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Differential antioxidative response of ascorbate glutathione pathway enzymes and metabolites to chromium speciation stress in green gram (*Vigna radiata* (L.) R.Wilczek. cv CO 4) roots

Arun K. Shanker [a,*], M. Djanaguiraman [a], R. Sudhagar [b], C.N. Chandrashekar [a], G. Pathmanabhan [a]

[a] *Department of Crop Physiology, Tamil Nadu Agricultural University, Lawly Road, Coimbatore 641003, Tamil Nadu, India*
[b] *Department of Plant Breeding and Genetics, Tamil Nadu Agricultural University, Lawly Road, Coimbatore 641003, Tamil Nadu, India*

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Abstract

The response of the antioxidant enzymes and metabolites of the ascorbate glutathione pathway to oxidative stress caused by equal concentration (50 μM) of Cr(III) and Cr(VI) was studied in 15-day-old seedlings of green gram (*Vigna radiata* (L.) R.Wilczek. cv CO 4) for 5 days after imposition of stress. Significant increase in lipid peroxidation and H_2O_2 generation was seen 5 h after stress in Cr(VI) as against 12 h in Cr(III)-treated plants. High rate of uptake and translocation of Cr was seen in the first 12 h of treatment with roots retaining 80 times more Cr than the shoots in both the speciation. No significant increase in catalase (CAT) activity and monodehydroascorbate reductase (MDHAR) was observed under Cr(III). Superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities increased under Cr(VI) after 12 and 4 h, respectively. Under Cr(VI) stress there was a steep increase of 194.6 nM g^{-1} FW in the ascorbic acid (AA) content between 5 and 24 h of treatment. In contrast to reduced glutathione (GSH) content, which reduced after 24 h after treatment, oxidized glutathione (GSSG) increased steadily through the course of the experiment under both speciation. The rate of decline in the GSH/GSSG ratio was much faster in Cr(III) than Cr(VI). Results suggest differential response to AA and H_2O_2 signaling by Cr(III) and Cr(VI) and that AA in combination with APX was more effective in mitigating oxidative stress as against the role of GSH as an antioxidant.
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Keywords: Chromium toxicity; Lipid peroxidation; Superoxide dismutase; Ascorbate peroxidase; Reactive oxygen species; Glutathione metabolism

1. Introduction

Contamination of soil and ground water due to the use of chromium (Cr) in various anthropomorphic activities like leather, steel plating and dyeing industries have become a serious source of concern to plant and animal 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 complex electronic chemistry of Cr has been a major hurdle in unraveling its physiological interaction in plants. Cr has an electronic configuration of [Ar] $3d^5 4s^1$ and forms of biologically active Cr are the trivalent (Cr(III)) and the hexavalent (Cr(VI)) species.

Differential toxicity of Cr speciation to plants is well documented [1,2] wherein Cr(VI) has been found to be more toxic than Cr(III). 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 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 [3]. Chromium ion induced inactivation of mitochondrial electron transport and superoxide generation has been demonstrated in higher plants [4]. High concentrations of ROS such as singlet oxygen, hydroxyl ions and hydrogen peroxide at cellular level causes oxidative stress and this

* Corresponding author. Present address: National Research Centre for Agroforestry (NRCAF), Pahuj Dam, Gwalior Road, Jhansi 284003, Uttar Pradesh, India. Tel.: +91-9443044353; fax: +91-5172730364.
E-mail address: arunshanker@mailcan.com (A.K. Shanker).

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explains most of the visual Cr toxicity symptoms observed at whole plant level [5].

ROS may play two very different roles: exacerbating damage or signaling the activation of defense responses. Such a dual function has recently been demonstrated during several abiotic stress responses [6]. Rapid adaptive flux in cellular metabolism is an important prerequisite to counteract heavy metal stress in higher vascular plants. The synchronous action of various antioxidant enzymes, 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)) of the ascorbate glutathione pathway is a predominant mechanism of ROS quenching under heavy metal stress [7]. Apart from these enzymes low molecular weight antioxidant metabolites like ascorbic acid (AA) and reduced glutathione (GSH) play an important role in protecting plants from oxidative stress damage. Every enzyme in the ascorbate glutathione pathway was shown to be affected by Cu [8]. Formation of thiolate complexes by Cr has been reported [9].

