDA Content

MDA content was determined by the method of Heath and Packer (1968). Root and shoot were separately homogenized with 5% trichloroacetic acid (TCA; w/v), and the homogenate was used for MDA determination. One mL of 5% TCA and 4 mL of TBA reagent (0.5% in 20% TCA) were mixed and used as a blank. For correction of blank, 1 mL of homogenate and 4 mL of 20% TCA, and for sample 1 mL of homogenate and 4 mL of TBA reagent were mixed, after heating at 95°C for 30 min, the mixture was cooled and centrifuged at 4,000 rpm for 10 min. The absorbance was read at 532 nm and corrected for non-specific absorbance at 600 nm and for the absorbance at 532 nm of the correction blank. MDA content was calculated by using an extinction coefficient at 155 mM^{-1} cm^{-1}. MDA level is routinely used as an index of lipid peroxidation and was expressed as nmoL g^{-1} FW.

Determination of H_2O_2 Content

H_2O_2 content was determined according to Patterson et al. (1984). Root and shoot were separately ground in 6 mL ice-cold acetone and centrifuged at 8,000 rpm for 30 min at 4°C. Five hundred µL of supernatant was mixed with 1.5 mL of mixture of $CHCl_3$ and CCl_4 (1:3, v/v) and 2.5 mL of distilled water was added. The reaction mixture was centrifuged at 1,000 rpm for 1 min, the aqueous phase was used for H_2O_2 determination. To set controls, 100 µL of CAT (0.3 U) was added to the 1 mL of supernatant to remove the H_2O_2. For treatments, CAT solution was replaced by 100 µL MQ (Millipore, USA) water. The reaction mixtures were incubated at 37°C for 10 min, 1 mL of 200 mM phosphate buffered saline (PBS, pH 7.8) and 1 mL of 200 mM 4-(2-pyridylazo) resorcinol were added to samples. The reaction mixtures were incubated at 45°C for 20 min, and the absorbance was read at 508 nm. H_2O_2 content was expressed as µmoL g^{-1} FW.

Estimation of $O_2^{\cdot -}$ Content

$O_2\cdot -$ content was estimated according to the method of Able et al. (1998). Five hundred mg of root and shoot washed thoroughly with distilled water, blotted dry and then incubated in 200 µL of 0.12 mm XTT in 50 mM PBS (pH 8.2) for 20 min at 20°C and centrifuged at 15,000 rpm for 5 min. The A_{450} of supernatant was measured and expressed as change in OD min^{-1} g^{-1} FW using the molar extinction coefficient for the XTT formazan product of 23,600 M^{-1} cm^{-1} (Sutherland and Learmonth, 1997). $O_2\cdot -$ estimation was carried out in duplicate, verification of $O_2\cdot -$ detection was confirmed by adding SOD at the start of the assay (Beauchamp and Fridovich, 1971).

Extraction and Assays of Ascorbate-Glutathione Cycle Related Antioxidative Enzymes

Five hundred mg of root and shoot were separately macerated with 10 mL of 50 mM potassium PBS (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP, w/v) in pre-cooled mortar and pestle and centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was used for enzyme analysis. Protein content was determined according to Lowry et al. (1951) using BSA as a standard.

CAT (EC 1.11.1.6) activity was determined according to Aebi (1984). Two mL of assay mixture comprised of 1.8 mL of 50 mM PBS (pH 7.0) and 200 µL of enzyme extracts. The reaction was started with the addition of 1 mL of 30 mM H_2O_2 prepared in 50 mM PBS (pH 7.0). Decrease in absorbance was read at 240 nm. CAT activity was expressed as U g^{-1} FW. APX (EC 1.11.1.11) activity was determined according to Nakano and Asada (1981). Two hundred mg of root and shoot were separately homogenized in 50 mM ice-cold PBS (pH 7.8) containing 2 mM ascorbate and 5 mM EDTA and centrifuged at 10,000 rpm for 30 min at 4°C. The reaction mixture contained 2.7 mL of 50 mM PBS (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 100 µL of 0.1 mM H_2O_2 and 50 µL of enzyme extracts. The decrease in absorbance was read at 290 nm. APX activity was expressed as U g^{-1} FW. SOD (EC 1.15.1.1) activity was determined according to Beauchamp and Fridovich (1971). The reaction mixture contained 0.24 mM riboflavin, 2.1 mM methione, 1% (v/v) Triton X-100, 1.72 mM nitroblue tetrazolium chloride (NBT; in 50 mM potassium PBS, pH 7.8). SOD activity was expressed as U g^{-1} FW. One U of SOD is defined as the amount of enzyme required to cause 50% inhibition in the rate of NBT photo-reduction.

MDHAR (EC 1.6.5.4) activity was determined according to Hussain et al. (1984). The reaction mixture comprised of 90 mM PBS (pH 7.5), 0.01 mM EDTA, 0.0125% (v/v) Triton X-100, 2.5 mM ascorbate, 0.25 U ascorbate oxidase (SigmaAldrich, USA), 0.2 mM NADH, and 50 µL of enzyme extracts. The decrease in absorbance was read at 340 nm due to NADPH oxidation using an absorbance coefficient of 6.2 mM^{-1} cm^{-1}. Monodehydroascorbate (MDHA) formed by ascorbate oxidase was used as standard. One enzyme U equaled to nMNADPH oxidized g^{-1} min^{-1} FW. MDHAR activity was expressed as U g^{-1} FW. DHAR (EC 1.8.5.1) activity was determined by the method of Doulis et al. (1997). The assay mixture contained 90 mM PBS (pH 7.0), 0.1 mM EDTA, 5 mM GSH, and 50 µL of enzyme extracts. The reaction was initiated by the addition of freshly prepared 0.2 mM dehydroascorbate (DHA). DHAR activity was measured similar to MDHAR by measuring the reduction of DHA at 265 nm after accounting for the non-enzymatic reduction of DHA by GSH. One enzyme U equaled to nM ascorbate produced g^{-1} min^{-1} FW. DHAR activity was expressed as U g^{-1} FW. GR (EC 1.6.4.2) activity was determined according to Schaedle and Bassham (1977). One ml reaction mixture comprised of 50 mM potassium PBS (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM glutathione disulphide (GSSG), and 200 µL of enzyme extracts. The reaction was initiated by addition of NADPH. The reaction was followed by the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 3 min. Corrections were made for the background absorbance at 340 nm without NADPH. GR activity was expressed as U g^{-1} FW.

