

COMPARATIVE STUDY ON THE EFFECT OF NITRIC OXIDE, CHROMIUM AND EDTA ON OXIDATIVE STRESS RESPONSES IN AGATHI (*SESBANIA GRANDIFLORA* L. PERS)

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Abstract. Heavy metal contaminations are a major cause of global environmental pollutions. The release of heavy metals into the environment has increased the threat of serious health issues for living organism. Among the known heavy metals, chromium is released into the environment by various anthropogenic activities causing various ill effects on the life forms. Hence in the present study which was carried out in Vellore Institute of Technology, Tamil Nadu, India and the phytoextraction of chromium enhanced with metal chelator ethylenediamine tetraacetic acid was studied in *Sesbania grandiflora* in VIT, India. To assess this, various treatments were prepared with combinations of chromium and chelator. The antioxidant enzyme activities such as Superoxide dismutase, Catalase, Ascorbate peroxidase and Glutathione reductase activity were determined in the presence of metal and chelator. The results showed an increase in all the antioxidant enzymes in the presence of metal and chelator due to the increased metal uptake. To reduce the stress levels created by the metal and chelator, sodium nitroprusside a nitric oxide donor was exogenously sprayed on to the plants and a comparative study was made in the presence and absence of sodium nitroprusside. The results indicated that the presence of sodium nitroprusside had a positive role in combating the stress created by the metal and chelator which was evident by the reduced antioxidant enzyme levels in spite of metal uptake. Hence sodium nitroprusside could be considered for its wider application against metal stress conditions to combat oxidative stress. The plant *Sesbania grandiflora* showed higher withstanding capacity to the heavy metal stress and was effective in extracting the metal out of the contaminated soil.

Keywords: heavy metal, *Sesbania grandiflora*, superoxide dismutase, catalase, sodium nitroprusside, chelator, oxidative stress

Introduction

Heavy metals pose a critical concern to human health due to their increasing usage as industrial inputs (Alloway, 1995; Diels et al., 2002). Chromium (Cr) is an environmental pollutant that ranks seventh in abundance in the earth crust. The major contributors of Cr contamination are the leather tanning, electroplating, and stainless steel industries. India is one of the largest producers of leather, and nearly 80% of the tanneries are engaged in the chrome-tanning process. Chromium being toxic interferes with metabolic processes in plants, which causes several morphological and physiological changes causing reduced growth, impaired photosynthesis and can lead to death of the plant. Chromium exists in two forms Cr (III) and Cr (VI). Among them Cr (VI) exists in the forms of chromate anions (CrO_4^{2-}), dichromate ($\text{Cr}_2\text{O}_7^{2-}$) and chromium trioxide (CrO_3) which are considered the most toxic forms of chromium, since they possess high oxidizing potential, high solubility, and mobility across the

membranes in living organisms and in the environment (Azmat and Khanum, 2005). Clean up of contaminated soils needs special attention because these soils support agricultural crops and so the consumption of crop plants grown on these soils may create serious health hazards.

Plants sequester heavy metals in roots and/or shoots and, therefore, significantly contribute to metal removal from the environment through the mechanism of phytoremediation (Jadia and Fulekar, 2009). The uptake and translocation of such heavy metal by plants can be made bio available by the addition of natural or synthetic chelators (Schmidt, 2003; Quartacci et al., 2006) like, ethylenediamine tetraacetic acid (EDTA), ethylenediamine di (o-hydroxyphenylacetic acid) (EDDHA) and hydroxyethylethylene-diaminetriacetic acid (HEDTA). Among them, EDTA is most effective chelating agent which enhances the accumulation of metals, by mobilizing metal cations, water soluble complexes (Lestan et al., 2008) there by modifying the availability of the metals in soils to the above ground parts of plants (Garba et al., 2012b), and increasing the uptake of heavy metals.

One of the mechanisms that make a plant tolerant to heavy metal stress is the presence of strong antioxidant defense system (Metwally et al., 2005; Qureshi et al., 2005; Israr et al., 2006 and Diwan et al., 2008). Heavy metals stress causes molecular damage to plants, either directly or indirectly through reactive oxygen species (ROS) formation. Further, plants growing in chromium-stressed environment suffer due to potential risk from reactive oxygen species (ROS) like superoxide ($O_2^{\cdot-}$), hydroxyl radicals ($\cdot OH$) and hydrogen peroxide (H_2O_2). However, plants possess their own defense system to combat these toxicity problems like cellular antioxidants and antioxidant enzymes that play a vital role in protecting the plant from various physiological damage caused by ROS (Mittler, 2002., Baek and Skinner, 2003) that make a plant species tolerant to heavy metal stress (Metwally et al., 2005; Qureshi et al., 2007; Israr et al., 2006; Diwan et al., 2008). Antioxidant system consists of enzymatic and non-enzymatic compounds. Enzymatic antioxidants include superoxide dismutase, catalase, peroxidases, glutathione reductase and ascorbate reductase. Non-enzymatic antioxidants are ascorbate, glutathione, tocopherol and carotenoids (Mittler, 2002; Candan and Tarhan, 2003).

Nitrite oxide (NO) is a lipophilic molecule that diffuses through membranes. Although first described as a signal molecule in animals, accumulating evidence shows that NO is an important signal molecule involved in plant response to biotic and abiotic stresses (Delledonne et al., 1998; Carlos and Lorenzo, 2001; Uchida et al., 2002).

Based on the above observation, the objective of the present study is to investigate whether exogenous sodium nitroprusside (SNP) plays a positive role in protecting *Sesbania grandiflora* against antioxidative stress created by chromium in the presence of EDTA.

Materials and methods

Soil spiking and experimental setup

The study was done in Vellore Institute of Technology (VIT), Tamil Nadu, India. The experiments were conducted during the months of June-August. Pot experiments were conducted using the soil samples taken from VIT garden. The soil was air-dried and sieved. The study was a comparison made by conducting two experiments (Exp), namely Exp 1 and Exp 2. Five pots each containing 2.7 kg of soil + 0.3 kg of manure

was mixed along with five different concentrations of chromium and ethylenediamine tetraacetic acid (EDTA) in case of Exp 1. The study was done to assess the efficiency of chromium accumulation by *Sesbania grandiflora*. This was studied in comparison with Exp 2 containing in addition 10 ml of 250 μ M of SNP as foliar spray along with chromium and EDTA. The various treatments that were considered in Exp 1 and Exp 2 are given in the following treatment tables:

Treatment name (Exp 1)	Concentrations
C1	20 mg Cr Kg ⁻¹
C2	40 mg Cr Kg ⁻¹
C3	60 mg Cr Kg ⁻¹
C4	80 mg Cr Kg ⁻¹
C5	100 mg Cr Kg ⁻¹
C1+EDTA	20 mg Cr Kg ⁻¹ + 0.35 mM EDTA
C2+EDTA	40 mg Cr Kg ⁻¹ + 0.35 mM EDTA
C3+EDTA	60 mg Cr Kg ⁻¹ + 0.35 mM EDTA
C4+EDTA	80 mg Cr Kg ⁻¹ + 0.35 mM EDTA
C5+EDTA	100 mg Cr Kg ⁻¹ + 0.35 mM EDTA

Treatment Name (Exp2)	Concentrations
C1	60 mg Cr Kg ⁻¹
C2	100 mg Cr Kg ⁻¹
C3	60 mg Cr Kg ⁻¹ + 0.35 mM EDTA
C4	100 mg Cr Kg ⁻¹ + 0.35 mM EDTA
C5	60 mg Cr Kg ⁻¹ + 250 μ M SNP
C6	100 mg Cr Kg ⁻¹ + 250 μ M SNP
C7	60 mg Cr Kg ⁻¹ + 0.35 mM EDTA + 250 μ M SNP
C8	100 mg Cr Kg ⁻¹ + 0.35 mM EDTA + 250 μ M SNP
C9	Control + 250 μ M SNP
C10	Control

Control plants were free of chromium and EDTA (Exp 1) and contained SNP in case of Exp 2. All the treatments were done in triplicates. The control and the treated soil were left to equilibrate for 3 weeks during which it was occasionally turned and mixed to ensure thorough mixing. The seeds of *Sesbania grandiflora* were sterilized in 3% formaldehyde and sown in plastic trays for one week to attain the seedling stage (Fig. 1a). About 6 seedlings were transferred to the spiked soil pots after three weeks of incubation. Totally 33 pots from Exp 1 and Exp 2 were maintained in a randomized block design (Fig. 1b). Both the experiments were performed at the same time period. The harvested plants were collected during every morning at the end of 30, 60 and 90 days and were stored at -20 °C for a short duration and all the enzyme assays were performed within a week's time from the harvest.

Enzyme extraction and activity of antioxidant enzymes

500 mg of fresh leaves was weighed and homogenized in 3 ml of extraction buffer containing 0.5 ml of 50 mM potassium phosphate buffer (pH 7.5), 0.5 ml of Triton X-100, 1 g of polyvinyl pyruvate (PVP) and 3.72 mg of EDTA. The homogenate were

filtered and centrifuged at $15,000 \times g$ for 20 min at 4 °C. The supernatant was used for assaying superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) activities.



Figure 1. a One week old seedling of *Sesbania grandiflora*; **b** randomized block design of treatment pots

Superoxide dismutase (SOD) (EC1.15.1.1)

SOD activity was assayed using a modified NBT method according to Beyer and Fridovich (1987). The 2 ml assay reaction mixture contained 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 9.9 mM L-methionine, 55 μ M nitro blue tetrazolium (NBT), and 0.025% Triton-X100. 40 μ l of diluted (2 \times) sample and 20 μ l of 1 mM riboflavin were added and the reaction was initiated by illuminating the samples under a 15 W fluorescent tube. Duplicate tubes with the same reaction mixture were kept in the dark and used as blank. Absorbance of the samples was measured immediately after the reaction was stopped at 560 nm against blank. The enzyme activity grams^{-1} f.w of a sample was determined from a standard curve obtained by using pure SOD.

Native polyacrylamide gel electrophoresis (PAGE) of crude extract (25 μ g) combined with loading buffer (containing neither SDS nor P-mercaptoethanol) was performed in a mini-gel system (Medox) at 150-200 V. SOD activity in native polyacrylamide gels was determined by the nitroblue tetrazolium (NBT) method of Beauchamp and Fridovich (1971). SOD activity was visible after several minutes as an achromatic band against a blue background.