Although there are several reports on oxidation by Cr(VI) of different cellular thiols in solutions such as GSH [10,11], there is a distinct 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. Roots accumulate several magnitudes higher Cr under both speciation as compared to shoots [12]. The present study was taken up to investigate the effect of equal concentrations of Cr(III) and Cr(VI) in nutrient media on the antioxidant defense system including the enzymes and metabolites of ascorbate glutathione pathway in roots of green gram (*Vigna radiata* (L.) R.Wilczek. cv CO 4).

2. Materials and methods

2.1. Plant material and hydroponic experiment

Green gram (*V. radiata* (L.) R.Wilczek. cv CO 4) 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 0.1N HCl or 0.1N KOH. The experiment was in completely randomized design with five replications. Growth chamber conditions were: photosynthetic photon flux density of 430 $\mu M\,m^{-2}\,s^{-2}$, 16 h of light, 8 h of dark and a relative humidity of 60%. 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 μM each. Plants were harvested after 2, 4, 12, 24, 48, 72 and 120 h of Cr treatment for estimation of enzyme activities and 5, 12, 24, 120 h of Cr treatment for estimation of total Cr content, lipid peroxidation, H_2O_2, superoxide anion 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 g fresh weight samples which were frozen in liquid nitrogen and stored at −70 °C.

2.2. Chromium content and plant growth

Measurement of chromium content ($\mu g\,g^{-1}$) was made on individual plants. Roots and shoots were separated and oven dried for 3 days at 80 °C. Samples were then ground into fine powder using a grinding mill. The conditions used for digestion were according to [13]. Five milliliters 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 1 h at 150 °C, tubes were then removed allowed to cool and 2 ml of 30% 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% and $CaCl_2$ was added at 0.5% 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.

2.3. Enzyme 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% 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.

2.3.1. Catalase (CAT) (EC 1.11.3.6)

CAT (EC 1.11.3.6) was estimated according to [14]. One milliliter 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% 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 μM H_2O_2 destroyed mg^{-1} $protein^{-1}$ min^{-1}.

2.3.2. Superoxide dismutase (SOD) (EC 1.15.1.1)

SOD (EC 1.15.1.1) was determined by nitroblue tetrazolium (NBT) method [15] by measuring the photoreduction of NBT at 560 nm. One unit of SOD activity equaled to the amount required to inhibit photoreduction of NBT by 50%.

2.3.3. Ascorbate peroxidase (APX) (EC 1.11.1.11)

APX (EC 1.11.1.11) enzyme activity was measured according to [16] following the oxidation of ascorbate to dehydroascorbate spectrophotometrically. The enzyme activity was calculated from the observed rates of absorbance decrease and the millimolar extinction coefficient for ascorbate which is 0.80 and 0.055 mM^{-1} cm^{-1} at 298 and 310, respectively. One unit of enzyme activity was that which catalyzed the oxidation of 1 μM ascorbate min^{-1}. Activity was expressed in mU g^{-1} fresh weight.

2.3.4. Monodehydroascorbate reductase (MDHAR) (EC 1.6.5.4)

MDHAR (EC 1.6.5.4) enzyme activity was measured in the supernatant at 25 °C as described by [17]. MDHAR was assayed spectrophotometrically by following the decrease in absorbance at 340 nm due to NADPH oxidation using an absorbance coefficient of 6.2 mM^{-1} cm^{-1}. Monodehyroascorbate formed by ascorbate oxidase was used as standard. One enzyme unit equaled to nM NADPH oxidized g^{-1} fresh weight min^{-1}. The enzyme activity was expressed in mU g^{-1} fresh weight.

2.3.5. Dehydroascorbate reductase (DHAR) (EC 1.8.5.1)

DHAR (EC 1.8.5.1) enzyme activity was measured similar to MDHAR by measuring the reduction of dehydroascorbate at 265 nm according to [18]. One enzyme unit equaled to nM ascorbate produced g^{-1} fresh weight min^{-1}. The activity was expressed in mU g^{-1} fresh weight.