Extraction and Determination of Metabolites

AA and DHA contents were determined according to Cakmak and Marschner (1992). Five hundred mg of root and shoot were separately macerated in ice-cold extraction buffer (40% (v/v) methanol, 0.75% (w/v) *m*-phosphoric acid, 16.7 mM (w/v) oxalic acid, and 0.127 mM acetone) and centrifuged at 10,000 rpm for 30 min at 4°C. One hundred µL of supernatant was transferred to 900 µL of mobile phase (24.25 mM acetic acid (pH 4.8), 0.1 mM acetone, 0.015% (w/v) *m*-phosphoric acid, 0.04% (w/v) octylamine, and 15% (v/v) methanol) for determination of AA. DHA content was calculated as the difference of total ascorbate and AA. AA and DHA contents were expressed as µmoL g^{-1} FW. GSH and GSSG contents were determined according to Anderson (1985). Five hundred mg of root and shoot were homogenized separately with 5 mL of 0.15% (w/v) sodium ascorbate and centrifuged at 10,000 rpm for 15 min at 0°C. Supernatants were incubated for 4 min at 100°C to denature proteins and centrifuged at 10,000 rpm for 30 min at 4°C. One mL of reaction mixture contained 200 µL thylakoid suspension, 0.2 mM NADPH, 0.6 mM 5,5'-dithiobis(2-nitrobenzoic acid; DTNB), and 50 U of GR. Reduction of DTNB was followed at 412 nm for 2 min at 30°C. To

measure the amount of GSSG in the supernatant, 2 μM of PVP was added to 100 μL of the supernatant and mixed vigorously for 1 min. Reduction rate of DTNB was measured after 20 and 40 min as in the total glutathione assay. Total glutathione and GSSG were quantified by comparing with the standard curves obtained with the reduced and oxidized forms of glutathione (SigmaAldrich, USA). GSH content was calculated by subtracting the amount of GSSG from total glutathione. Contents of GSH and GSSG were expressed as nmoL g^{-1} FW.

Estimation of NPT Content

NPT content was measured with Ellman's reagent (Ellman, 1959). Five hundred mg of root and shoot were separately macerated in 1 mL of ice-cold 5% (w/v) sulfosalicylic acid and centrifuged at 10,000 rpm for 30 min at 4°C. Three hundred μL of the supernatant was mixed with 1.2 mL of 100 mM PBS (pH 7.6) and after a stable absorbance was reading at 412 nm, 25 μM DTNB solution (6 mM DTNB dissolved in 5 mM EDTA, 100 mM PBS, pH 7.6) was added. Increase in absorbance was read at 412 nm. NPT content was expressed as nmoL g^{-1} FW.

Data Analysis

Three replicates were taken for estimation of contents of Cr, MDA, metabolites, NPT, ROS, and enzyme assays from each set of experiments (n=10). The mean values ± SE are given in all the Tables and Figures. The data were analyzed statistically using a general linear model for analysis of variance (ANOVA) using SigmaPlot 8.0 (Jandel Scientific Corporation). The significance of differences between control and treatments were compared at 0.05 and 0.01.

RESULTS

Uptake of Cr(III) and Cr(VI) and Their Effects on Plant Growth

In the present study, we have investigated Cr(III)- and Cr(VI)-induced oxidative damage occurs in 7-d-old black gram seedlings in Hoagland nutrient medium. The present results showed that the employed morphometric parameters were sensitive and useful for evaluating the Cr(III)- and Cr(VI)-induced oxidative damage in black gram seedlings with various experimental conditions (Table 1; Fig. 1). There was only a marginal decrease in root and shoot length, DW of root and shoot and total leaf area by the treatment with 50 and 75 μM of both the speciation of Cr, which possibly indicated week growth inhibition activity of Cr(III) and Cr(VI) at these all the above concentration (Table 1; Fig. 1). Cr(III) and Cr(VI) beyond 75 μM was growth inhibiting where in changes in morphometric parameters were more pronounced at 100 and 125 μM of Cr(III)- and Cr(VI)-treatments. Decrease in root length, shoot length, root DW, shoot DW and total leaf area was much same as 100 and 125 μM of Cr(III)- and Cr(VI)-treatments (Table 1; Fig. 1). Therefore, 100 μM of Cr(III) and Cr(VI) were used for further treatments and analysis in the present investigation.

