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Full Length Research Paper

Chromium-induced accumulation of peroxide content, stimulation of antioxidative enzymes and lipid peroxidation in green gram (*Vigna radiata* L. cv. Wilczek) leaves

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Chromium (Cr)-induced oxidative damage and changes in contents of chlorophyll, protein, peroxide and malondialdehyde (MDA) and activities of enzymatic antioxidants were investigated in 4-day-old green gram (*Vigna radiata* L. cv. Wilczek) seedlings. Cr increased the contents of peroxide and MDA but decreased the contents of chlorophyll and proteins. Cr reduced the activities of catalase (CAT) and glutathione reductase (GR), but increased the activity of superoxide dismutase (SOD) in green gram leaves. Lipid peroxidation is considered to be an important mechanism of Cr-induced oxidative damage in green gram leaves. The peroxidation of lipids can be initiated by free radicals. The effects of Cr-induced oxidative damage and increase in the contents of peroxide and MDA in green gram leaves can be minimized by pretreatment with ascorbic acid (AA) and reduced glutathione (GSH) or both.

Key words: antioxidative enzymes, Cr-toxicity, green gram, oxidative damage, lipid peroxidation.

INTRODUCTION

Heavy metals are known to cause irreversible damage to a number of vital metabolic constituents and important biomolecules. They also cause irreversible injury to the plant cell and cell membrane. Trace elements are necessary for the normal metabolic functions of the plant, but at relatively higher concentration these metals are toxic and may severely interfere with physiological and biochemical functions (Chandra and Kulshreshtha, 2004; Parmar and Chanda, 2005). The toxic threshold level of the metal in the plant tissue is defined by the 'stress point' for metal toxicity and beyond this level the physiological state of the cell will be irreversibly damaged in plants and finally its leads to cell death. Chromium (Cr) is a naturally occurring element and its one of the components of the earth's crust. It is one of the heavy metal pollutants produced from tanning and pigment industries. It is easily absorbed by the plants from the soil and atmosphere, accumulate in the organs of the plants and show their cytotoxic and phytotoxic effects (Parmar and Chanda, 2005).

In the external environment, plants are under Cr stress. An important response to Cr stress by aerobic cells is the production of reactive oxygen species (ROS), like superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), alkoxy radical ($RO^{\cdot}$), singlet oxygen ($^1O_2$), and toxic hydrogen peroxide (H_2O_2) molecules (Breusegem et al., 2001). These ROS produced in the plant cells are detoxified by both enzymatic (catalase [CAT], peroxidase [POX], superoxide dismutase [SOD] and glutathione reductase [GR]) and non-enzymatic (ascorbate [ASC], glutathione, α-tocopherol [α-TOC] and carotenoids [CAR]) antioxidative systems. ROS, if not detoxified, cause serious damage to chlorophyll, protein, membrane lipids

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Abbreviations: CAT, Catalase; **Cr**, chromium; **GR**, glutathione reductase; **MDA**, malondialdehyde; **POX**, peroxidase; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase.

and nucleic acids (Alscher et al., 1997). H_2O_2 is a constituent of oxidative plant metabolism. It is a product of peroxisomal and chloroplastic oxidative reactions (Del Rio et al., 1992). It can react with $O_2^{\cdot -}$ radicals to form more reactive $OH^{\cdot}$ radicals in the presence of trace amounts of Fe or Cr. The $OH^{\cdot}$ radicals initiate self-propagating reactions leading to peroxidation of membrane lipids, destruction of chlorophyll and proteins (Halliwell, 1987; Bowler et al., 1992). Lipid peroxidation is considered to be an important mechanism of Cr-induced oxidative damage in plants. The peroxidation of lipids can be initiated by ROS (Bowler et al., 1992). The protective mechanisms of both enzymatic and non-enzymatic antioxidative systems are important components to protect the plants cells from oxidative damage caused by ROS. This is based on the fact that activity of one or more of these enzymes is generally increased in plants when exposed to heavy metal stress conditions (Alscher et al., 1997; Karuppanapandian et al., 2006a, b, c). SOD catalyzes the dismutation of $O_2^{\cdot -}$ radical to O_2 and H_2O_2. The produced H_2O_2 is then detoxified by CAT. GR plays a part in the control of endogenous H_2O_2 through an oxido-reduction cycle involving glutathione and ascorbate (Halliwell, 1987).