Catalase (CAT) (EC 1.11.1.6)

The Catalase assay was performed by the method of (Luck, 1974). The enzyme extract (0.02 ml) was added to the reaction mixture containing 3 ml of hydrogen peroxide (H_2O_2) and 0.067 M phosphate buffer (pH 7.0) and the optical density change was measured at 240 nm, the time taken for decrease in the absorbance from 0.45 to 0.4 is noted as ΔT . The activity of the enzyme is expressed in the terms of μ mole of H_2O_2 consumed/min/ mg protein. The activity was determined by calculating the concentration of H_2O_2 using the extinction coefficient 0.036 per μ mole per ml.

Ascorbate peroxidase (APX) (EC1.11.1.11)

The APX activity was determined by the method followed by Nakano and Asada (1987). The 1 ml reaction mixture contained 0.1 ml of 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.3 mM H₂O₂ and 50 µl of enzyme extract. The oxidation of ascorbic acid was measured by the decrease in absorbance at 290 nm for 3 min using a spectrophotometer. The enzyme activity was calculated using the extinction coefficient 2.8 mM⁻¹ cm⁻¹ and expressed in enzyme units (mg protein)⁻¹. One unit of enzyme is the amount necessary to decompose 1 µmol of substrate per min at 25 °C.

Glutathione reductase (GR) (EC 1.6.4.2)

The activity of GR was measured by monitoring the glutathione dependent oxidation of (Nicotinamide adenine dinucleotide phosphate) NADPH described by Rao et al. (1996). 1 ml reaction mixture contained 0.1 ml of 100 mM potassium phosphate buffer (pH 7.5), 0.1 ml of 1 mM EDTA, 0.02 ml of 0.2 mM NADPH, 0.05 ml of 0.5 mM oxidized glutathione and 50 µL of enzyme extract. The reaction was allowed to run for 3 min and the absorbance was measured at 340 nm using a spectrophotometer. The enzyme activity was calculated using extinction coefficient 6.2 mM⁻¹ cm⁻¹ and expressed in enzyme units (mg protein⁻¹). One unit of enzyme is the amount necessary to decompose 1 µmol of NADPH per min at 25 °C.

Determination of ascorbate oxidase activity (AAO) (EC 1.10.3.3)

Assay of ascorbic acid oxidase activity was carried out according to Oberbacher and Vines (1963). 0.1 ml of the enzyme extract was added to 3 ml of the substrate solution (8.8 mg of ascorbic acid in 300 ml phosphate buffer, pH 5.6). The absorbance change was measured at 265 nm for every 30 s for a period of 5 min. One enzyme unit is equivalent to an absorbance change of 3.58 per minutes.

Non-enzymatic antioxidants glutathione estimation

The level of glutathione in the treatments with and without EDTA containing chromium was determined according to Tukendorf and Rauser (1990), using Ellman's reagent. About 500 mg of the plant material was homogenized with mortar and pestle in 5 ml of cold extraction buffer containing, 0.1 M Tris-HCl (pH 8.0), 10 mM MgCl₂, 3 mM Sodium-EDTA. The homogenate was centrifuged for 30 min at 15 000 g at 4 °C. The reaction mixture contained 1 ml of the supernatant, 2 ml of 0.4 M Tris-HCl buffer (pH 8.9), 50 µl of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) DTNB. All the samples were incubated for 1 h at 37 °C. The absorbance was measured at 412 nm after 2 min in a spectrophotometer ($\Sigma 412 = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Statistical analysis

The data observed in the experiment were statistically analyzed for the mean, standard deviation (SD), standard error (S.E.) and least significant difference (LSD) to determine the critical level of significance ($P \leq 0.05$) of the differences between means of treatments.

Results and discussion

Effects of Cr, EDTA and SNP on superoxide dismutase activity

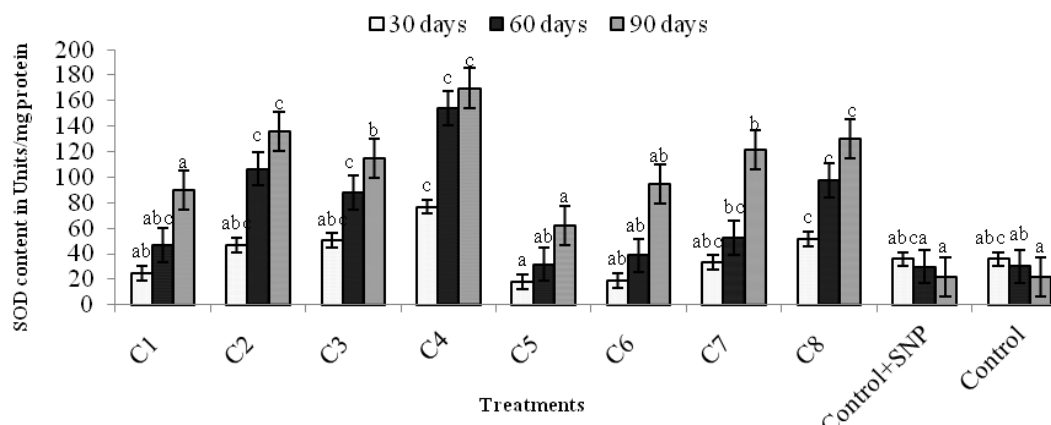
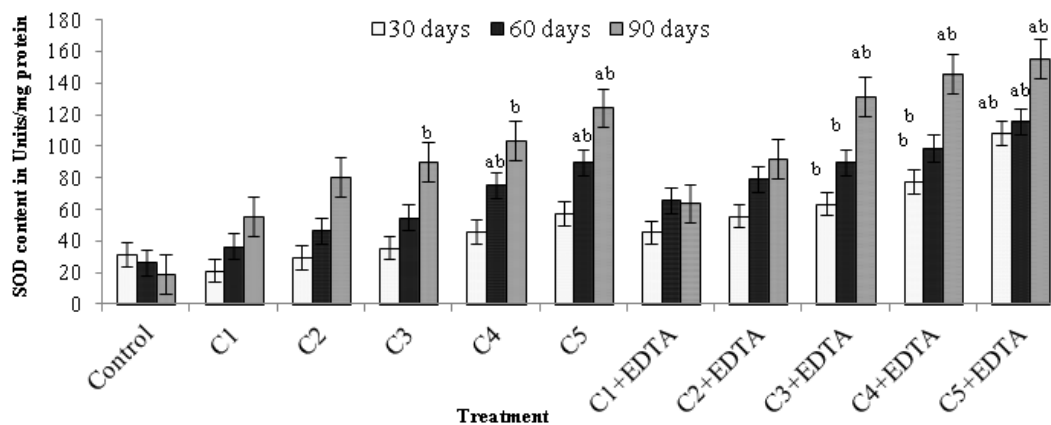
The results of SOD activity are presented in *Figure 2a* showing the effect of different concentration of chromium with and without EDTA on superoxide dismutase activity (Units $\text{mg}^{-1}\text{protein}$) in *Sesbania grandiflora* and in *Figure 2b* showing the effect of chromium and EDTA in the presence of SNP on superoxide dismutase activity (Units $\text{mg}^{-1}\text{protein}$) in *Sesbania grandiflora* respectively. The results after 30 days time period from *Figure 2a* showed an increase in the SOD activity with increase in the concentration of Cr. When compared with control plants the C5 treatment plants showed 80.7% increase with respect to the SOD activity. Further in the presence of chelator EDTA, the SOD activity was observed to be increased in all the treatments with EDTA when compared with control. Significant difference was noted for the treatment with highest concentration of Cr at both the levels $P \leq 0.05$ and $P \leq 0.01$. During 60 days of growth significant difference at both $P \leq 0.05$ and $P \leq 0.01$ were observed for the treatments C4, C5 and C5+EDTA, whereas treatments C3+EDTA and C4+EDTA showed significant difference only at one level $P \leq 0.05$. The highest SOD activity was represented by the treatment C5+EDTA at 60 days of growth with 116.01 units/mg protein. Whereas in absence of EDTA the C5 plants showed 22.48 % decrease in the SOD activity, this justifies the role of EDTA in enhanced metal uptake and increased oxidative stress, thereby increasing the SOD antioxidant enzymes. At 90 days there was a significant difference for C3+EDTA, C4+EDTA and C5+EDTA at both $P \leq 0.05$ and $P \leq 0.01$, whereas in the absence of EDTA, C3 and C4 showed difference only at $P \leq 0.05$, whereas C5 showed significant difference at both the significant levels. Hence the significant difference between the treatments Cr without and with EDTA shows that, in the presence of EDTA the concentration of Cr is higher and it also increased with the time period. The elevation of SOD activity in general is a clear indication to detoxify the O^{-2} and other ROS in downstream pathways under metal stress. But in the presence of EDTA, the levels of SOD are still higher.

SOD activity increased with 150 $\mu\text{g/g}$ Cr in the presence of EDTA in *Cucumis utilisissimus* (Sinam et al., 2011). The increase in SOD activity after 12 hr of application of 50 $\mu\text{mol/L}$ of Cr (VI) was also reported in *Vigna radiata* cv. CO₄ (Shankar et al., 2005). In maize the SOD activity increases with 300 μM of Cr in Sartaj variety (Maiti et al., 2012).

The result represented in *Figure 2b* depicts the effects of exogenous nitric oxide in the presence of chromium and EDTA. From the figure it was clear that, when the concentration of chromium is increased from 60 mg/Kg Cr to 100 mg/Kg Cr, the SOD activity also increases corresponding to the heavy metal increase enhanced by EDTA, which is vivid from the treatments C1-C4. This phenomenon of increase in SOD activity corresponding to the metal increase has been reported by different authors. Due to oxidative stress created by the metal the plant has adapted a defensive mechanism by increased production of anti-oxidative enzymes, among the known antioxidative enzymes, SOD is considered to be the first line of defense for plants anti oxidative defense system. In the presence of SNP, this condition is reversed in the present study. From this it is concluded that, C3 and C4 plants when compared with that of C7 and C8 plants, the presence of SNP in latter in spite of metal and EDTA concentration it decreased the SOD activity in those plants (i.e. C7 and C8) by 34.4% and 32.90% respectively at the end of 30 days growth period. Significant difference was depicted in

activity of SOD in C3 and C7 for varying time periods. This shows that in the presence of SNP the SOD activity have been reduced. Based on the literature (Ferreira et al., 2010) the decreased SOD activity during treatment with SNP clearly states that nitric oxide (NO) action has created lower substrate availability and created a reversed effect. This fact was detected in the present study with the plants supplied with 250 μ M of SNP.