2.3.6. Glutathione reductase (GR) (EC 1.6.4.2)

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 GSH [19]. 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 U ml^{-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 mU g^{-1} fresh weight wherein unit activity is the amount of enzyme which reduces 1 μM of GSSG in 1 h at 24 °C.

2.4. Metabolite estimation

One gram of tissue was ground with inert sand in 5 ml of 6.5% *m*-phosphoric acid containing 1 mM NaEDTA with a mortar and pestle. Total ascorbate (AA) and reduced ascorbate was estimated according to [20]. Dehydroascorbate (DHA) was calculated from the difference between the total and reduced ascorbate. GSH, GSSG and total glutathione were assayed by the enzymatic GSSG recycling method advocated by [21]. 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 nM g^{-1} fresh weight.

2.5. 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 [22]. The pink chromogen was measured at 532 and 600 nm for the correction of blank. Lipid peroxidation was expressed as melondialdehyde content in μM g^{-1}. The H_2O_2 level was colorimetrically measured as described by [23]. H_2O_2 was extracted by homogenizing 0.5 g of root 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 nM g^{-1} DW. Superoxide anion was estimated according to [24] and expressed as change in OD min $^{-1}$ g^{-1} DW.

2.6. 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 ± S.E. are given in all the tables and figures. The data were analyzed statistically using a general linear model for analysis of variance (repeated measures Anova) [25]. Significance between control and treatments were compared at 0.05 and 0.01 probability levels.

3. Results

3.1. Plant growth and Cr accumulation

Cr(III) did not affect shoot growth both in terms of shoot length, shoot dry weight and total leaf area whereas root length and root dry weight were significantly affected by Cr(III) (Table 1). Cr(VI) treatments exhibited a significant

Table 1
Growth parameters of green gram as influenced by Cr speciation in nutrient medium after 120 h of treatment

Parameter	Control	Cr(III) (50 μM)	Cr(VI) (50 μM)
Shoot length (cm)	9.48 (±0.6)	8.28 (±0.6)	6.91 (±0.5)[a]
Root length (cm)	6.97 (±0.7)	4.73 (±0.5)[a]	4.06 (±0.9)[b]
Total leaf area (cm^2)	16.3 (±2.1)	14.3 (±2.8)	10.7 (±2.3)[b]
Shoot dry weight (g)	0.028 (±0.003)	0.015 (±0.005)	0.012 (±0.006)[a]
Root dry weight (g)	0.096 (±0.009)	0.067 (±0.012)[a]	0.053 (±0.018)[b]

Figures in parenthesis are S.E.M.
[a] Significant at 0.05.
[b] Significant at 0.01.

reduction in all the growth parameters observed. Reduction in leaf area was by 34.36% due to Cr(VI) as against a 12.27% due to Cr(III). The reduction in root dry weight under Cr(VI) was 0.043 g as against 0.029 g under Cr(III) in comparison with control. Root length, root dry weight and total leaf area were most significantly affected by Cr(VI).

Roots and shoots varied highly in their ability to accumulate Cr regardless of the speciation in the nutrient medium. There was 71.2 and 78.1% increase in Cr uptake by roots between 5 and 12 h of treatment of Cr(III) and Cr(VI), respectively. Thereafter there was a decline in the rate of uptake (Fig. 1). Total Cr in Cr(III)-treated roots was 437 $\mu g\, g^{-1}$ as against 633 $\mu g\, g^{-1}$ in Cr(VI)-treated roots after 120 h of treatment. Shoots accumulated 70–80 times lesser total Cr in tissue as compared to roots. Increase in the rate of uptake of shoots at the initial hours of treatment was similar to that of roots under both speciation. After 120 h of treatment, total Cr in shoots were 5.34 and 7.86 $\mu g\, g^{-1}$ in Cr(III)- and Cr(VI)-treated plants.