In the present study, we investigated the effects of Cr-induced degradation in chlorophyll and proteins, accumulation of peroxide and malondialdehyde (MDA) contents and stimulation of activities of CAT, SOD and GR. These effects can be minimized by pretreatment with ascorbic acid (AA) and reduced glutathione (GSH) or both in green gram (***Vigna radiata*** L.) leaves.

MATERIALS AND METHODS

Plant material and its growth condition

Healthy and uniform green gram (***V. radiata*** L. cv. Wilczek; Tamil Nadu Agricultural University, Coimbatore, India) seeds were germinated aseptically after surface sterilization with 70% ethanol, followed by a treatment with 0.1% $HgCl_2$ solution for 5 min each. Subsequently, seeds were thoroughly washed 5 times in sterile double distilled water, and germinated in petridishes in the darkness containing Whatman No. 1 filter paper moistened with Hoagland nutrient solution (Hoagland and Arnon, 1950). After 48 hr of germination, seedlings were transferred to plastic glasses containing Hoagland nutrient solution at pH 5.8 and kept in growth chamber. The growth chamber was maintained at 25 ± 1°C with 16 hL/8 hD and 150 µmol s^{-1} m^{-2} light intensity. Relative humidity was 35% during day and 60% during night. Cr (in the form of potassium dichromate, $K_2Cr_2O_7$) was added to the Hoagland nutrient solution. Seedlings with H_2O-treatment served as the control of this experiment. After 48 h of germination, seedlings were used for biochemical analysis.

Estimation of chlorophyll and protein

Chlorophyll estimation was carried out by following the method of Arnon (1949). 100 mg of leaves were homogenized with mortar and pestle in 15 ml of 80% acetone and centrifuged at 3,000 rpm for 15 min. Supernatant used for chlorophyll estimation and then absorbance was read at 645 and 663 nm. After absorbance was taken, the content of Chl ***a***, Chl ***b*** and total Chl were estimated. Proteins were extracted by homogenizing 500 mg of fresh leaves in 10 ml of 50 mM prechilled Tris-HCl (pH 8.0) and centrifuged at 10,000 rpm for 15 min at 4°C and an aliquot from the supernatant was mixed with an equal volume of ice cold 10% trichloroacetic acid (TCA; w/v) and incubated at 0°C for 1 h to precipitate the proteins. The protein pellet was collected by centrifugation at 5,000 rpm for 15 min at 4°C and dissolved in 1 M NaOH. Protein content was estimated by the procedure of Lowry et al. (1951) with BSA used as a standard. Protein content was quantified immediately after harvest.

Peroxide estimation

100 mg of fresh leaves were homogenized with 5% TCA and centrifuged at 17,000 rpm at 4°C for 10 min. The supernatant used for peroxide estimation by following ferri-thiocyanate methods by Sagisaka (1976). Reaction mixture contained 2 ml leaf extract, 0.4 ml of 50% TCA, 0.4 ml ferrous ammonium sulphate and 0.2 ml potassium thiocyanate. The absorbance of ferri-thiocyanate complex was read at 480 nm and compared to H_2O_2 standard. Peroxide content was expressed as µmol g FW^{-1}.

MDA determination

MDA content was determined by the thiobarbituric acid (TBA) as described by Heath and Packer (1968). After homogenizing the leaves with 5% TCA, the homogenate is directly used for MDA estimation. 1 ml of 5% TCA and 4 ml of TBA reagent (0.5% in 20% TCA) was 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 in a water bath the mixture was cooled and centrifuged 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 FW^{-1}.

Assays of antioxidative enzymes

For the extraction and assays of enzymes, 500 mg of leaves were macerated with 10 ml of 50 mM potassium phosphate buffer solution (potassium-PBS; pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP) in pre-cooled mortar and pestle and centrifuged at 15,000 rpm for 30 min at 4°C and the supernatant used for the following enzyme assays.

CAT (EC 1.11.1.6) activity was determined according to the method of Aebi (1984). 2 ml of assay mixture comprised of 1.8 ml of PBS (pH 7.0) and 200 µl of enzyme extract. The reaction was started with the addition of 1 ml of 30 mM H_2O_2 prepared in PBS (pH 7.0). Absorbance was read at 240 nm during the reaction. The CAT activity was expressed as U g FW^{-1}. SOD (EC 1.15.1.1) activity was assayed according to the method of Beauchamp and Fridovich (1971). The reaction mixture contained 0.24 mM riboflavin, 2.1 mM methione, 1% Triton X-100, 1.72 mM nitroblue tetrazolium chloride (NBT; in 50 mM sodium-PBS, pH 7.8). SOD activity was expressed as U g FW^{-1}. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition in the rate of NBT photo-reduction. GR (EC 1.6.4.2) activity was determined according to the method of Schaedle and Bassham (1977). The reaction was following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 3 min in 1 ml of an assay mixture containing 50 mM potassium-PBS (pH 7.8), 2 mM