Similar results were reported in ryegrass and in *Arabidopsis* under Cu stress (Dong et al., 2014; Cobbett, 2000). Reports in sunflower and rice leaves under Cd stress by Laspina et al. (2005) and Hung and Kao (2003) have detected that in the presence of SNP, the SOD levels decreases and this low SOD activity was due to decrease in lipoperoxide levels. It increased the photosynthetic ability of the plant and confers the plant protection against oxidative stress. Similar to the above results (Caro and Puntarulo, 1998) observed the lowered levels of substrates for SOD due to NO presence in soybean embryo. Reports also suggested that nitric oxide play role even as an antioxidant thereby directly quenching the super oxide anion and regulates the oxidative damage as seen in *Triticum aestivum* roots grown under Pb induced toxicity (Kaur et al., 2015). Thus based on the above reports by various authors it could be correlated in the present work, that NO gradually scavenged the superoxide radical when SNP is supplied exogenously in the treatments C5 and C6. Even in the presence of combined effect of Cr and EDTA in the treatments C7 and C8, presence of SNP had a positive role in attenuating the oxidative stress generated by the metal and chelator.



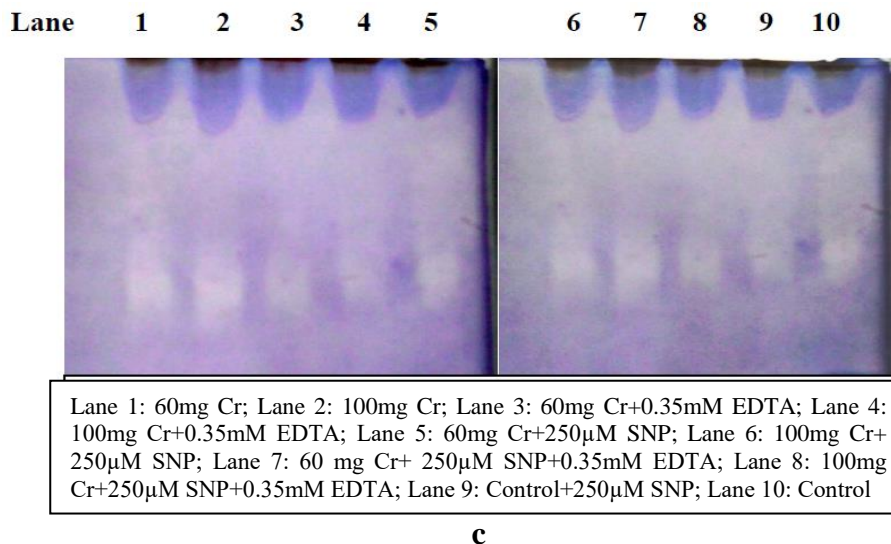


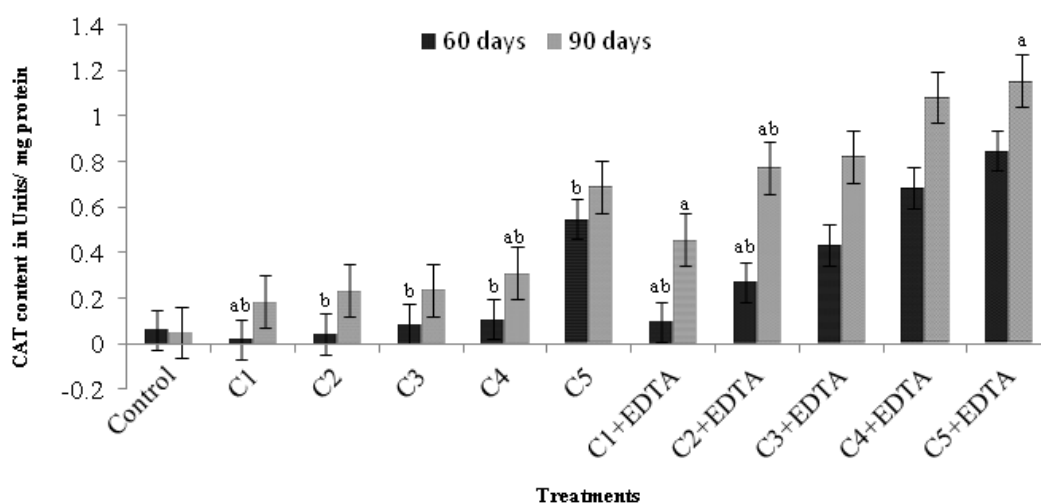
Figure 2. a Effect of different concentration of chromium with and without EDTA on Superoxide dismutase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Significance at $P \leq 0.05 = b$, significance at $P \leq 0.01$ and $P \leq 0.05 = ab$.) **b** Effect of chromium and EDTA in the presence of SNP on superoxide dismutase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Lower case letters that are different denote the treatments which are significantly different. Same letter indicates the treatments which are not significantly different.) **c** Superoxide dismutase (SOD) isoenzymes activities in native gels in leaves of *Sesbania grandiflora* under various combinations of chromium, EDTA and SNP

SOD enzyme was distinguished by native Polyacrylamide gel electrophoresis (PAGE) experiment to determine the isoenzymes of SOD in the presence of SNP-treatment. The finding in *Figure 2c* shows the superoxide dismutase (SOD) isoenzymes activities in native gels in leaves of *Sesbania grandiflora* under various combinations of chromium, EDTA and SNP, this shows the presence of one manganese superoxide dismutase (Mn-SOD) in both the control and chromium treated plants. When plants were exposed to higher concentration of chromium with 100 mg Cr Kg^{-1} , it was observed that MnSOD was apparently affected in SNP treated plants which is clear from the bands which were clearly less intense (lane 8) than the other treatments (lane 4) in the absence of SNP.

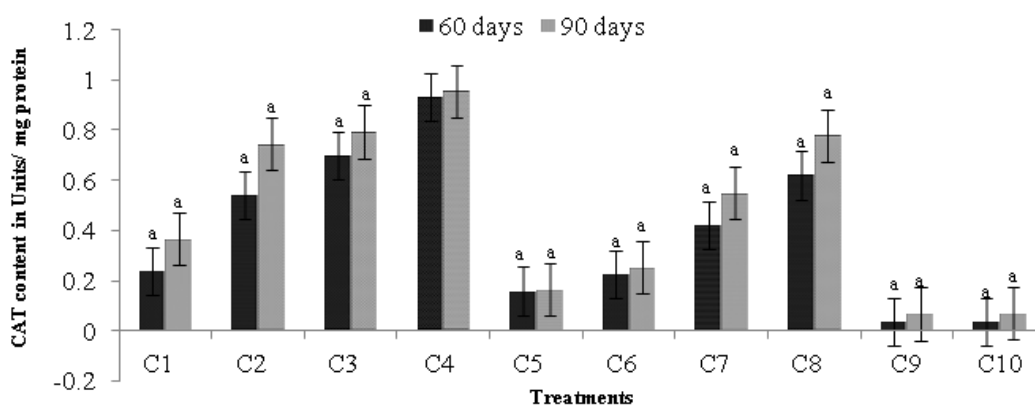
Effects of Cr, EDTA and SNP on catalase activity

Catalase, a tetrameric heme-containing enzyme, that degrade H_2O_2 rapidly and more sensitive to excess Cr, where it readily binds to thiol groups and inactivates the thiol-containing enzyme (Luna et al., 1994). Similar to SOD activity, the catalase activity in the study represented in *Figure 3a* shows the effect of different concentration of chromium with and without EDTA on catalase activity (Units mg^{-1} protein) in *Sesbania grandiflora*. The activity was found to be increased with higher concentrations of Cr in the presence of EDTA. When compared with the control, the highest catalase activity was noted for higher concentration of Cr in presence of EDTA (C5+EDTA) which was observed to be 55.5% increased than non-EDTA plants (C5) at 60 days growth period. The CAT activity also increased with the time period from 60 days compared to 90 days due to the persistent effect of chromium action. Hence under this metal stress reactive

oxygen species (ROS) production is elevated leading to oxidative damage. Statistically it was noted that at 60 days significant change was exhibited at $P \leq 0.05$ and $P \leq 0.01$ for the treatments C1, C1+EDTA and C2+EDTA. Similarly at 90 days C4 and C2+EDTA showed significant difference ($P \leq 0.05$ and $P \leq 0.01$). Statistically also EDTA proves its higher metal enhancement activity, since from *Figure 3* it is clear that, at 60 and 90 days of the growth the treatment C2+EDTA alone showed significant difference at the p values $P \leq 0.05$ and $P \leq 0.01$, stating that the presence of EDTA with higher concentration of Cr had influence on the increased activity. The combined action of SOD and CAT is critical in mitigating the effects of oxidative stress, since SOD show customary action on the superoxide anion converting it to H_2O_2 which is another reactive intermediate and later H_2O_2 gets converted it to water and oxygen (Mates, 2000). Catalase enzyme increases with increasing concentration of Cr in *Ocimum tenuiflorum* and *Sesbania sesban* (Rai et al., 2004; Bakiyaraj et al., 2014).



a



b

Figure 3. a Effect of different concentration of chromium with and without EDTA on catalase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Significance at $P \leq 0.05 = b$, significance at $P \leq 0.01$ and $P \leq 0.05 = ab$.) **b** Effect of chromium and EDTA in the presence of SNP on catalase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)

The results of catalase activity in the presence of chromium and EDTA, and exogenous application of SNP are represented in *Figure 3b* showing the effect of chromium and EDTA in the presence of SNP on catalase activity expressed in (Units mg^{-1} protein) in *Sesbania grandiflora*. From the figure it is clear that, the catalase activity increased with increase in chromium and EDTA levels, which is apparent from the treatments C2 and C4. But the treatments C5-C8 with SNP the activity of catalase decreased, with corresponding increase in the concentration of chromium.