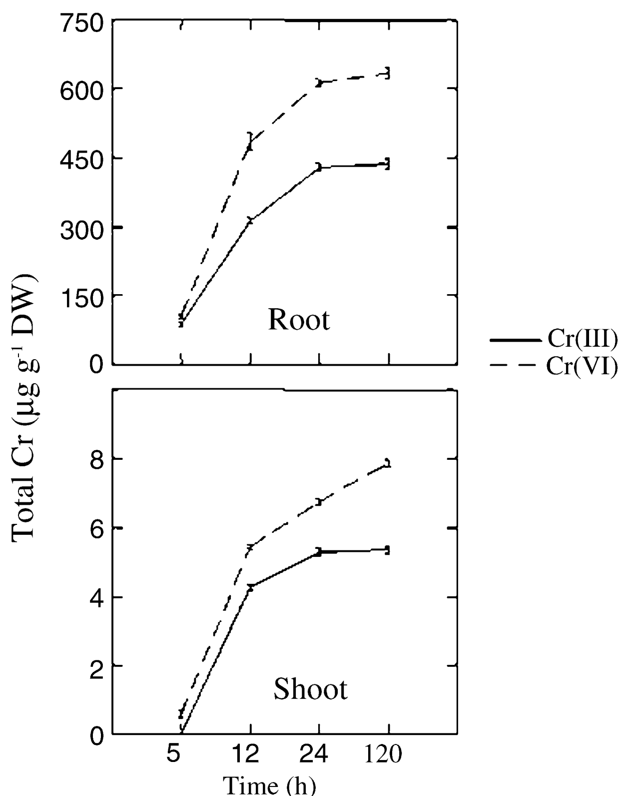

Fig. 1. Time course changes in total Cr concentration in root and shoot of green gram as influenced by Cr speciation in nutrient medium. Vertical bars represent S.E.M. Cr not detected at 0 h.

3.2. Lipid peroxidation and ROS

Cr(VI) induced higher malondialdehyde formation, H_2O_2 and superoxide anion production than Cr(III). Malondialdehyde recorded significant increase under Cr(VI) treatments at 5 h and continued to be significantly higher till 120 h wherein the increase was 22.8 and 14.2 $\mu M\, g^{-1}$ more than control and Cr(III) treatments, respectively (Table 2). Unlike Cr(VI), Cr(III) exhibited significant increase in malondialdehyde only after 12 h of treatment. Although Cr(III) increased H_2O_2 production there was a significant increase only at 12 h after treatment. In contrast Cr(VI)-treated roots of green gram showed significant increase in H_2O_2 from 5 h of treatment onwards whereas there was no significant increase at 120 h. A similar trend was seen in the case of superoxide anion production wherein a significant increase of 2.16 was seen even at 120 h.

3.3. Enzyme activity

The presence of Cr in the medium increased catalase activity irrespective of the speciation. Significant increase however was observed only in Cr(VI) treatment at 120 h (Table 3). There was no significant increase in CAT activity under Cr(III) stress. On the other hand, significant increase in the activity of SOD was seen as early as 12 h after treatment and continued to be significant at 120 h (60.1 enzyme units). In contrast, Cr(III) addition to the nutrient medium although did increase the SOD activity, significant increase was seen only at 24 h. Increase in APX activity was highly significant after 12 h of Cr(VI) treatment in comparison with a similar increase only at 72 h in the case of Cr(III). The earliest significant increase among all the enzymes studied was seen after 2 h in DHAR and GR in the roots of Cr(VI)-treated green gram (Table 4). On the other hand, Cr(III) treatment induced significant changes in the activity of these enzymes much latter at 24 and 48 h, respectively. Cr(III) did not influence the activity of MDHAR and Cr(VI)-treated roots exhibited increase at 4 and 12 h after which it did not vary significantly in comparison to control.

Table 2
Time course changes in lipid peroxidation, H_2O_2 and superoxide anion production in green gram roots as influenced by Cr speciation in nutrient medium