In the present study, among the tested concentration of Cr(III) and Cr(VI) in the range of 50 to 125 μM, 100 μM was found to be optimal (or stress point) to induce oxidative damage in 7-d-old black gram seedlings (Table 1; Fig. 1). In black gram seedlings, shoot growth was not significantly affected by 100 μM of Cr(III)-treatment with respect to shoot length, shoot DW, and total leaf area, whereas significant reduction was observed in root length and root DW under 100 μM of Cr(III)-treatment (Table 1; Fig. 1). One hundred μM of Cr(VI)-treatment showed a significant reduction in all the morphometric growth parameters in black gram seedlings (Table 1; Fig. 1). Total leaf area was significantly affected 39.61% reduction due to 100 μM of Cr(VI) as against 14.29% reduction due to 100 μM of Cr(III)-treatment compared to control. The reduction in root DW was 44 mg under 100 μM of Cr(VI)-treatment as against 31 mg under 100 μM of Cr(III)-treatment was compared to control (Table 1; Fig. 1). Root length, root DW, and total leaf area were most significantly affected by Cr(VI) than Cr(III). Root and shoot varied highly in their ability to accumulate Cr regardless of the speciation in the nutrient medium. The Cr uptake was 3.25- and 4.28-fold increased in roots between 6 and 12 h of 100 μM of Cr(III)- and Cr(VI)-treatments, respectively. Thereafter, sharp decline in the rate of Cr uptake was observed (Fig. 2). Total Cr in 100 μM of Cr(III)-treated roots was 362 mg

Table 1. Growth characteristics of black gram seedlings grown for 7-d under Cr(III)- and Cr(VI)-treatments in Hoagland nutrient medium after 120 h of treatment.

Treatments	Root length (cm)	Shoot length (cm)	Root DW (mg)	Shoot DW (mg)	Total leaf area (cm^2)
H_2O control	6.02 ± 0.72	10.86 ± 0.63	93 ± 7	32 ± 8	15.4 ± 1.2
Cr(III) (50 μM)	6.00 ± 0.70	10.77 ± 0.61	91 ± 7	32 ± 7	15.3 ± 1.2
Cr(VI) (50 μM)	5.98 ± 0.69	10.76 ± 0.60	91 ± 7	31 ± 8	15.2 ± 1.2
Cr(III) (75 μM)	5.96 ± 0.68	10.75 ± 0.59	90 ± 7	31 ± 6	15.1 ± 1.0
Cr(VI) (75 μM)	5.92 ± 0.67	10.73 ± 0.58	89 ± 6	31 ± 7	15.0 ± 1.0
Cr(III) (100 μM)	4.96 ± 0.59[a]	9.08 ± 0.59	62 ± 7[a]	28 ± 7	13.2 ± 1.0
Cr(VI) (100 μM)	4.01 ± 0.50[b]	7.32 ± 0.50[a]	49 ± 7[b]	17 ± 6[a]	9.3 ± 0.9[b]
Cr(III) (125 μM)	4.96 ± 0.58[a]	9.07 ± 0.58	62 ± 7[a]	28 ± 7	13.2 ± 1.0
Cr(VI) (125 μM)	4.00 ± 0.49[b]	7.31 ± 0.50[b]	49 ± 7[b]	17 ± 6[a]	9.2 ± 0.9[a]

The data are means of 5 different determinations. Values in parenthesis are SEM.
[a]Significant at 0.05
[b]Significant at 0.01

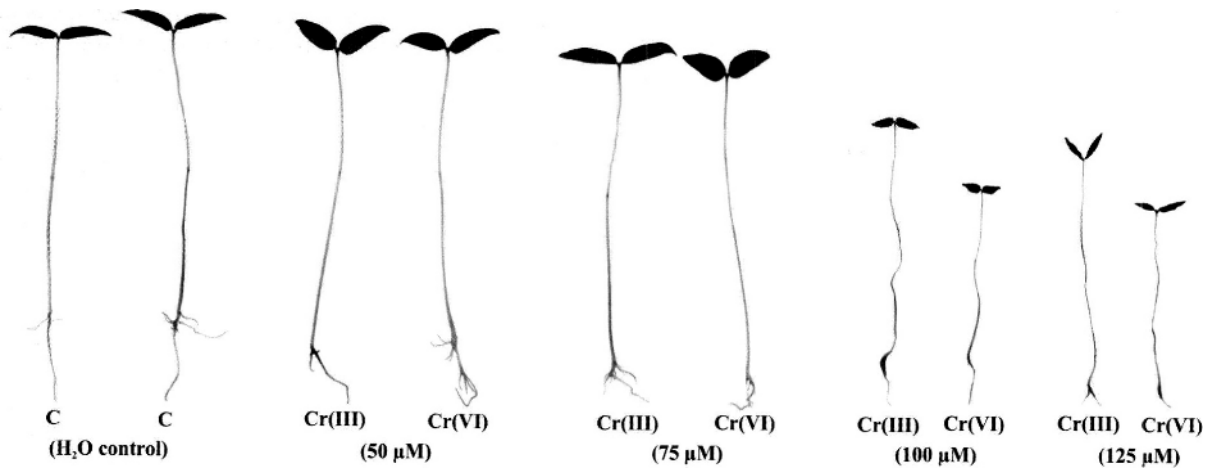

Figure 1. Effects of various concentration of Cr(III)- and Cr(VI)-treated black gram seedlings grown for 7-d in Hoagland nutrient medium.