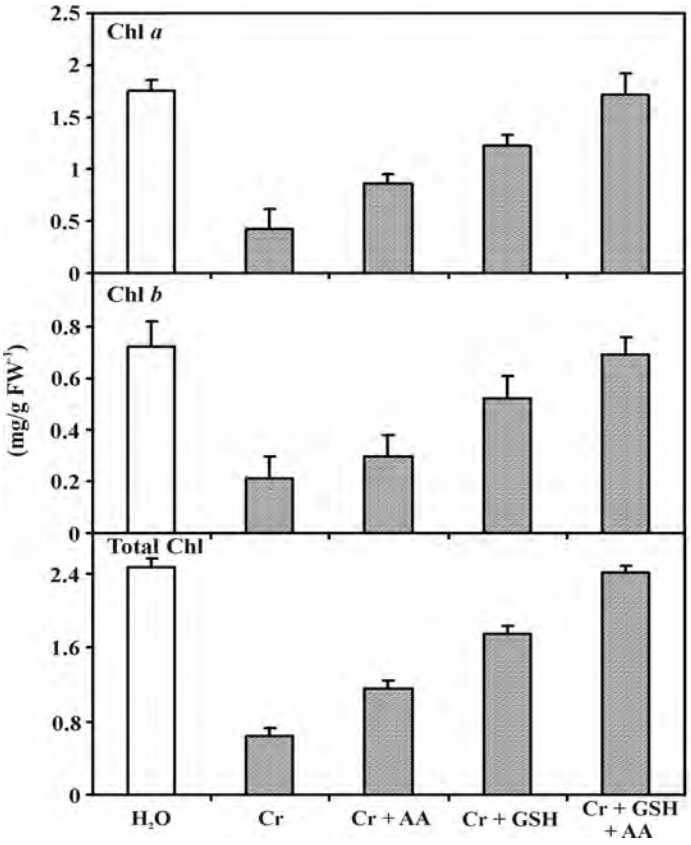

Figure 1. Effect of 300 µM Cr on the contents of Chl ***a***, Chl ***b*** and total Chl in green gram leaves pretreated with AA and GSH at 10 mM. The contents of Chl ***a***, Chl ***b*** and total Chl were measured after 48 h of treatment. Values are means ± SD.

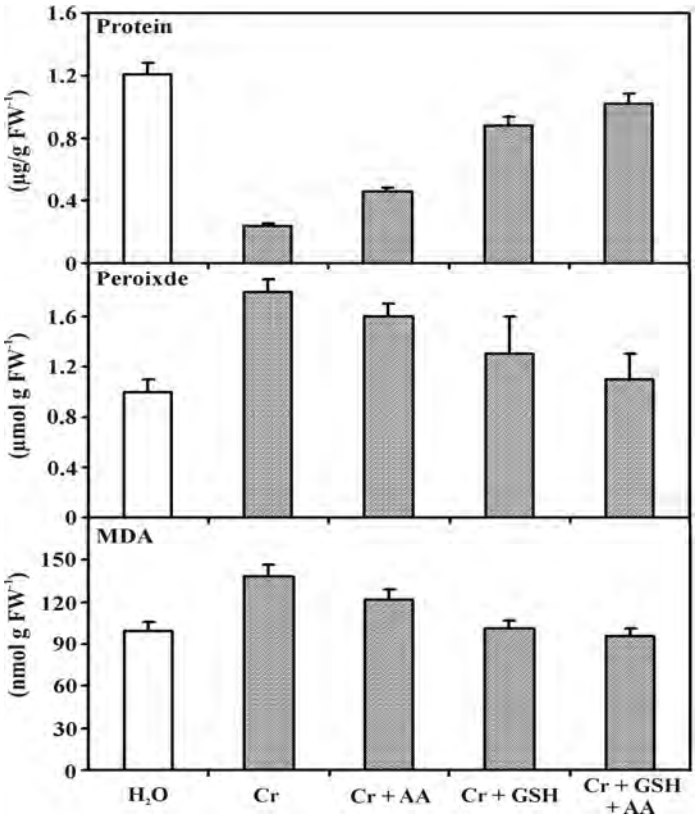

Figure 2. Effect of 300 µM Cr on the contents of protein, peroxide and MDA in green gram leaves pretreated with AA and GSH at 10 mM. The contents of protein, peroxide and MDA were measured after 48 h of treatment. Values are means ± SD.