Time (h)	Malondialdehyde (μM g^{-1} DW)			H_2O_2 (nM g^{-1} DW)			Superoxide anion ($O_2^{\bullet -}$) ΔOD min^{-1} g^{-1} DW		
	Control	Cr(III) (50 μM)	Cr(VI) (50μ M)	Control	Cr(III) (50 μM)	Cr(VI) (50 μM)	Control	Cr(III) (50 μM)	Cr(VI) (50 μM)
0	7.4 (±1.4)	7.4 (±1.8)	7.4 (±1.8)	5.4 (±0.5)	5.4 (±0.5)	5.4 (±0.5)	0.42 (±0.03)	0.42 (±0.03)	0.42 (±0.03)
5	9.3 (±0.9)	14.6 (±1.6)	21.4 (±1.8)[a]	6.3 (±0.7)	9.3 (±0.5)	16.8 (±1.0)[b]	1.13 (±0.08)	2.44 (±0.07)[a]	2.87 (±0.10)[b]
12	9.8 (±1.3)	17.8 (±1.7)[a]	24.6 (±2.1)[a]	6.7 (±1.0)	14.8 (±0.7)[a]	24.6 (±1.0)[b]	1.14 (±0.09)	2.63 (±0.10)[a]	3.21 (±0.09)[b]
24	10.7 (±1.3)	22.9 (±2.0)[a]	28.3 (±1.9)[a]	7.0 (±1.1)	12.4 (±1.1)	22.5 (±1.2)[a]	1.26 (±0.12)	2.78 (±0.08)	3.48 (±0.14)[a]
120	15.8 (±1.6)	24.4 (±1.9)	38.6 (±2.3)[b]	11.1 (±1.3)	13.7 (±0.6)	18.4 (±1.5)	1.41 (±0.10)	2.56 (±0.12)	3.57 (±0.16)[a]

Figures in parenthesis are S.E.M.
[a] Significant at 0.05.
[b] Significant at 0.01.

Table 3
Time course changes in CAT, SOD and APX activities in green gram roots as influenced by Cr speciation in nutrient medium (±S.E.M.)

Time (h)	CAT (μM H_2O_2 destroyed mg^{-1} $protein^{-1}$ min^{-1})			SOD (one unit of SOD activity equals the amount required to inhibit photoreduction of NBT by 50%)			APX (mU g^{-1} fresh weight) (one unit of enzyme activity was that which catalyzed the oxidation of 1 μM ascorbate min^{-1})		
	Control	Cr(III) (50 μM)	Cr(VI) (50 μM)	Control	Cr(III) (50 μM)	Cr(VI) (50 μM)	Control	Cr(III) (50 μM)	Cr(VI) (50 μM)
0	24.8 ± 1.4	24.8 ± 1.4	24.8 ± 1.4	36.3 ± 1.3	36.3 ± 1.3	36.3 ± 1.3	6420 ± 23	6420 ± 23	6420 ± 23
2	25.7 ± 1.2	28.3 ± 1.3	31.4 ± 1.2	38.5 ± 1.2	43.2 ± 1.2	46.8 ± 1.1	6428 ± 16	6949 ± 19	7230 ± 21
4	25.9 ± 1.1	30.1 ± 1.4	35.3 ± 1.2	40.1 ± 1.3	44.8 ± 1.2	46.9 ± 1.3	6843 ± 24	7186 ± 14	7436 ± 26[a]
12	24.9 ± 1.4	31.4 ± 1.6	34.3 ± 1.2	40.8 ± 1.5	45.2 ± 1.3	48.5 ± 1.1[a]	7024 ± 14	7346 ± 13	7984 ± 13[b]
24	30.3 ± 1.2	33.1 ± 1.5	35.7 ± 1.3	42.8 ± 1.1	48.8 ± 1.1[a]	52.3 ± 1.2[a]	7143 ± 21	7438 ± 13	8140 ± 19[b]
48	29.9 ± 1.1	34.2 ± 1.3	36.2 ± 1.4	41.3 ± 1.0	46.3 ± 1.8[a]	51.8 ± 1.3[a]	7184 ± 20	7949 ± 15[a]	8244 ± 24[b]
72	26.8 ± 1.0	32.4 ± 1.4	33.4 ± 1.2	44.4 ± 1.4	50.8 ± 0.8[a]	56.4 ± 1.3[b]	7644 ± 25	8238 ± 13[b]	8638 ± 13[b]
120	30.3 ± 0.9	34.1 ± 1.1	38.2 ± 1.0[a]	43.1 ± 1.3	58.2 ± 1.4[a]	60.1 ± 1.3[b]	7700 ± 18	8834 ± 12[b]	8680 ± 17[a]

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

Table 4
Time course changes in DHAR, MDHAR and GR activities in green gram roots as influenced by Cr speciation in nutrient medium (±S.E.M.)