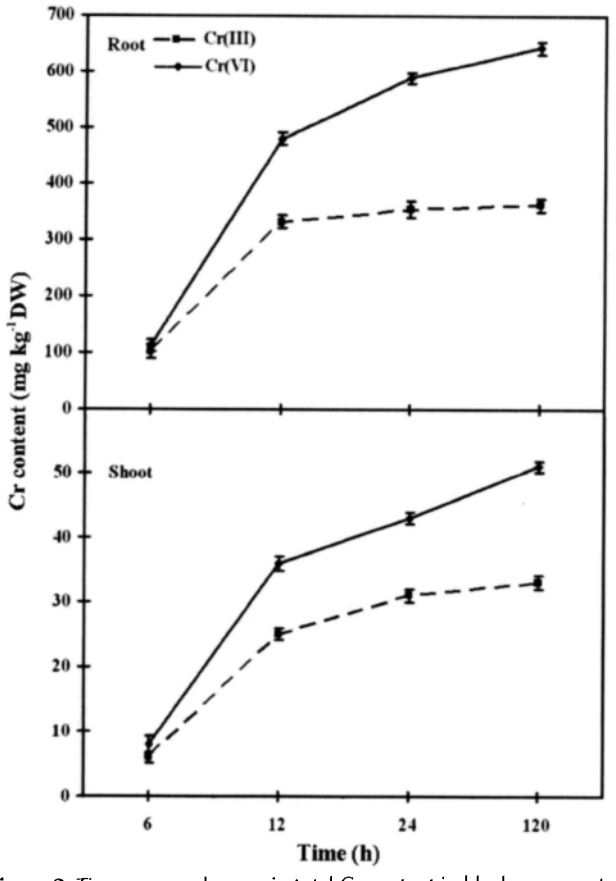

Figure 2. Time course changes in total Cr content in black gram roots and shoots grown for 7-d under Cr(III)- and Cr(VI)-treatments in Hoagland nutrient medium. Vertical bars represent SEM. Cr not detected at 0 h.

kg^{-1} DW as against 643 mg kg^{-1} DW in 100 µM of Cr(VI)-treated roots after 120 h of treatment. Shoots accumulated 10- to 12-fold lesser Cr in tissue as compared to the roots. The increasing rate of Cr uptake by shoots at the initial hours of treatment was similar to that of roots under 100 µM of Cr(III)- and Cr(VI)-treatments. Total Cr in shoots were 33 and 51 mg kg^{-1} DW after 120 h of 100 µM of Cr(III)- and Cr(VI)-treatments, respectively.

Effect of Cr(III) and Cr(VI) on Lipid Peroxidation and ROS

Accumulation of MDA and generation of H_2O_2 and $O_2^{\cdot -}$ was higher under Cr(VI)- than Cr(III)-treated black gram roots. A significant increase was observed in MDA level under Cr(VI)-treatment at 6 h and continued to be significantly higher till 120 h, wherein the increase was 59.3 and 79.3 nmoL g^{-1} FW higher than Cr(III)-treatment and control, respectively (Table 2). Unlike Cr(III), Cr(VI) exhibited significantly increased the MDA content only after 12 h of treatment. Cr(III)-treated black gram roots increased the H_2O_2 production, however, a significant increase was observed only at 12 h of treatment. In contrast, Cr(VI)-treated black gram roots significantly increased the H_2O_2 production after 6 h of treatment, however, there was no significant increase at 120 h of treatment. A similar trend was observed in the case of $O_2^{\cdot -}$ production, wherein a significant increase of 3.86 OD min^{-1} g^{-1} FW was observed even at 120 h under Cr(VI)-treatment.

Responses of Antioxidative Enzymes to Cr Exposure

The presence of Cr in the nutrient medium increased the CAT activity irrespective of the Cr(III)- and Cr(VI)-treatments. There was no significant increase was observed in CAT activity under Cr(III)-treated black gram roots, but significant increase was observed only under Cr(VI)-treatment at 120 h (Table 3). APX activity was significantly increased after 12 h of Cr(VI)-treatment, whereas increased APX activity was observed only at 48 h under Cr(III)-treatment. On the other hand, SOD activity was significantly increased only after 12 h of Cr(VI)-treatment and continued to be significant upto 120 h (63.4 U g^{-1} FW). In contrast, Cr(III) addition to the nutrient medium increased the SOD activity, slight increase was observed only at 24 h of treatment. The earliest onset of activity among all the enzymes studied was seen after 3 h in DHAR and GR under Cr(VI)-treated black gram roots (Table 4). On the other hand, Cr(III)-treatment induce the increased activities of these enzymes much latter at 24 and 48 h, respectively. Cr(III) did not influence the MDHAR activity, whereas Cr(VI)-treated black gram roots exhibited increased MDHAR activity at 6 and 12 h, after which it did not vary

Table 2. Time course changes in contents of MDA, H_2O_2 and $O_2^{\cdot -}$ in black gram roots grown for 7-d under Cr(III)- and Cr(VI)-treatments in Hoagland nutrient medium. The data are means ± SE of 5 independent determinations.

Time (h)	MDA (nmoL g^{-1} FW)			H_2O_2 (μmoL g^{-1} FW)			$O_2^{\cdot -}$ (ΔOD min^{-1} g^{-1} FW)		
	H_2O control	Cr(III) (100 μM)	Cr(VI) (100 μM)	H_2O control	Cr(III) (100 μM)	Cr(VI) (100 μM)	H_2O control	Cr(III) (100 μM)	Cr(VI) (100 μM)
0	52.6 ± 6.2	52.6 ± 6.2	52.6 ± 6.2	2.1 ± 0.09	2.1 ± 0.09	2.1 ± 0.09	0.71 ± 0.09	0.71 ± 0.09	0.71 ± 0.09
6	62.4 ± 6.6	82.4 ± 9.1	121.4 ± 11.8[a]	3.6 ± 0.18	4.8 ± 0.32	8.6 ± 0.92[b]	1.42 ± 0.12	1.73 ± 0.11[a]	3.16 ± 0.13[b]
12	69.4 ± 7.0	109.6 ± 10.8[a]	142.8 ± 16.3[a]	3.9 ± 0.18	8.9 ± 0.71[a]	12.2 ± 1.02[b]	1.43 ± 0.10	2.92 ± 0.12[a]	3.50 ± 0.12[b]
24	78.8 ± 8.2	122.9 ± 13.2[a]	149.4 ± 17.1[a]	4.1 ± 0.24	7.4 ± 0.82	11.0 ± 1.01[a]	1.55 ± 0.11	3.07 ± 0.15[b]	3.77 ± 0.16[a]
120	99.4 ± 10.2	148.7 ± 15.6[a]	168.7 ± 18.2[b]	7.3 ± 0.61	8.0 ± 0.72	9.9 ± 0.96[a]	1.70 ± 0.13	2.85 ± 0.14[b]	3.86 ± 0.18[a]

[a]Significant at 0.05
[b]Significant at 0.01

Table 3. Time course changes in activities of CAT, APX, and SOD in black gram roots grown for 7-d under Cr(III)- and Cr(VI)-treatments in Hoagland nutrient medium. The data are means ± SE of 5 independent determinations.