According to Ferreira et al. (2010), the cause of the decrease in CAT activity was due to reduced substrates for CAT activity as observed in SNP-pretreated soybean plants. This fact was paralleled with the present study that the decreased H_2O_2 production as a consequence of the NO effect, approve protection against ROS in SNP administered C7 and C8 plants. Our study is in concordance with the above statement that, in the presence of SNP the catalase activity is reduced in C5 and C6. This is also applicable to the treatments C7 and C8, where in spite of chromium and EDTA the catalase activity was reduced in these plants. Thus when a comparison was made between C3 and C4 plants with their counter parts C7 and C8 plants, presence of SNP in latter, reduced the CAT activity by 31.6% in case of 60 mg Cr, SNP and EDTA and 18.94% in case of 100 mg Cr, SNP and EDTA at 90 days growth period. Hence compared with the control the antioxidant activity was decreased in SNP treated plants which clearly explains the potential of NO, which confers resistance to heavy metal stress created oxidative damage in *Sesbania grandiflora*. And moreover the reduction of catalase activity and its decreased levels to normalcy was experienced after addition of SNP indicating the cell response to NO action and thus decreasing the ROS generation inside the cell. Similar results were reported by Dalurzo et al. (1996) in Pea leaves and (Cobbett, 2000) in *Arabidopsis* showing the decline in CAT activity.

Effects of Cr, EDTA and SNP on ascorbate peroxidase (APX) activity

The APX activity for Exp.1 is presented in *Figure 4a* showing the effect of different concentration of chromium with and without EDTA on ascorbate peroxidase activity (Units mg^{-1} protein) in *Sesbania grandiflora*. From the figure, it is clear that the APX activity increased in the treatments of chromium in the presence of EDTA than experienced by the treatment in the absence of EDTA. The levels of APX increased in 30 days of plant growth, and when compared with the control, the value of 4.6 units/mg protein observed in C5+EDTA plants, is 10.96% higher than C5 plants with 0.51 units/mg protein of APX. A significant difference in the treatments was observed for the higher concentration at both the levels $P \leq 0.05$ and $P \leq 0.01$ in the presence of EDTA (C4+EDTA and C5+EDTA). After 30 days, the APX activity increased with increase in the chromium concentration. This increase in the enzyme activity could be correlated with action of ascorbate peroxidase that was more efficient in destroying H_2O_2 under Cr stress in the presence of chelator. Statistically at 60 days, there was a significant difference noted for all the treatments at both $P \leq 0.05$ and $P \leq 0.01$, except for C2+EDTA and C5+EDTA which showed significant difference only at one level $P \leq 0.05$. Among the EDTA treated plants C1+EDTA was the only treatment that showed significant difference at $P \leq 0.05$, whereas all the treatments in the absence of EDTA significantly differed ($P \leq 0.05$ and $P \leq 0.01$). When compared with the control, the progressive level of APX showed an increase in the activity of APX in the treatment C5+EDTA at 90 days with 8.6 units/mg protein of the enzyme. The study is in agreement with the reports of (Karuppanapandian et al., 2006a) that, the antioxidant

enzyme increase was due the antioxidative capacity stimulated by Cr, which caused the conversion of H_2O_2 to H_2O and O_2 . The high levels of APX produced, due to the higher concentrations of Cr in the presence of EDTA, uses ascorbic acid as a reductant in the first step of the ascorbate-glutathione cycle (Mittova et al., 2003 and Smirnov, 2000). Similar results were reported by Rai et al. (2006) in sugarcane plants exposed to hexavalent chromium. Cr and Al induced H_2O_2 accumulations in wheat and green gram seedlings were also found to significantly elevate the APX activity (Sharma and Sharma, 1996). APX plays an important role in scavenging H_2O_2 which is a systemic signal for the induction of APX (Morita et al., 1999).

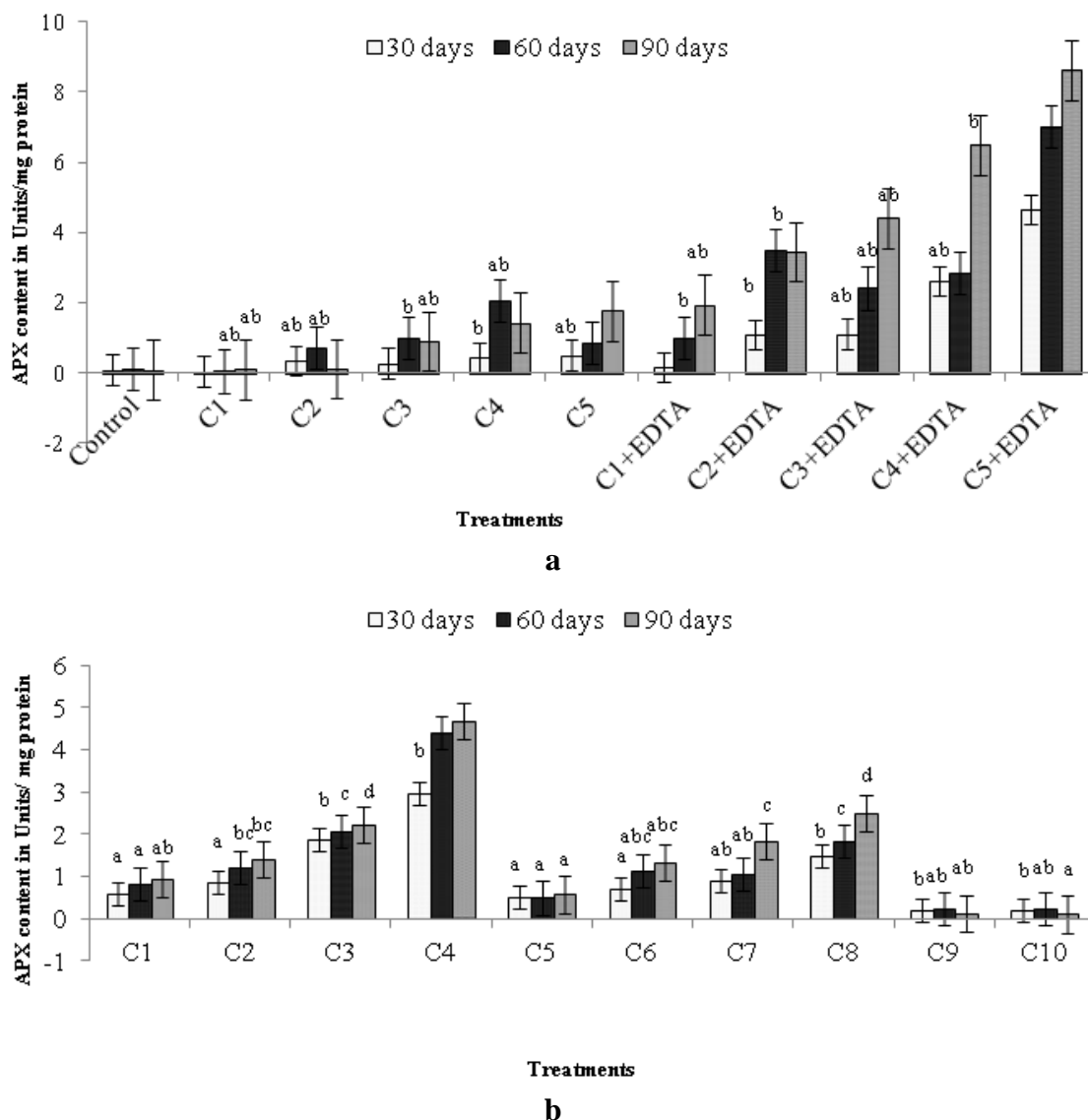


Figure 4. a Effect of different concentration of chromium with and without EDTA on ascorbate peroxidase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Significance at $P \leq 0.05 = b$, significance at $P \leq 0.01$ and $P \leq 0.05 = ab$.) **b** Effect of chromium and EDTA in the presence of SNP on ascorbate peroxidase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)

The effects of APX activity in the presence of chromium and EDTA, with exogenous application of 250 μM SNP are represented in *Figure 4b* showing the effect of chromium and EDTA in the presence of SNP on ascorbate peroxidase activity (Units mg^{-1} protein) in *Sesbania grandiflora*. From Exp.1, it was concluded that, the APX activity was found increased at higher chromium levels with EDTA, due to higher metal accumulation which was assisted in presence of EDTA. From Exp.1 also it is evident that, in order to overcome the oxidative stress, the APX activity increased in EDTA treated plants as a plant adaptive defence mechanism, which is evident from the treatments C3 and C4 (60 and 100 mg Cr kg^{-1} with 0.35 mM EDTA). The increased APX activity was observed for higher concentrations of chromium with increase in time period (C5, C5+EDTA), whereas C2, C4 and C2+EDTA plants exhibited lesser variation in APX activity during 90 days. Similarly the plants supplied with 250 μM SNP, showed decreased APX activity. This is obvious when comparison is made between C3 and C4 and C7 and C8 plants, that presence of SNP has decreased the APX activity by 17.56% (between C3 and C7) and 47% (between C4 and C8). Significant difference was depicted in C8 during 60 and 90 days compared to C4. This decrease in APX activity from the present study could be correlated with Hiner et al. (2000) and Talukdar (2012b) that, low availability of reduced ascorbate, its co-factor, or inhibition of its isoforms by excess H_2O_2 or both, caused reduced APX activity in *Lathyrus sativus* by the latter. Hence in the present study the lower ascorbate levels created by the nitric oxide have reduced the stress levels and hence a reduced APX activity was observed. Similar results were reported in wheat roots (Singh et al., 2008).