Time (h)	DHAR (mU g^{-1} fresh weight) (one enzyme unit equals to nM ascorbate produced g^{-1} fresh weight min^{-1})			MDHAR (mU g^{-1} fresh weight) (one enzyme unit equals to nM NADPH oxidised g^{-1} fresh weight min^{-1})			GR (mU g^{-1} fresh weight) (one enzyme unit equals the amount of enzyme which reduces 1 μM of GSSG in 1 h at 24 °C)		
	Control	Cr(III) (50 μM)	Cr(VI) (50 μM)	Control	Cr(III) (50 μM)	Cr(VI) (50 μM)	Control	Cr(III) (50 μM)	Cr(VI) (50 μM)
0	256 ± 13	256 ± 13	256 ± 13	532 ± 10	532 ± 10	532 ± 10	628 ± 5	628 ± 5	628 ± 5
2	232 ± 12	246 ± 11	258 ± 18[a]	514 ± 12	541 ± 12	553 ± 13	603 ± 8	615 ± 16	629 ± 7[a]
4	248 ± 16	263 ± 14	272 ± 13	546 ± 11	554 ± 10	597 ± 11[a]	645 ±12	659 ± 13	668 ± 18[a]
12	250 ± 12	274 ± 13	283 ± 11[a]	563 ± 18	600 ± 14	622 ± 15[a]	663 ± 8	674 ± 10	681 ± 12
24	267 ± 11	304 ± 14[a]	309 ± 10[a]	583 ± 9	612 ± 9	624 ± 10	674 ± 10	704 ± 12	716 ± 10[a]
48	272 ± 10	302 ± 16	311 ± 12[a]	576 ± 20	610 ± 16	625 ± 9	668 ± 14	708 ± 13[a]	724 ± 9[b]
72	278 ± 16	312 ± 13	325 ± 11[b]	561 ± 17	613 ± 14	624 ± 13	671 ± 9	757 ± 9[b]	785 ± 11[b]
120	281 ± 18	326 ± 16[a]	338 ± 15[b]	578 ± 16	600 ± 8	631 ± 14	689 ± 10	752 ± 12[a]	789 ± 10[a]

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

3.4. Metabolites

Ascorbic acid content in the reduced form fluctuated around the control values throughout the course of the experiment in the case of Cr(III)-treated roots (Fig. 2). Under Cr(VI) stress, however, there was a steep increase of 194.6 nM g^{-1} FW in the AA content between 5 and 24 h of treatment thereafter the metabolite content stabilized till 120 h. DHA calculated in terms of the difference between total and reduced ascorbate in Cr(VI) was more than twice the amount observed in Cr(III) throughout the progress of the experiment. No DHA was observed in control till 24 h. Total glutathione and reduced glutathione exhibited similar pattern of increase under both Cr speciation treatments

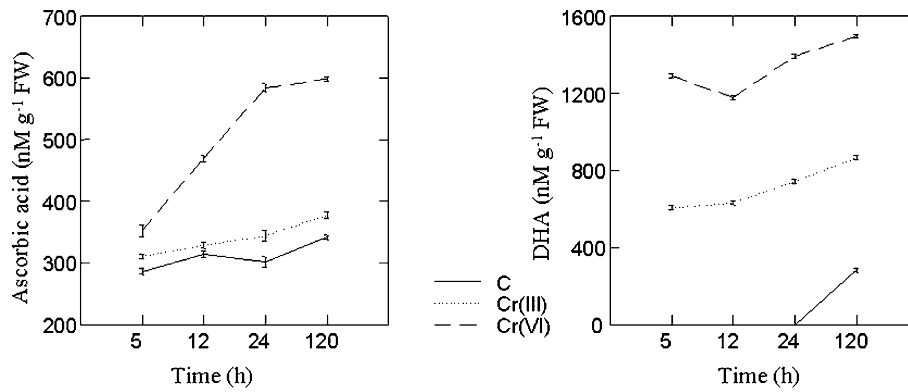

Fig. 2. Time course changes in ascorbic acid and DHA content in roots of green gram as influenced by Cr speciation in nutrient medium. Vertical bars represent SEM. Values for AA and DHA were 289.32 and 0 nM g^{-1} FW at 0 h, respectively.