Time (h)	CAT (U g^{-1} FW)			APX (U g^{-1} FW)			SOD (U g^{-1} FW)		
	H_2O control	Cr(III) (100 μM)	Cr(VI) (100 μM)	H_2O control	Cr(III) (100 μM)	Cr(VI) (100 μM)	H_2O control	Cr(III) (100 μM)	Cr(VI) (100 μM)
0	35.8 ± 1.9	35.8 ± 1.9	35.8 ± 1.9	0.82 ± 0.05	0.82 ± 0.05	0.82 ± 0.05	39.2 ± 1.8	39.2 ± 1.8	39.2 ± 1.8
3	36.4 ± 2.1	38.4 ± 1.8	42.2 ± 2.1	0.91 ± 0.05	1.08 ± 0.06	1.41 ± 0.11	41.6 ± 1.7	46.2 ± 1.7	49.8 ± 1.8
6	36.9 ± 2.0	39.4 ± 2.4	46.6 ± 2.0	1.02 ± 0.06	1.39 ± 0.06	1.64 ± 0.11	43.4 ± 1.5	47.5 ± 1.2	49.9 ± 1.7[a]
12	35.9 ± 2.1	40.5 ± 2.1	45.2 ± 1.9	1.12 ± 0.05	1.62 ± 0.11	2.09 ± 0.15[a]	43.9 ± 1.2	48.8 ± 1.8	51.4 ± 1.4[b]
24	41.2 ± 2.5	42.1 ± 1.8	46.3 ± 2.6	1.36 ± 0.08	1.68 ± 0.12[a]	2.38 ± 0.14[a]	45.6 ± 1.4	51.4 ± 1.7[b]	55.2 ± 1.8[b]
48	40.3 ± 1.9	43.2 ± 1.9	47.2 ± 2.1	1.46 ± 0.06	2.18 ± 0.16[a]	2.49 ± 0.15[a]	44.3 ± 1.3	49.7 ± 1.7[a]	54.6 ± 1.8[b]
72	37.4 ± 1.7	41.1 ± 2.1	44.1 ± 2.4	1.64 ± 0.08	2.46 ± 0.16[a]	2.60 ± 0.16[b]	47.8 ± 1.5	53.4 ± 1.5[b]	59.2 ± 1.4[b]
120	41.3 ± 2.4	43.2 ± 1.7	49.8 ± 2.5[a]	1.85 ± 0.08	2.98 ± 0.16[a]	2.69 ± 0.16[b]	46.4 ± 1.4	55.1 ± 1.7[b]	63.4 ± 1.8[a]

[a]Significant at 0.05
[b]Significant at 0.01

Table 4. Time course changes in activities of DHAR, MDHAR, and GR in black gram roots grown for 7-d under Cr(III)- and Cr(VI)-treatments in Hoagland nutrient medium. The data are means ± SE of 5 independent determinations.

Time (h)	DHAR (U g^{-1} FW)			MDHAR (U g^{-1} FW)			GR (U g^{-1} FW)		
	H_2O control	Cr(III) (100 μM)	Cr(VI) (100 μM)	H_2O control	Cr(III) (100 μM)	Cr(VI) (100 μM)	H_2O control	Cr(III) (100 μM)	Cr(VI) (100 μM)
0	38.3 ± 1.1	38.3 ± 1.2	38.3 ± 1.2	42.6 ± 1.4	42.6 ± 1.4	42.6 ± 1.4	48.2 ± 1.7	48.2 ± 1.7	48.2 ± 1.7
3	19.2 ± 1.1	27.4 ± 1.2	41.4 ± 1.2[a]	24.5 ± 1.2	52.7 ± 1.2	64.2 ± 1.3	23.1 ± 1.4	35.4 ± 1.6	49.4 ± 1.7
6	30.4 ± 1.2	42.7 ± 1.5	57.4 ± 1.4[a]	56.6 ± 1.1	65.3 ± 1.2	111.8 ± 1.4[a]	62.4 ± 1.6	79.4 ± 1.9	88.3 ± 1.6[a]
12	36.5 ± 1.3	53.4 ± 1.2	68.3 ± 1.3[a]	73.2 ± 1.2	107.4 ± 1.3	138.4 ± 1.3[a]	83.4 ± 2.1	94.3 ± 1.8	101.4 ± 1.5[a]
24	48.2 ± 1.1	84.5 ± 1.6[a]	95.2 ± 1.7[a]	94.5 ± 1.3	124.3 ± 1.2	141.1 ± 1.5[b]	98.5 ± 2.2[a]	124.2 ± 2.2[a]	136.3 ± 1.7[a]
48	53.4 ± 1.2	81.3 ± 1.4	98.1 ± 1.6[a]	85.6 ± 1.4	121.9 ± 1.3	143.6 ± 1.4[b]	88.8 ± 2.2[a]	129.4 ± 2.2[a]	144.9 ± 1.9[b]
72	59.2 ± 1.2	92.4 ± 1.5[a]	111.4 ± 1.4[b]	73.2 ± 1.4	126.7 ± 1.4	141.2 ± 1.3[b]	91.4 ± 2.4[a]	177.4 ± 2.1[b]	205.4 ± 2.2[b]
120	62.9 ± 1.4	107.6 ± 1.7[a]	126.2 ± 1.6[b]	89.3 ± 1.3	106.8 ± 1.5	157.4 ± 1.5[b]	107.2 ± 2.5[a]	172.3 ± 2.0[a]	209.6 ± 2.1[a]

[a]Significant at 0.05
[b]Significant at 0.01

significantly when compared to control.