Effects of Cr, EDTA and SNP on glutathione reductase (GR) activity

The results for GR activity after 30, 60 and 90 days are presented in *Figure 5a* showing the effect of different concentration of chromium with and without EDTA on glutathione reductase activity (Units mg^{-1} protein) in *Sesbania grandiflora*. The figure denoted an increasing GR activity corresponding to the increasing concentration of the chromium and increased further in the presence of EDTA. The treatment C5 was recorded with 0.38 units/mg protein of GR content which corresponds to a decreased percentage of 58.24%, when compared with that of C5+EDTA plants with 0.91 units/mg protein. Statistically there was significant difference for the treatments C5, C3+EDTA and C5+EDTA after 30 days at $P \leq 0.05$ and $P \leq 0.01$. During 60 days of growth C4, C5 and C3+EDTA showed significant difference. At 90 days there was statistical difference for $P \leq 0.05$ and $P \leq 0.01$ in all the treatments with and without EDTA, except for C1. Thus the determination of GR content from Exp.1 concludes that, the GR levels increased with the concentration of the treatments and it is duration dependent. Similar results were also reported in Indian mustard (Diwan et al., 2009) stating that the activity of GR under Cr treatment was concentration as well as duration dependent.

GR is a flavoenzyme, oxidoreductase, which catalyze the conversion of oxidized glutathione to reduced glutathione, which is found in both prokaryotes and eukaryotes. It is a prospective enzyme for the (Ascorbate glutathione) ASH-GSH cycle and plays a vital role in defense system against ROS by sustaining the reduced status of (Glutathione) GSH (Romero-Puertas et al., 2006; Gill and Tuteja, 2010).

GR content increased under Cr stress in *Vigna radiata* roots (Shankar et al., 2004). In another study (Nehnevajova et al., 2012), the activity of GR was strongly increased in young seedlings exposed to heavy metals and the enzyme activities were particularly

pronounced in mutant lines of sunflower. Increased GR activity was noted (Thounaojan et al., 2012) in Cu treated *Oryza sativa* over control and, the roots showed greater activity than shoot. According to Yadav et al. (2010) chromium stress induced the production of the GR and Cr detoxification in *Jatropha curcus* plant.

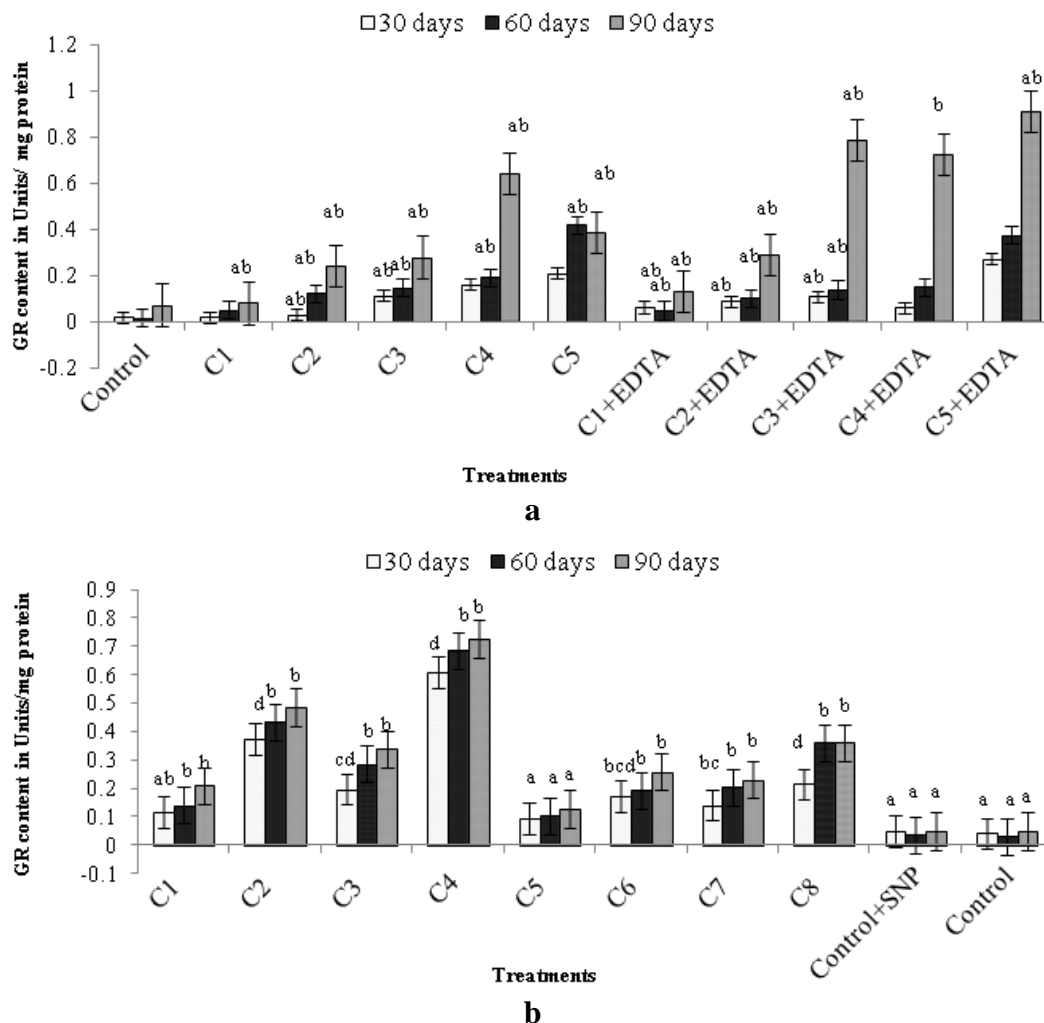


Figure 5. a Effect of different concentration of chromium with and without EDTA on glutathione reductase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Significance at $P \leq 0.05 = b$, significance at $P \leq 0.01$ and $P \leq 0.05 = ab$.) **b** Effect of chromium and EDTA in the presence of SNP on glutathione reductase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)

According to Diwan et al. (2012) APX and GR maintain the H_2O_2 level. The increased level of H_2O_2 when exported to cytosol, create an adverse effect due to formation of hydroxyl radicals through the metal-catalyzed Haber–Weiss reaction. In the present study the increased activities of APX and GR in higher concentrations in Cr treated plants in the presence of EDTA shows that they were functioning concurrently to remove H_2O_2 .

Glutathione reductase catalyzes the reduction of GSH, a molecule involved in many metabolic regulatory and anti oxidative processes in plants. GR catalyses the NADPH dependent reaction of disulphide bond of GSSG (oxidized glutathione). It is thus important for maintaining the GSH (reduced glutathione) pool (Reddy and Raghavendra, 2006). GR and GSH play crucial role in determining the tolerance of a plant under various stress (Chalapathi Rao and Reddy, 2008).

The GR activity with SNP treated plants are represented in *Figure 5b* showing the effect of chromium and EDTA in the presence of SNP on glutathione reductase activity (Units mg^{-1} protein) in *Sesbania grandiflora*. It depicts that there is an elevation of the scavenging enzyme in the treatments C3 and C4 in order to counter act Cr-induced stress in the presence of chelator compared to its absence in C1 and C2. According to Shigeoka et al. (2002) APX and GR are the two main enzymes of the ascorbate-glutathione cycle, which are localized mainly in chloroplasts and play an important role in combating oxidative stress. This is proved in the present study that in plants treated with Cr and EDTA, there is a considerable increase in the GR content.

Further comparing with other treatments, it is slightly reversed upon addition of SNP. The treatment plants C5 and C6 supplied with 250 μM of SNP in the presence of 60 and 100 mg kg^{-1} , the oxidative stress created by the metal is reduced. In treatments C7 and C8 despite the presence of EDTA and Cr, addition of SNP reduced the stress by showing reduction in the enzyme activity which reduced the stress by 33.3% (C3 and C7) and 50% (C4 and C8) respectively. In other words, SNP as a NO donor has an ameliorating effect on Cr and EDTA induced stress. Supporting facts by Singh et al. (2008) reported similar reduction in GR activity upon SNP treatment against Cd toxicity in wheat roots.

Effects of Cr, EDTA and SNP on ascorbic acid oxidase activity

The activities of Ascorbic acid oxidase determined in the leaves of *Sesbania grandiflora* are presented in *Figure 6a* showing the effect of different concentration of chromium with and without EDTA on ascorbic acid oxidase activity (units mg^{-1} protein) in *Sesbania grandiflora*. From the results it is clear that when the concentration of the chromium increases from 20-100 mg, there is an increase in the activity of the antioxidant enzyme ascorbic acid oxidase. Moreover when compared with the control, the activity of the enzyme increased by 89.4%, 115.7%, 105.2%, 363.1% and 410.5% in the presence of EDTA in C1+EDTA to C5+EDTA plants respectively corresponding to chromium concentration noted after 60 days of growth. When compared with C5 plants, C5+EDTA showed 94% increase in ascorbic acid oxidase activity. Statistical significant difference was noted in all the treatments at $P \leq 0.05$. After 30 days of growth, treatments C3+EDTA and C4+EDTA showed significant difference at both the levels $P \leq 0.05$ and $P \leq 0.01$. At 60 days of growth C2, C3, and C4 showed significant difference at $P \leq 0.05$ and $P \leq 0.01$. Similarly after 90 days, significant difference was exhibited by the treatments C1, C2 and C3. Thus the present study shows that EDTA had an influence on the ascorbic acid oxidase enzyme concentration determination in the presence of increasing concentrations of Cr.

Ascorbate is a universal soluble antioxidant that is involved in various physiological mechanisms of plant, which exists in photosynthetic organisms. The ascorbate is considered to be the most important substrate for decomposition of hydrogen peroxide H_2O_2 detoxification (Singh et al., 2005). High AO activity is associated with the cell wall ascorbate and cell wall localized ascorbate oxidase (AO) that function to control

growth and expanding cells and as a model that links wall ascorbate and ascorbate oxidase to cell wall extensibility (Smirnoff, 1996).