Na_2EDTA, 0.15 mM NADPH, 0.5 mM GSSG and 200 µl of enzyme extract. The reaction was initiated by adding NADPH. Corrections were made for the background absorbance at 340 nm, without NADPH. GR activity was expressed as U g FW^{-1}.

Data analysis

All experiments were performed thrice with five replicates each. Similar results and identical trends were observed each time and the data presented here are from a single experiment.

RESULTS AND DISCUSSION

In the present study, we have investigated Cr-induced oxidative damage that occurs in 4-day-old green gram seedlings. Among the tested concentrations of Cr, in the range of 10 µM to 1 mM, 300 µM was found to be minimal the concentration (or stress point) to induce oxidative damage in green gram leaves. Increasing levels of degradation in chlorophyll and proteins were observed in 300 µM Cr-treated green gram leaves after 48 h of treatement. 300 µM Cr treatment showed a reduction in contents of Chl ***a***, Chl ***b*** and total Chl (Figure 1). Under heavy metal stress condition, rye seedlings showed relatively increasing degradation in the content of Chl ***a*** + ***b*** in the detached leaves (Krupa et al., 1996). Chlorosis (loss of chlorophyll), which appears in the leaves of Cr-treated seedlings, has been proposed as an indirect effect of Cr, probably due to the retardation of Fe and Zn translocation (Fontes and Cox, 1998). It has been shown that heavy metals can reduce chlorophyll formation by reducing the uptake of Fe^{2+} and Mg^{2+} (Gallengo et al., 1996) and by reacting with essential thiol groups in both the protochlorophyllide reductase protein and other enzymes involved in the light dependent synthesis of 5-aminolaevulinic acid (Gallengo et al., 1996; Fontes and Cox, 1998). The effect of Cr on the loss of chlorophyll could have resulted from the effects of free radicals produced by treatment with Cr ions. The same phenomenon has been observed with exposure to high concentrations of Cr and Al where photo-synthetic pigments (Chl ***a*** + ***b***) decreased (Anderson et al., 1973; Karuppanapandian et al., 2006a, b). Treatment of green gram seedlings with 300 µM Cr resulted in decreasing the proteins level (Figure 2). Cr-induced ROS formation may also cause protein damage. Excess Cr may replace other metals in metalloprotein or may interact directly with SH- groups of proteins (Fontes and Cox, 1998). The effects of Cr on the loss of chlorophyll and proteins can be minimized by pretreatment with

AA and GSH at 10 mM concentration (Figures 1 and 2).

Figure 2 shows that MDA level in Cr-treated green gram leaves was higher than that of the H_2O-treated control throughout the entire duration of the experiment. This indicates that Cr-induced oxidative damage in green gram leaves are linked to lipid peroxidation. The increase in lipid peroxidation in Cr-treated green gram leaves may be a reflection of the decline of antioxidative enzymes. Similar results were earlier obtained in our laboratory with Al and Cr (Karuppanapandian et al., 2000a, c). Verkleij and Schat (1990) and Gallengo et al. (1996) reported a similar increase in lipid peroxidation when plants were treated with Cr. It has been shown that Cr can react with $O_2^{\cdot -}$ radical to form more reactive $OH^{\cdot}$ radical. Since lipid peroxidation is generally considered to be induced by free radicals, it is possible that the effect of Cr on the oxidative damage in green gram leaves is mediated through earlier, especially $OH^{\cdot}$ radical. If this suggestion is correct, then the promotive effect of Cr on the oxidative damage in green gram leaves should be prevented by the addition of free radical scavengers. Thus, Figure 2 shows the effect of pretreatment of green gram leaves with free radical scavengers, such as AA and GSH for 48 h reduced Cr-induced oxidative damage and lipid peroxidation. GSH was the best scavenger and that is consistent with the report of van Assche and Clijsters (1990). Figure 2 shows that content of peroxide in 300 µM Cr-treated green gram leaves was increased compared to the H_2O-treated leaves. The peroxide content was minimized by pretreatment with AA and GSH at 10 mM.