Effect of Cr Speciation on Ascorbate-Glutathione Cycle Metabolites

AA content was fluctuated around the control values throughout the progress of the experiment in the case of Cr(III)-treated black gram roots (Fig. 3). There was a significant increase of 5.82 μmoL g^{-1} FW in AA content was recorded between 6 and 24 h of treatment, and thereafter the AA content stabilized till 120 h under Cr(VI)-treatment. DHA content was calculated in terms of difference between total and reduced ascorbate, in Cr(VI)-treatment, DHA content was observed more than 2-fold when compared to Cr(III)-treatment throughout the course of the experiment. No DHA was observed in control till 24 h. Total glutathione and GSH contents exhibited similar pattern of increase was observed under Cr(III)- and Cr(VI)-treatments (Fig. 4). Both these metabolites were highest at 24 h under Cr(III)- and Cr(VI)-treatments (269 and 329 nmoL g^{-1} FW of GSH and 81.4 and 84.1 nmoL g^{-1} FW of total glutathione in Cr(III)

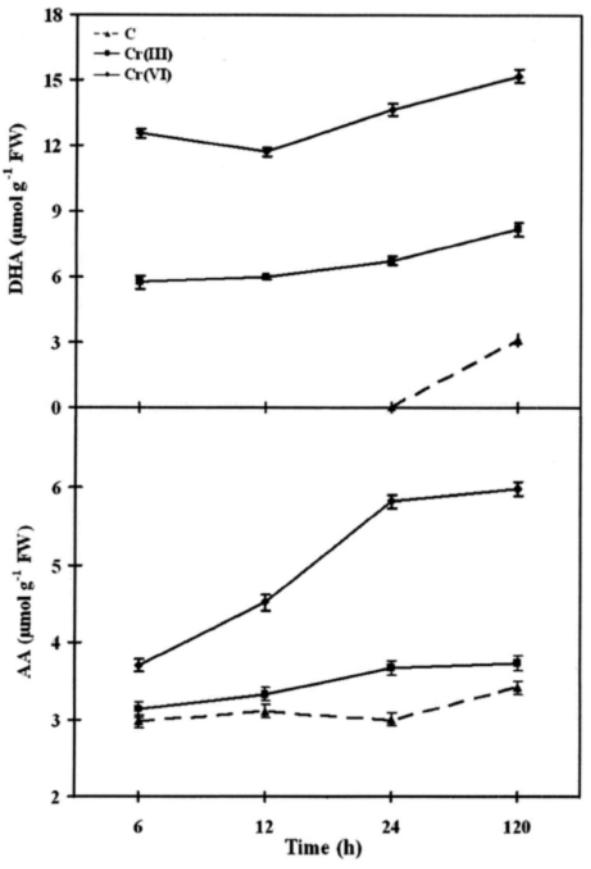

Figure 3. Time course changes in contents of AA and DHA in black gram roots grown for 7-d under Cr(III)- and Cr(VI)-treatments in Hoagland nutrient medium. Vertical bars represent SEM. Values for AA and DHA were 2.98 and 0 μmol g^{-1} FW at 0 h, respectively.

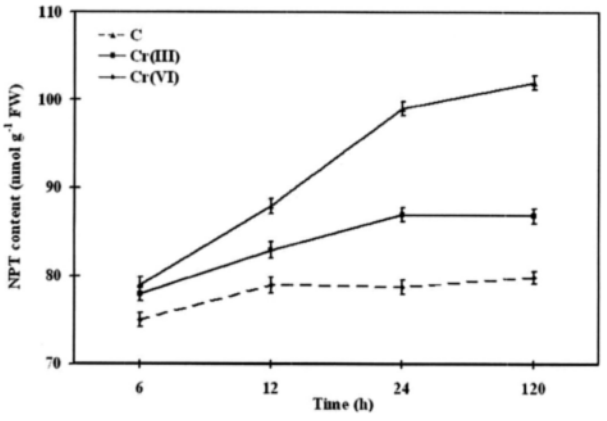

Figure 5. Time course changes in total NPT content in black gram roots grown for 7-d under Cr(III)- and Cr(VI)-treatments in Hoagland nutrient medium. Vertical bars represent SEM. Value of NPT was 49 nmoL g^{-1} FW at 0 h.

and Cr(VI), respectively). GSSG was 3-fold lesser in control and treated when compared to GSH. In contrast to GSH content, which reduced after 24 h of treatment, GSSG increased steadily throughout the course of the experiment. GSH/GSSG ratio of 4.05 and 4.29 was observed at 6 h after treatment and it declined to 2.67 and 3.29 at 120 h under Cr(III)- and Cr(VI)-treatments, respectively. The rate of decline in the ratio was much faster in Cr(III)- than Cr(VI)-treated black gram roots. The rate of decline in the ratio was lesser in control than the Cr(III)- and Cr(VI)-treatments.