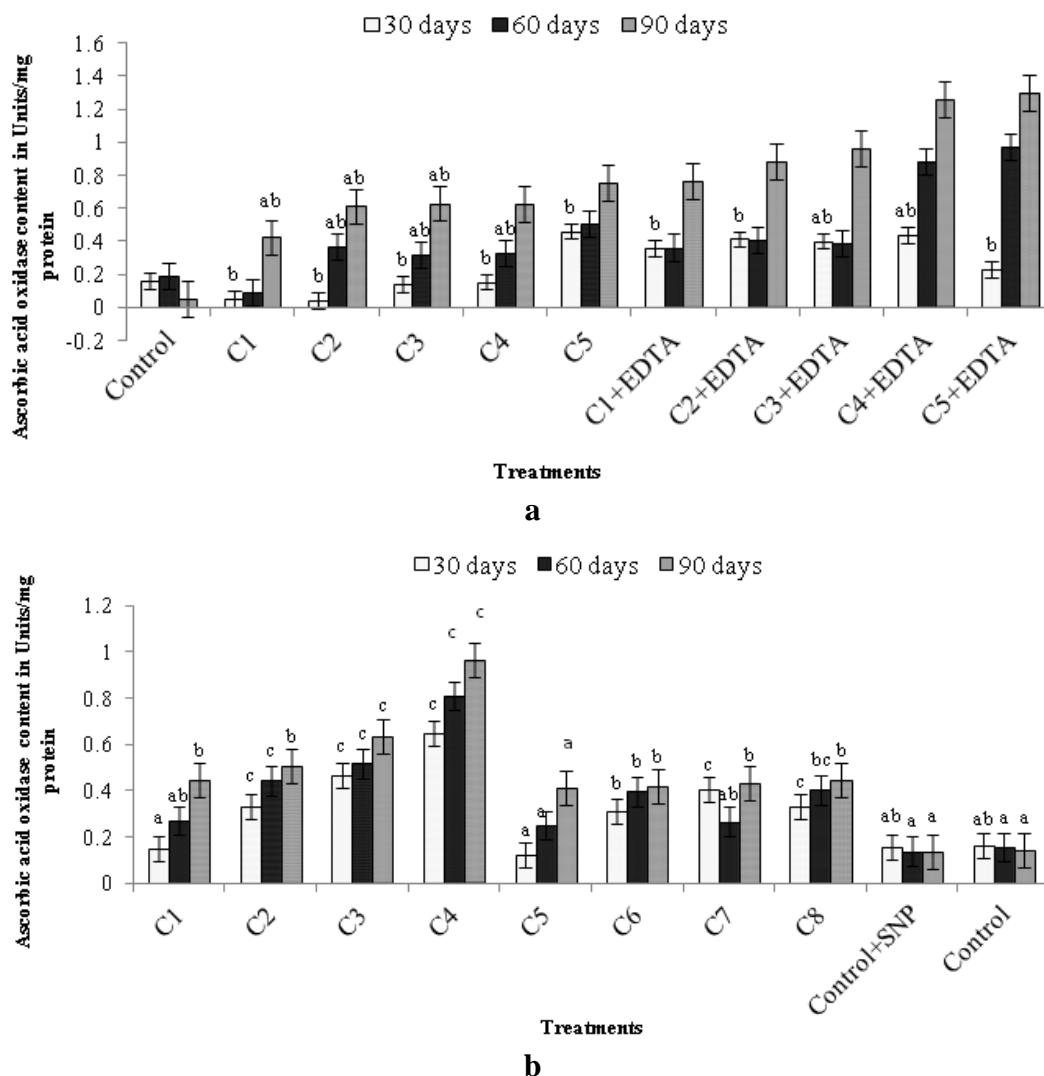


Figure 6. a Effect of different concentration of chromium with and without EDTA on ascorbic acid oxidase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Significance at $P \leq 0.05 = b$, significance at $P \leq 0.01$ and $P \leq 0.05 = ab$.) **b** Effect of chromium and EDTA in the presence of SNP on ascorbic acid oxidase activity (units mg^{-1} protein) in *Sesbania grandiflora*. (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)

Ascorbate is considered to be the primary as well as a secondary antioxidant, which binding to the metals, affects their movement across biological membranes, or act as a reductant. Reports suggested that, the ascorbate content in *Vigna aconitifolia* roots increased significantly under cadmium stressed conditions (Vijendra et al., 2016). Similarly the activity of ascorbate oxidase enzyme was determined for a period of three months for the plants treated with 60 and 100 mg of Cr with 0.35 mM EDTA and 250 μM SNP. The result represented in *Figure 6b* explains the effect of chromium and EDTA in the presence of SNP on ascorbic acid oxidase activity (Units mg^{-1} protein) in

Sesbania grandiflora. The figure articulates that, the enzyme activity increased from 30 to 90 days in treatments (C1-C4) in the presence of chromium and EDTA as a defensive mechanism for the plant to withstand the oxidative stress formed due to the reactive oxygen species (ROS) liberated due to high metal concentrations. But in the treatments (C5-C8) the result showed that, the presence of nitric oxide in the plants under the treatments showed decline in the enzyme levels, where the C8 plants showed a decreased percentage of 54.1% when compared with C4 plants in the absence of SNP, stating the fact that nitric oxide can counteract Cr and EDTA induced oxidative stress.

Such effects were confirmed by the other antioxidant enzymes studied and such studies reported that NO can detoxify or scavenge ROS directly or it can also react with $O_2^{\cdot-}$ and generate peroxynitrite ($ONOO^-$) (Martinez et al., 2000; Neill et al., 2003). Thus SNP protects plants against Cr+ EDTA induced oxidative stress. The SNP effect in the present study represents the antioxidant property of NO for suppression of high levels of Cr-triggered ROS.

Effects of Cr, EDTA and SNP on glutathione activity

Glutathione (GSH), a tri-peptide is most abundant low molecular weight thiol in all mitochondria-bearing eukaryotes including plants. In plants, GSH is involved in defence against ROS (Foyer and Noctor, 2005; Mullineaux and Rausch, 2005), sequestration of heavy metals (Cobbett and Goldsbrough, 2002) and is one of the major source of non-protein thiols in most plant cells (Freeman et al., 2004). The glutathione content in the chromium treated plants with and without EDTA in the present study represented in *Figure 7a* shows the effect of different concentration of chromium with and without EDTA on glutathione content ($\mu\text{g. FW}^{-1}$) in *Sesbania grandiflora*. When compared with the control the glutathione content increased with the increase in the concentration of Cr in the presence of EDTA. The highest glutathione content was recorded for the treatment with 100 mg of Cr + 0.35 mM EDTA with 280.63 μg .

With increase in time period from 30 to 90 days the glutathione content was found to be increased by 92.9% in C5+EDTA plant. All the treatments showed significant difference at both levels $P \leq 0.05$ and $P \leq 0.01$. But at 60 days of growth all the treatments showed significant difference at both levels $P \leq 0.05$ and $P \leq 0.01$ except C5 and C5+EDTA. This validates that, higher concentrations of Cr have influence on glutathione concentration in plant under heavy metal stress and this effect being more pronounced in the presence of EDTA. Thus at higher concentration of Cr/chelator the level of glutathione increased with time.

Furthermore, elevated GSH concentration is correlated with the ability of plants to withstand metal-induced oxidative stress. Enhanced antioxidant activity in the leaves and chloroplast of *Phragmites australis* was mainly associated with a large pool of GSH resulting in protection of many photosynthetic enzymes against the thiophilic bursting of Cd (Pietrini et al., 2003). Similarly, increased concentration of GSH has been reported in *Pisum sativum*, *Sedum alfredii* and *Vigna mungo* (Metwally et al., 2005; Sun et al., 2007; Molina et al., 2008).

Various non enzymatic antioxidants play an important role in plant defense mechanism under heavy metal stress. In *Oryza sativa* there was no observed significant change in the ascorbate (AsA) content in developing seedlings under Cr (VI) (Panda, 2007); however, the reduced glutathione (GSH) content increased by 30% and 52% at 50 mM and 100 mM of Cr (VI), respectively, after 48 h of exposure. Elevated expression of genes encoding these enzymes under Cr (VI) toxicity suggests defensive

role of glutathione under heavy metal stress according to Zulfiqar et al. (2011), these observations show the involvement of glutathione metabolism in the alleviation of Cr (VI) toxicity by providing protection against oxidative stress.

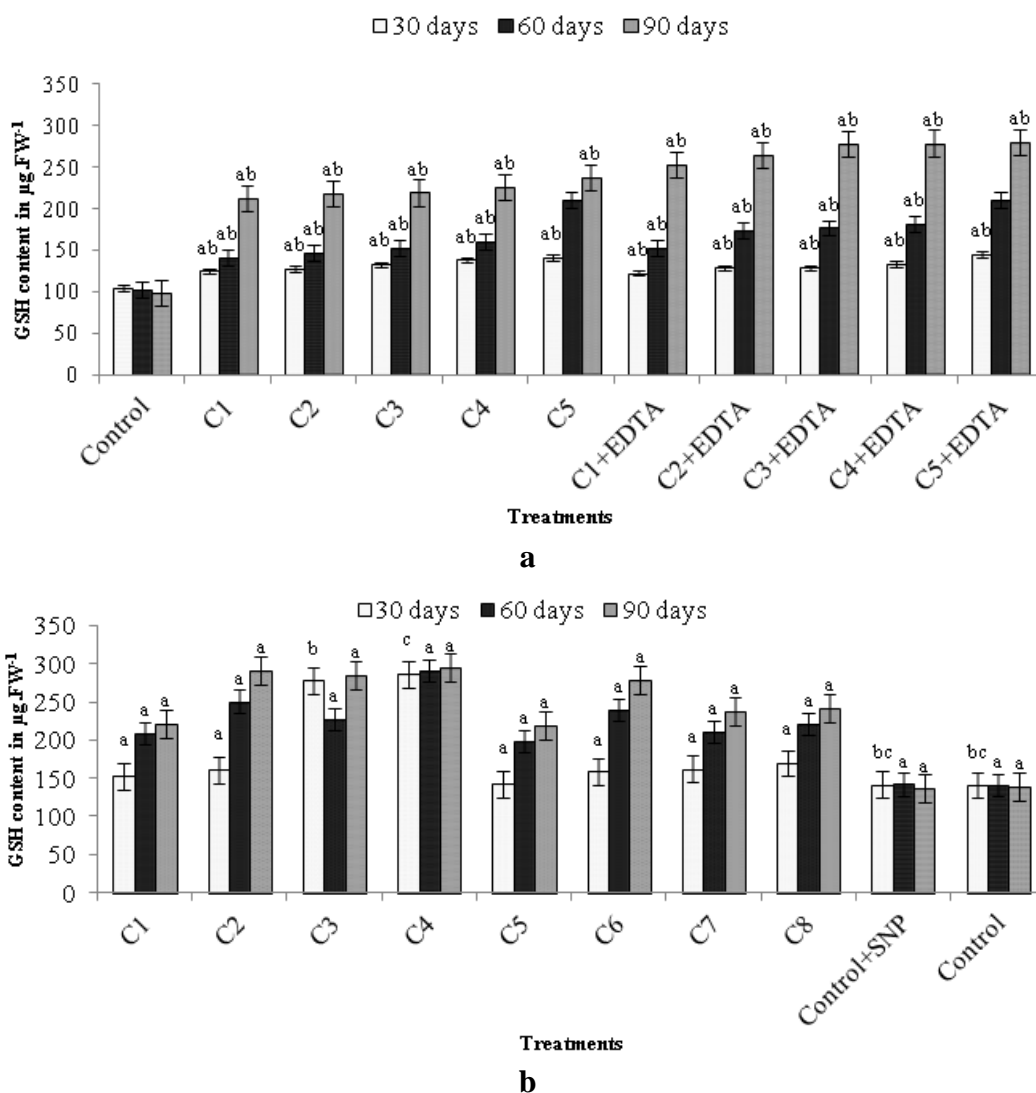


Figure 7. a Effect of different concentration of chromium with and without EDTA on glutathione content ($\mu\text{g.FW}^{-1}$) in *Sesbania grandiflora*. (Significance at $P \leq 0.05 = b$, significance at $P \leq 0.01$ and $P \leq 0.05 = ab$.) **b** Effect of chromium and EDTA in the presence of SNP on glutathione content ($\mu\text{g.FW}^{-1}$) in *Sesbania grandiflora*. (Lower case letters that are different denote the treatments which are significantly different. Same letters indicate the treatments which are not significantly different)