In the present study, 300 µM Cr increased the activity of SOD in leaves of green gram (Figure 3). The increase of SOD activity can be considered as an indirect evidence for enhanced production of free radicals. It has been reported that excess Cr- and Al-increased the activity of SOD in higher plants during oxidative damage (Chongpraditnum et al., 1992; Karuppanapandian et al., 2006a, c). However, van Assche and Clijsters (1990) reported that SOD activity did not seem to be affected by excess Cr^{2+} ions. 300 µM Cr-decreased the activities of CAT and GR compared to the control (Figure 3). These results are consistent with those of Verkleij and Schat (1990). Gallengo et al. (1996) reported that Cr did not affect CAT activity. Such a variation in response of these enzymes to Cr stress could be due to the variability of plant species in producing free radicals (Parmar and Chanda, 2005).

GR catalyzes the reduction of oxidized glutathione (GSSG) in an NADPH-dependent reaction. GR, therefore, plays an essential role in the protection of chloroplasts against oxidative damage by maintaining a high GSH/GSSG ratio. In the present investigation, GR activity is decreased in green gram leaves exposed to excess Cr (Figure 3). These results are in harmony with those of Panda and Patra (2000) and suggest a decrease in GSH/GSSG ratio. This would explain why Cr treatment

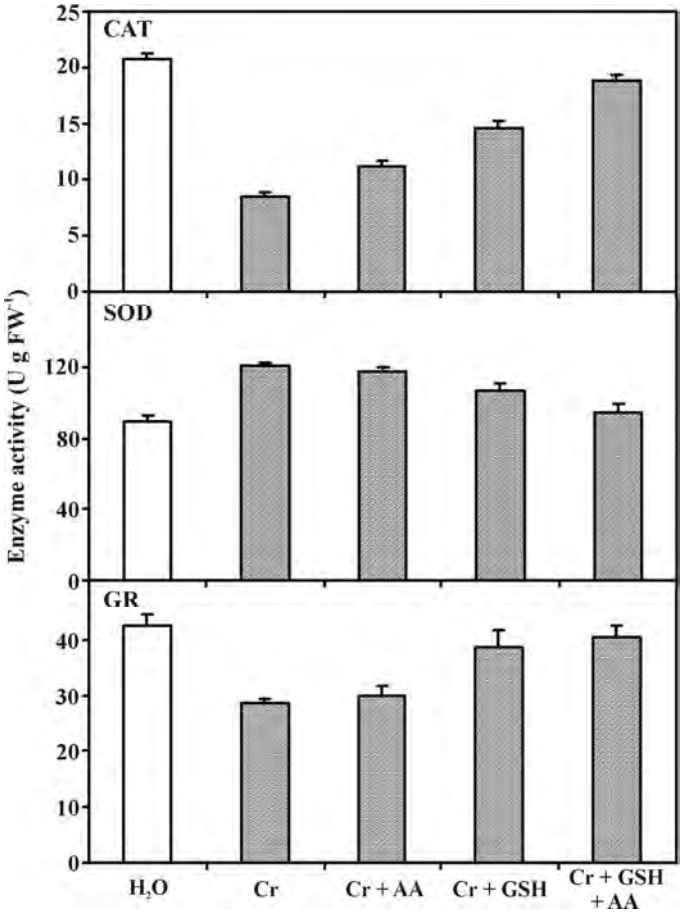

Figure 3. Effect of 300 µM Cr on the activities of CAT, SOD and GR in green gram leaves pretreated with AA and GSH at 10 mM. The enzyme activities were measured after 48 h of treatment. Values are means ± SD.

resulted in oxidative damage in green gram leaves. Pretreatment of green gram leaves with 10 mM AA + 10 mM GSH increased the activity of CAT, SOD and GR compared to the control (Figure 3). These results are in agreement with those of Parmer and Chanda (2005). When green gram leaves were pretreat-ed with the free radical scavengers AA (destruction of ROS, particularly H_2O_2) and GSH (scavenger of free radicals) and then treated with 300 µM Cr for 48 h, they were able to prevent the decrease in the activities of CAT, SOD and GR (Figure 3). GSH was better scavenger than AA and a mixture of the two compounds showed the best prevention. van Assche and Clijsters (1990) reported similar results. GSH and AA are the main antioxidants present in plant cells. GSH can react with $^1O_2$ and $OH^{\cdot}$ radicals and protects the thiol groups of enzymes (Prasad, 1998). The present results suggest that the Cr-induced oxidative damage in green gram leaves may be caused by an enhanced production of ROS and stimulation of ROS scavenging systems and subsequent high lipid peroxidation.

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