Effect of Cr Speciation on NPT

NPT content was fluctuated around the control values

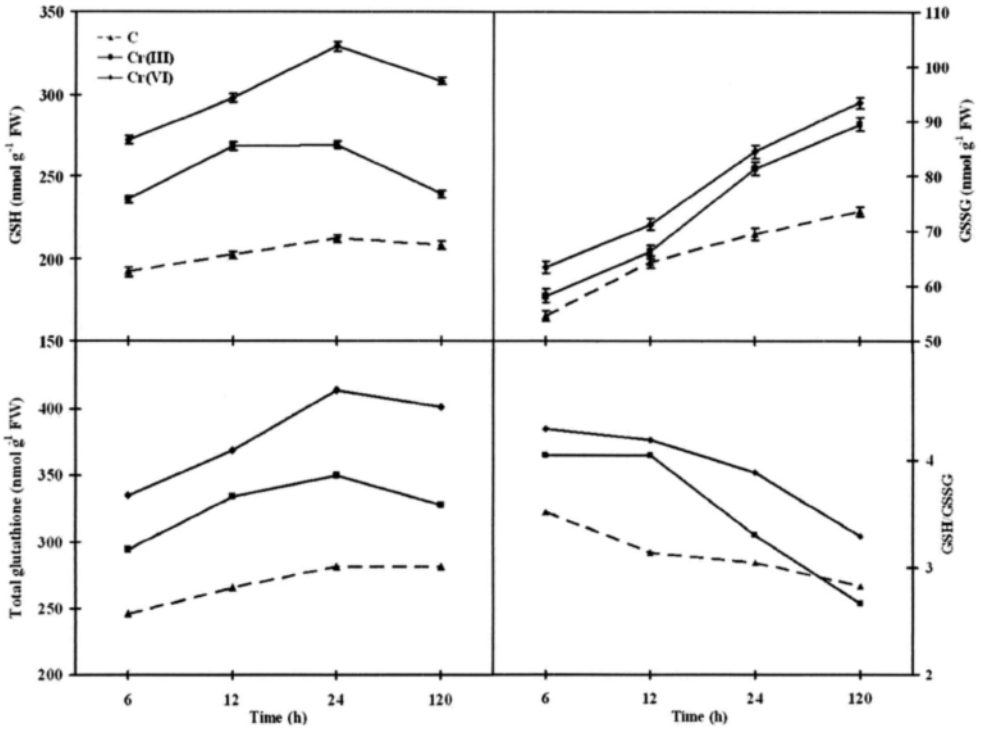

Figure 4. Time course changes in contents of GSH, GSSG, total glutathione, and GSH/GSSG in black gram roots grown for 7-d under Cr(III)- and Cr(VI)-treatments in Hoagland nutrient medium. Vertical bars represent SEM. Values of GSH, GSSG, and total glutathione were 178, 42.5, and 220.5 nmoL g^{-1} FW at 0 h, respectively.

throughout the course of the experiment in the case of Cr(III)-treated black gram roots (Fig. 5). There was a significant increase of 99 nmoL g^{-1} FW in NPT content was observed between 6 and 24 h of Cr(VI)-treatment, and thereafter, NPT content stabilized till 120 h. Figure 5 exhibited the changes in NPT content showed a pattern similar to that of AA over the time of Cr(III)- and Cr(VI)-treatments.

DISCUSSION

The toxicity of Cr in soil and to plants depends on its speciation and bioavailability. Cr exists as in the Cr(III) and Cr(VI) (Zayed and Terry, 2003), whereas intermediate states Cr(IV) and Cr(V) are metastable and rarely encountered by plants. Cr(III) is largely present in soil as relatively unavailable, insoluble oxides of Cr and Cr-Fe. The prevailing view is that the Cr(VI) is more toxic and more soluble than Cr(III). There is conflicting reports on the uptake and translocation of Cr(III) and Cr(VI) by plant roots (Skeffington et al., 1976). However, both forms, such as, Cr(III) and Cr(VI) are now reported to be observed by plants. The uptake of Cr(III) is a passive process, whereas Cr(VI) uptake is active and mediated by sulfate carrier (Skeffington et al., 1976). In general, plants have a low capacity to absorb and translocate Cr. Different vegetable crops vary in their ability to accumulate Cr in tissues (Zayed et al., 1998).

In the present study, black gram seedlings absorbed both speciation of Cr and showed toxicity symptoms at a range of equal concentration (100 µM) of each. Cr(VI) was readily absorbed by the plants when compared to Cr(III), but there was a distinct restriction of translocation of Cr(III) and Cr(VI) to shoots. In earlier, similar results have been reported in vegetable crops (Zayed et al., 1998). Cr(VI) is actively absorbed by plants and it is metabolically driven process, in contrast to Cr(III), which is passively taken up and retained by cation exchange sites of the cell wall (Murphy et al., 1999). Therefore, plants can accumulate higher amount of Cr(VI) than Cr(III). Poor translocation of Cr to the shoots could be due to sequesterization of Cr in the vacuoles of the root cells to render it non-toxic which may be a natural toxicity response of the plants. Since Cr is toxic, plants may not possess any specific mechanism of Cr transport and assimilation.

The phytotoxic effect of Cr(VI) was more pronounced in shoots as well as roots, whereas Cr(III) was toxic only to the roots. This could be mainly due to the high concentration of ROS produced by Cr(VI) which might be resulted in membrane damage as evidenced by lipid peroxidation in the form of accumulation of MDA was observed in the present study (Table 2). It was reported that Cr(VI) is a strong oxidant with a high redox potential in range of 1.33 and 1.38 eV (Kotas and Stasicka, 2000). Electron microscopy studies indicated extensive oxidative damage to the outer root cells under Cr(VI)-treatment in nutrient medium (Toppi et al., 2004). Therefore, the normal mechanism of selective inorganic nutrient uptake might have been destroyed Cr(VI). Oxidative damage thus might permit larger quantities of Cr(VI) to enter the roots passively and further translocation of Cr(VI) to shoot causing oxidative damage to the mitochondrial and photosynthetic apparatus eventually reflected in poor growth. In contrast, Cr(III) is kinetically inert to ligand substitution and therefore can form substitution inert metalloprotein complexes *in vivo*, thus greatly reducing its toxicity. The toxicity of Cr(III) has been reported to be by indirect effects, such as, changes in pH and/or inhibition of ion transport (Dixit et al., 2002). In the present study, it was noted that Cr(III)-increased the $O_2^{\cdot -}$ production after 6 h of treatment and H_2O_2 production and MDA accumulation after 12 h of treatment. H_2O_2 could act as an oxidizing agent and might oxidize Cr(III) to Cr(VI) (Rock et al., 2001). On the other hand, Cr(III) could be endogenously reduced to Cr(II) by biological reductants, such as, 1-cysteine and NADPH. In turn, the newly formed Cr(II) could react with H_2O_2 to producing $OH^{\cdot}$ radicals causing tissue damage in roots (Varquez et al., 1987).