A marked increase in GSH levels in *Zea mays*, *Brassica spp.* and *L. esculentum* upon exposure to 5–10 mg l⁻¹ Cr (VI) (Sanita di Toppi et al., 2002). A positive correlation between GSH and Cr levels has also been observed in *Salvia natans* (Dhir et al., 2009) and *Brassica juncea* (Diwan et al., 2010).

GSH is one of the crucial metabolites in plants which are considered as most important intracellular defense against ROS induced oxidative damage. GSH is necessary to maintain the normal reduced state of cells so as to counteract the inhibitory

effects of ROS induced oxidative stress. It is a potential scavenger of $^1\text{O}_2$, H_2O_2 and most dangerous ROS like OH^\cdot .

The observations made on the glutathione content with SNP treated plants represented in *Figure 7b* show the effect of chromium and EDTA in the presence of SNP on glutathione content ($\mu\text{g. FW}^{-1}$) in *Sesbania grandiflora* depicts that increased glutathione content with chromium and EDTA treated plants (C1-C4) was reduced in the presence of SNP. From the figure, it shows that treatments, C5 to C8 exhibited reduced glutathione content when compared with control and other treatment plants.

A comparison between the treatments in presence and absence of SNP showed that, presence of SNP in C7 and C8 plants showed a decreased percentage of glutathione content with 16.5% and 17.9%, with that of their respective counterparts C3 and C4 plant.

The reports indicate that the heat-treated *Triticum aestivum* plants showed a reduced GSSG content compared to heat treated plants, due to the SNP supplement (Hasanuzzaman et al., 2012). Literatures also suggest that during stress conditions the glutathione levels inside the cell increases as a consequence to confer resistant against oxidative stress created by heavy metal (Hasanuzzaman et al., 2017). This was paralleled with the present study that the GSH content was found to increase with higher concentrations of chromium in the presence of EDTA. But Exp.2 concluded that, though there was increase in GSH content in chromium and EDTA treated plants, the SNP supplemented plants showed a decreased GSH content than the normal.

This could be justified that the oxidative stress generated by metal and chelator was mitigated by the action of SNP, there by manifesting the key role of nitric oxide in acting as a direct scavenger of ROS and thus reduced the oxidative stress in *Sesbania grandiflora* subjected to metal stress induced by chelator. The reduced GSH levels in the present study could be attributed to the fact that, the reduced stress levels has limited the accumulation of GSH inside the plant due to NO activity.

Conclusion

The present study concludes that exogenous nitric oxide supply ameliorates the Cr-toxicity in the presence of chelator EDTA. Nitric oxide also helped in ameliorating oxidative stress by decreasing the antioxidant levels in response to increasing metal and chelator concentration. Hence SNP when supplemented to heavy metal accumulating plants will reduce the stress levels in plants. Further studies are required to elucidate the changes in molecular levels, to identify the isoenzymes or proteins that are involved in mediating the ameliorating action of NO.

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REFERENCES

- [1] Alloway, B. J. (1995): Soil Processes and the Behavior of Metals. – In: Alloway, B. J. (ed.) Heavy Metals in Soils. Blackie and Sons Limited, Glasgow, pp. 1-52.
- [2] Azmat, R., Khanum, R. (2005a): Effect of chromium on uptakes of minerals in bean plant. – Pak J Biolo Sci 8: 281-283.

- [3] Baek, K. H., Skinner, D. Z. (2003): Alteration of antioxidant enzyme gene expression during cold acclimation of near-isogenic wheat lines. – *Plant Science* 165: 1221-1227.
- [4] Bakiyaraj, R., Mahakavi, T., Baskaran, L. (2014): Chromium toxicity in *Sesbania sesban* (L.) Merr. – *International Letters of Natural Sciences* 4: 65-75.
- [5] Beauchamp, C., Fridovich, I. (1971): Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. – *Anal Biochem* 44: 276-287.
- [6] Beyer, W. F., Fridovich, I. (1987): Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. – *Analytical Biochemistry* 161: 559-566.
- [7] Candan, N., Tarhan, L. (2003): The correlation between antioxidant enzyme activities and lipid peroxidation levels in *Mentha pulegium* organs grown in Ca²⁺, Mg²⁺, Cu²⁺, Zn²⁺ and Mn²⁺ stress conditions. – *Plant Science* 165: 769-776.
- [8] Carlos, G. M., Lorenzo, L. (2001): Nitric oxide induces stomatal closure and the enhances adaptive plant responses against drought stress. – *Plant Physiology* 126: 1196-1204.
- [9] Caro, A., Puntarulo, S. (1998): Nitric oxide decreases superoxide anion generation by microsomes from soybean embryonic axes. – *Physiologia Plantarum* 104: 357-364.
- [10] Chalapathi Rao, A. S. V., Reddy, A. R. (2008): Glutathione Reductase: A Putative Redox Regulatory System in Plant Cells. – In: Khan, N. A., Singh, S., Umar, S. (eds.) *Sulfur Assimilation and Abiotic Stresses in Plants*. Springer, Netherlands, pp. 111-147.
- [11] Cobbett, C. S. (2000): Phytochelatins and their roles in heavy metal detoxification. – *Plant Physiology* 123: 825-832.
- [12] Cobbett, C., Goldsbrough, P. (2002): Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. – *Annual Review of Plant Biology* 53: 159-182.
- [13] Dalurzo, H. C., Sandalio, L. M., Gómez, M., Del Río, L. A. (1996): Effect of Cadmium on the Growth and Oxidative Metabolism of Pea Plants. – In: Puig-Parellada, P., Mitjavila, M. T. (eds.) *VII Biennial Meeting of the International Society for Free Radical Research. Abstracts Book*. University of Barcelona, Barcelona.
- [14] Delledonne, M., Xia, Y., Dixon, R. A., Lamb, C. (1998): Nitric oxide functions as a signal in plant disease resistance. – *Nature* 394: 585-588.
- [15] Dhir, B., Sharmila, P., Saradhi, P. P., Nasim, S. A. (2009): Physiological and antioxidant responses of *Salvinia natans* exposed to chromium-rich wastewater. – *Ecotoxicology and Environmental Safety* 72: 1790-1797.
- [16] Diels, L., Vander Lelie, N., Bastiaens, L. (2002): New development in treatment of heavy metal contaminated soils. – *Reviews in Environmental Science and Biotechnology* 1: 75-82.
- [17] Diwan, H., Ahmad, A., Iqbal, M. (2008): Genotypic variation in phytoremediation potential of Indian mustard for chromium. – *Environmental Management* 41: 734-741.
- [18] Diwan, H., Ahmad, A., Iqbal, M. (2009): Chromium-induced modulation in the antioxidant defense system during phenological growth stages of Indian mustard. – *International Journal of Phytoremediation* 12: 142-158.
- [19] Diwan, H., Khan, I., Ahmad, A., Iqbal, M. (2010): Induction of phytochelatins and antioxidant defence system in *Brassica juncea* and *Vigna radiata* in response to chromium treatments. – *Plant Growth Regulation* 61: 97-107.
- [20] Diwan, H., Ahmad, A., Iqbal, M. (2012): Chromium-induced alterations in photosynthesis and associated attributes in Indian mustard. – *Journal of Environmental Biology* 33: 239-244.
- [21] Dong, Y., Xu, L., Wang, Q., Fan, Z., Kong, J., Bai, X. (2014): Effects of exogenous nitric oxide on photosynthesis, antioxidative ability, and mineral element contents of perennial ryegrass under copper stress. – *Journal of Plant Interactions* 9: 402-411.
- [22] Ferreira, L. C., Cataneo, A. C., Remaeh, L. M. R., Corniani, N., Fumis, T. D. F., Souza, Y. A. D., Scavroni, J., Soares, B. J. A. (2010): Nitric oxide reduces oxidative stress