(Fig. 3). Both these metabolites were highest at 24 h after treatment under both Cr speciation (1890 and 2145 nM g^{-1} FW of reduced glutathione and 2200 and 2474 nM g^{-1} FW of total glutathione in Cr(III) and Cr(VI), respectively). GSSG was ten times lesser in treated and control in comparison to reduced glutathione. In contrast to GSH content, which reduced after 24 h after treatment, GSSG increased steadily through the course of the experiment. GSH/GSSG ratio was 7.81 and 7.91 at 5 h after treatment and it declined to 5.22 and 5.74 at 120 h in Cr(III) and Cr(VI), respectively. The rate of decline in the ratio was much faster in Cr(III) than Cr(VI). The rate of decline in the ratio was lesser in control than the speciation treatments.

4. Discussion

4.1. Cr content, translocation and toxicity

There is evidence that no conversion of Cr speciation takes place in the nutrient solution before absorption by plant roots [2]. 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. Similar results have been obtained by [12]. Cr(VI) is actively taken up and is a metabolically driven processes in contrast to Cr(III) which is passively taken up and retained by cation exchange sites of the cell wall [26]. This in part explains the higher

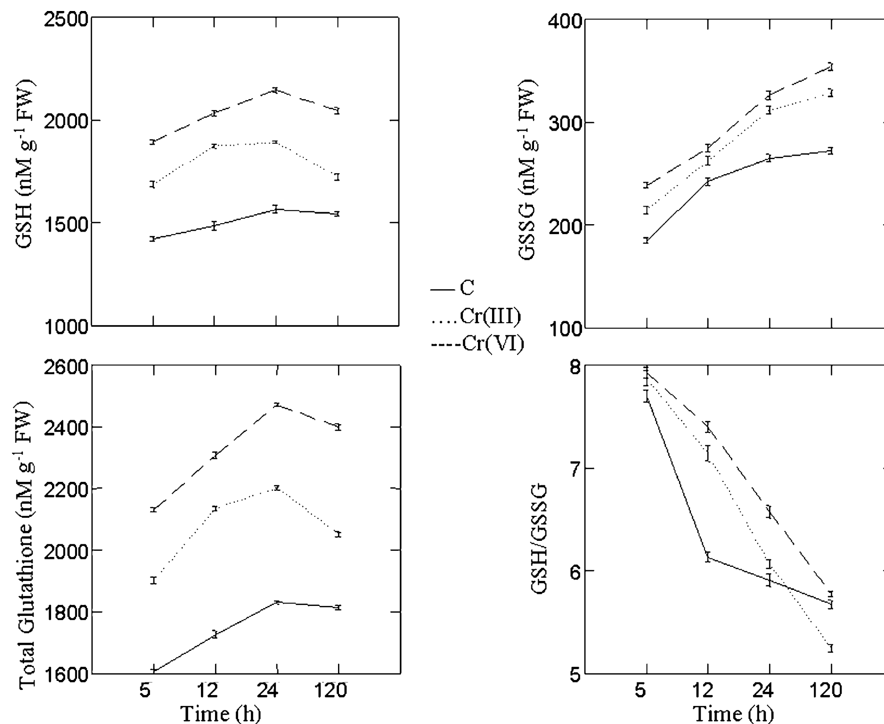

Fig. 3. Time course changes in GSH, GSSG, total glutathione and GSH/GSSG in roots of green gram as influenced by Cr speciation in nutrient medium. Vertical bars represent S.E.M. Values of GSH, GSSG and total glutathione were 1438, 168 and 1606 nM g^{-1} FW at 0 h, respectively.