ROS level in cellular compartments was determined by the interplay between the multiple ROS producing pathways and enzymes (e.g., respiration, NADPH oxidases, amine oxidases and cell wall-bound peroxidases) and the scavenging mechanisms essentially constitute the basic ROS cycle (Mittler, 2002; Liszkay et al., 2004; Karuppanapandian et al., 2006a,b, 2008). Fluctuations in levels of antioxidative enzymes and metabolites observed in untreated plants could be due to the fine metabolic tuning performed by this cycle in terms of increasing active quenching or suppressing metabolic activity responsible for ROS production (Karuppanapandian et al., 2006a,b,c, 2008). Further, recent evidence ascribed an alternative role of plant growth and cell cycle control to ascorbate and glutathione (Gratao et al., 2005). More specifically, AA-stimulated cell cycle activity and DHA blocked the normal cell cycle progression. Therefore, the time course changes in these compounds under control could have been due to a cellular machinery regulating cell cycle. The combined action of SOD and CAT is critical in mitigating the effects of oxidative stress, since the former merely acted on the $O_2^{\cdot -}$ converting it to another reactive intermediate (H_2O_2) and the latter acted on H_2O_2 converting it to water and O_2 (Liszkay et al., 2004). In the present study, it was noted that CAT did not participate in active H_2O_2 reduction irrespective of Cr(III)- and Cr(VI)-treatments, although SOD was active in scavenging the $O_2^{\cdot -}$ produced by Cr(III)- and Cr(VI)-treatments.

CAT and SOD activities under abiotic stress induced oxidative damage have been reported earlier (Pastori et al., 2000; Karuppanapandian et al., 2006a,b, 2008). In the present study, APX was more efficient in destroying H_2O_2 than CAT under Cr(III)- and Cr(VI)-treatments. The reason may be ascribed with the requirement of 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, unlike CAT which is present only in the peroxisome and has low substrate affinities (Willekens et al., 1997). Increase in APX activity was recorded after the increase in SOD activity in the case of Cr(III)-treated black gram roots, whereas under Cr(VI)-treatment increase in APX activity was observed before the increase in SOD activity. This suggests a differential response to AA signaling by Cr(III) and Cr(VI), which was evidenced by the higher concentration of AA in reduced form at very early in the roots under

Cr(VI) stress. Over expression of DHAR gene has shown to be increase of AA levels dramatically (Chen et al., 2003). In the present study, increase in DHAR activity was observed within 3 h after Cr(VI)-treatment. It can be correlated with higher ROS production by Cr(VI), which could acts as a signal transduction mechanism to increase DHAR synthesis, resulting in higher AA concentrations. The increased content of AA under Cr(VI)-treatment can be explained by the recycling function of the DHAR. DHA was rapidly and irreversibly hydrolyzed to 2,3-diketogulonic acid if not acted upon by DHAR (Morita et al., 1999). Increased DHAR activity could have been generated more AA from the DHA pool before hydrolysis.

The increase in DHA content under Cr(VI)-treatment in spite of no significant increase in MDHAR activity indicated that this metabolite was chiefly formed by non-enzymatic dispropotionation (Noctor and Foyer, 1998). This could be because of the short life of MDHA or the inhibition of the MDHAR by Cr ions. Glutathione pool dynamics was similar under Cr(III) and Cr(VI) although different in terms of amount of the metabolite present. The decline in total glutathione could not have been due to phytochelatin synthesis as the absence of phytochelatins in Cr-treated plants has been reported (Toppi et al., 2004). The depletion of GSH and total glutathione in spite of higher GR activities observed as time under stress progressed indicated 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 could be 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; Karuppanapandian et al., 2006a). Liu et al. (1995) have reported the presence of intermediate forms of Cr namely Cr(IV) and Cr(V) in roots exposed to high concentration of Cr(VI) by means of low-frequency electron paramagnetic resonance (EPR). It is possible that Cr(VI) could be reduced to Cr(V), Cr(IV) (unstable) and Cr(III) (stable) in the presence of ascorbate and GSH. The GSH-GSSG redox pair could function effectively only when there is an adequate supply of NADPH and GSH itself could serve as a cellular sensor to maintain the NADPH pool (Potters et al., 2002). The reduced rate of GSH/GSSG ratio decline under Cr(VI) indicated that maximum metabolic load was exerted to maintain a minimum redox buffer status of the cells, whereas under Cr(III) sufficient amount of AA was enough to counter oxidative stress.

In conclusion, the present study suggests that both the speciation of Cr is heavily accumulated in roots than in the shoots. Both the forms of Cr are toxic to the plants, however, the phytotoxicity and accumulation of Cr(VI) in the plant cells was more pronounced when compared to Cr(III). The degradation of lipids in the form accumulation of MDA, stimulation of ascorbate-glutathione cycle related enzymatic antioxidative systems, changes in contents of metabolites, and enhance the production of ROS, such as, H_2O_2 and $O_2\cdot^-$ radicals could be a characteristic feature of the mechanism of both the forms of Cr toxicity in black gram seedlings. These morphological and biochemical changes could be responsible for the inhibition of elongation of root and shoot and might have set off a sequence of reactions leading to plant cell death. However, more information at the subcellular and molecular levels is needed in order to get deeper insights into the mechanistic explanation of Cr phytotoxicity in plants.

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