accumulation of Cr(VI) by the plants. In addition it is known that P and Cr are competitive for surface sites and Fe, S and Mn are also known to compete with Cr for transport binding [27]. Hence it is possible that Cr effectively competed with these elements to gain rapid entry into the plant system. Poor translocation of Cr to the shoots could be due to sequesterization of most of the Cr in the vacuoles of 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. The toxic effect of Cr(VI) was pronounced in the shoots and roots as compared to Cr(III) which was only toxic to the roots. This was mainly due to high ROS intermediate production by Cr(VI) which resulted in membrane damage as evidenced by lipid peroxidation observed in the present study (Table 2). This could be due the fact that Cr(VI) is a strong oxidant with a high redox potential in range of 1.33 and 1.38 eV [3]. Electron microscopy studies indicate extensive oxidative damage to the outer root cells under Cr(VI) stress in hydroponic culture [28,29]. This may have caused serious impairment of uptake of mineral nutrients and water leading to deficiency in the shoot. In addition the normal mechanism of selective inorganic nutrient uptake may have been destroyed by this oxidative damage thus permitting larger quantities of Cr(VI) to enter the roots passively and further translocation of Cr(VI) to shoot causing oxidative damage to the photosynthetic and mitochondrial apparatus eventually reflecting in poor growth. In contrast Cr(III) 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. The toxicity of Cr(III) is reported to be due to indirect effects such as changes in pH and/or inhibition of ion transport [30]. Whereas in our study we found that Cr(III) did increase superoxide anion 5 h after treatment and H_2O_2 production and lipid peroxidation 12 h after treatment. Under appropriate conditions, H_2O_2 can act as an oxidizing agent and may oxidize Cr(III) to Cr(VI) [31] hence a possibility of such an endogenous oxidation cannot be ruled out. On the other hand, Cr(III) can be endogenously reduced to Cr(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 [32].

4.2. Antioxidant defence

Under normal conditions the levels of ROS in cellular compartments is 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 which essentially constitutes the basic ROS cycle [33]. Fluctuations in the antioxidative enzymes and metabolites observed in control could be because of the fine metabolic tuning performed by this cycle in terms of increasing active scavenging or suppressing metabolic activity responsible for ROS production. Further, recent evidence [34] ascribes an alternative role of plant growth and cell cycle control to ascorbate and glutathione. More specifically, AA stimulates cell cycle activity and DHA blocks the normal cell cycle progression. In this context the time course changes in these compounds under control could have been due to a cellular machinery regulating cell cycle.

Under stress ROS production is high resulting in oxidative damage. 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 [35]. In the present study, it was seen that catalase did not participate in active H_2O_2 reduction irrespective of speciation, although SOD was active in scavenging the superoxide produced by both the Cr species.

Differential CAT and SOD activity under abiotic stress induced oxidative damage has been reported [36]. APX was more efficient in destroying H_2O_2 than was catalase under both speciation of Cr. The reason for this could be that 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 [37]. Increase in APX activity was seen after the increase in SOD activity in the case of Cr(III)-treated plants whereas under Cr(VI) stress the increase in APX activity was before the increase in SOD activity. This suggests of a differential response to AA signaling by Cr(III) and Cr(VI). This was evident by the high increase in reduced AA very early in the roots under Cr(VI) stress. Over expression of DHAR gene has shown to increase AA levels dramatically [38]. In our study increase in DHAR activity was observed as early as 2 h after Cr(VI) treatment. It is possible that higher ROS generation by Cr(VI) acted as a signal transduction mechanism to increase DHAR synthesis resulting in higher AA amounts. The high increase in AA under Cr(VI) stress can be explained by the recycling function of the DHAR. DHA is rapidly and irreversibly hydrolyzed to 2,3-diketogulonic acid if not acted upon by DHAR. Increased DHAR activity could have generated more AA from the DHA pool before hydrolysis.

The increase in DHA under Cr(VI) stress inspite of no significant increase in MDHAR activity indicates that this metabolite was chiefly formed by non-enzymatic disproportionation [39]. 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 both speciations 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 phytochelatin in Cr stressed plants has been reported [29]. The depletion

of GSH and total glutathione in spite of higher GR activities observed as time under stress progressed 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 [40]. Liu et al. [41] have reported the presence of intermediate Cr species namely Cr(IV) and Cr(V) in roots exposed to high concentration of Cr(VI) by low-frequency electron paramagnetic resonance (EPR). It is possible that Cr(VI) was reduced to Cr(V), Cr(IV) (unstable) and Cr(III) (stable) in the presence of ascorbate and GSH as such interactions have been reported in animal systems [42]. 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 [34]. The reduced rate of GSH/GSSG ratio decline under Cr(VI) indicates 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.

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