- generated by lactofen in soybean plants. – Pesticide Biochemistry and Physiology 97: 47-54.
- [23] Foyer, C. H., Noctor, G. (2005): Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. – Plant Cell 17: 1866-1875.
- [24] Garba, S. T., Sunday, A., Manji, H., Tsaware, J (2012b): Ethylene diaminetetra acetate (EDTA)-assisted phytoremediation of heavy metal contaminated soil by *Eleusine indica* L. Gearth. – Journal of Environmental Chemistry and Ecotoxicology 4: 103-109.
- [25] Gill, S. S., Tuteja, N. (2010): Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. – Plant Physiol Biochem 48: 909-930.
- [26] Hasanuzzaman, M., Nahar, K., Alam, M. M., Fujita, M. (2012): Exogenous nitric oxide alleviates high temperature induced oxidative stress in wheat (*Triticum aestivum* L.) seedlings by modulating the antioxidant defense and glyoxalase system. – Australian Journal of Crop Science 6: 1314-1323.
- [27] Hasanuzzaman, M., Nahar, K., Anee, T. I., Fujita, M. (2017): Glutathione in plants: biosynthesis and physiological role in environmental stress tolerance – Physiol Mol Biol Plants 23(2): 249-268.
- [28] Hiner, A. N. P., Rodríguez-López, J. N., Arnao, M. B., Raven, E. L., García-Cánovas, F., Acosta, M. (2000): Kinetic study of the inactivation of ascorbate peroxidase by hydrogen peroxide. – Biochem J 348: 321-328.
- [29] Hung, K. T., Kao, C. H. (2003): Nitric oxide counteracts the senescence of rice leaves induced by abscisic acid. – Journal of Plant Physiology 160: 871-879.
- [30] Israr, M., Sahi, S. V., Jain, J. (2006): Cadmium accumulation and antioxidative responses in the *Sesbania drummondii* allus. – Arch Environ Cont Toxicol 50: 121-127.
- [31] Jadia, C. D., Fulekar, M. K. (2009): Phytoremediation of heavy metals: Recent techniques. – African Journal of Biotechnology 8: 921-928.
- [32] Karuppanapandian, T., Sinha, P. B., Haniya, A. M. K., Mamoharan, K. (2006a): Differential antioxidative responses of ascorbate-glutathione cycle enzymes and metabolites to chromium stress in green gram (*Vigna radiata* L. Wilczek) leaves. – Journal of Plant Biology 49: 440-447.
- [33] Kaur, G., Singh, H. P., Batish, D. R., Mahajan, P., Kohli, R. K., Rishi, V. (2015): Exogenous nitric oxide (NO) interferes with lead (Pb)-induced toxicity by Detoxifying reactive oxygen species in hydroponically grown wheat (*Triticum aestivum*) roots. – PloS One 10: 1-18.
- [34] Laspina, N. V., Groppa, M. D., Tomaro, M. L., Benavides, M. P. (2005): Nitric oxide protects sunflower leaves against Cd-induced oxidative stress. – Plant Science 169: 323-330.
- [35] Lestan, D., Luo, C. L., Li, X, D. (2008): The use of chelating agents in the remediation of metal contaminated soils: a review. – Environmental Pollution 153: 3-13.
- [36] Luck, H. (1974): Catalase. – In: Begmeyer, J., Garbi, M. (eds.) Methods of Enzymatic Analysis. Vol II. Academic Press, New York, pp.895-897.
- [37] Luna, C. M., Gonzalez, C. A., Trippi, V. S. (1994): Oxidative damage caused by an excess of copper in oat leaves. – Plant Cell Physiology 35: 11-15.
- [38] Martinez, G. R., Di Mascio, P., Bonini, M. G., Augusto, O., Briviba, K., Sies, H., Maurer, P., Rothlisberger, U., Herold, S., Koppenol, W. H. (2000): Peroxynitrite does not decompose to singlet oxygen ($^1\text{O}_2$) and nitroxyl (NO^-). – Proceedings of the National Academy of Sciences 97: 10307-10312.
- [39] Mates, J. M. (2000): Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. – Toxicology 153: 83-104.
- [40] Maiti, S., Ghosh, N., Mandal, C., Das, K., Dey, N., Adak, M. K. (2012): Responses of the maize plant to chromium stress with reference to antioxidation activity. – Brazilian Journal of Plant Physiology 24: 203-212.

- [41] Metwally, A., Safronova, A. B., Dietz, K. J. (2005): Genotypic variation of the response to cadmium toxicity in *Pisum sativum* L. – *J Exp Bot* 56: 167-178.
- [42] Mittler, R. (2002): Oxidative stress, antioxidants and stress tolerance. – *Trends Plant Science* 7: 405-410.
- [43] Mittova, V., Theodoulou, F. L., Kiddle, G., Gomez, L., Volokita, M., Tal, M., Foyer, C. M., Guy, M. (2003): Coordinate induction of glutathione biosynthesis and glutathione metabolizing enzymes is correlated with salt tolerance in tomato. – *FEBS Letter* 554: 417-421.
- [44] Molina, A. S., Nievas, C., Chaca, M. V. P., Garibotto, F., Gonza'lez, U., Marsa, S. M., Luna, C., Gime'nez, M. S., Zirulnik, F. (2008): Cadmium-induced oxidative damage and antioxidative defense mechanisms in *Vigna mungo* L. – *Plant Growth Regul* 56: 285-295.
- [45] Morita, S., Kaminaka, H., Masumura, T., Tanaka, K. (1999): Induction of rice cytosolic ascorbate peroxidase mRNA by oxidative stress; the involvement of hydrogen peroxide in oxidative stress signalling. – *Plant and Cell Physiology* 40: 417-422.
- [46] Mullineaux, P., Rausch, T. (2005): Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. – *Photosynthesis Research* 86: 459-474.
- [47] Nakano, Y., Asada, K. (1981): Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. – *Plant and Cell Physiology* 22: 867-880.
- [48] Nehnevajova, E., Lyubenova, L., Herzig, R., Schröder, P., Schwitzgubel, J. P., Schmulling, T. (2012): Metal accumulation and response of antioxidant enzymes in seedlings and adult sunflower mutants with improved metal removal traits on a metal contaminated soil. – *Environmental and Experimental Botany* 76: 39-48.
- [49] Neill, S. J., Desikan, R., Hancock, J. T. (2003): Nitric oxide signalling in plants. – *New Phytologist* 159: 11-35.
- [50] Oberbacher, M. F., Vines, H. M. (1963): Spectrophotometric assay of ascorbic acid Oxidase. – *Nature* 197: 1203-1204.
- [51] Panda, S. K. (2007): Chromium-mediated oxidative stress and ultrastructural changes in root cells of developing rice seedlings. – *Journal of Plant Physiology* 164: 1419-1428.
- [52] Pietrini, F., Iannelli, M. A., Pasqualini, S., Massacci, A. (2003): Interaction of cadmium with glutathione and photosynthesis in developing leaves and chloroplasts of *Phragmites australis* (Cav.) Trin. ex Steudel. – *Plant Physiology* 133: 829-837.
- [53] Quartacci, M. F., Argilla, A., Baker, A. J. M., Navari-Izzo, F. (2006): Phytoextraction of metals from a multiply contaminated soil by Indian mustard. – *Chemosphere* 63: 918-925.
- [54] Qureshi, M. I., Israr, M., Abdin, M. Z., Iqbal, M. (2005): Responses of *Artemisia annua* L. to lead and salt-induced oxidative stress. – *Environ Exp Bot* 53: 185-193.
- [55] Qureshi, M. I., Abdin, M. Z., Qadir, S., Iqbal, M. (2007): Lead-induced oxidative stress and metabolic alterations in *Cassia angustifolia* Vahl. – *Biologia Plantarum* 51: 121-128.
- [56] Rao, M. V., Paliyath, G., Ormrod, D. P. (1996): Ultraviolet-B-and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. – *Plant Physiology* 110: 125-136.
- [57] Rai, V., Vajpayee, P., Singh, S. N., Mehrotra, S. (2004): Effect of chromium accumulation on photosynthetic pigments, oxidative stress defense system, nitrate reduction, proline level and eugenol content of *Ocimum tenuiflorum* L. – *Plant Science* 167: 1159-1169.
- [58] Rai, R. K., Srivastava, M. K., Khare, A. K., Kishor, R., Shrivastava, A. K. (2006): Oxidative stress response and glutathione linked enzymes in relation to growth of sugarcane plants exposed to hexavalent chromium. – *Sugar Tech* 8: 116-123.
- [59] Reddy, A. R., Raghavendra, A. S. (2006): Photo Oxidative Stress. – In: Madhava Rao, K. V., Raghavendra, A. S., Reddy, K. J. (ed.) *Physiology and Molecular Biology of Stress Tolerance in Plants*. Springer, Netherlands, pp. 157-186.

- [60] Romero-Puertas, M. C., Corpas, F. J., Sandalio, L. M., Leterrier, M., Rodriguez-Serrano, M., del Rio, L. A., Palma, J. M. (2006): Glutathione Reductase from pea leaves: response to abiotic stress and characterization of the peroxisomal isozyme. – *New Phytol* 170: 43-52.
- [61] Sanita di Toppi, L., Fossati, F., Musetti, R., Mikerezi, I., Favali, M. A. (2002): Effect of hexavalent chromium on maize, tomato, and cauli-flower plants. – *J Plant Nutr* 25: 701-717.
- [62] Schmidt, U. (2003): Enhancing phytoextraction: the effect of chemical soil manipulation on mobility, plant accumulation, and leaching of heavy metals. – *J Environ Qual* 32: 1939-1954.
- [63] Shanker, A. K., Djanaguiraman, M., Sudhagar, R., Chandrashekar, C. N. and Pathmanabhan, G. (2004): Differential antioxidative response of ascorbate glutathione pathway enzymes and metabolites to chromium speciation stress in green gram (*Vigna radiata* (L.) R. Wilczek. cv CO₄) roots. – *Plant Science* 166: 1035-1043.
- [64] Shanker, A. K., Cervantes, C., Loza-Tavera, H., Avudainayagam, S. (2005): Chromium toxicity in plants. – *Environment International* 31: 739-753.
- [65] Sharma, D. C., Sharma, C. P. (1996): Chromium uptake and toxicity effects on growth and metabolic activities in wheat, *Triticum aestivum* L. cv. UP 2003. – *Indian Journal of Experimental Biology* 34: 689-691.
- [66] Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y., Yoshimura, K. (2002): Regulation and function of ascorbate peroxidase isoenzymes. – *Journal of Experimental Botany* 53: 1305-1319.
- [67] Sinam, G., Sinha, S., Mallick, S. (2011): Effect of chromium on accumulation and antioxidants in *Cucumis utilissimus* L. Response under enhanced bioavailability condition. – *Journal of Environmental Sciences* 23: 506-512.
- [68] Singh, A., Agrawal, S. B., Rathore, D. (2005): Amelioration of Indian urban air pollution phytotoxicity in *Beta vulgaris* L. by modifying NPK nutrients. – *Environmental Pollution* 134: 385-395.
- [69] Singh, H. P., Batish, D. R., Kaur, G., Arora, K., Kohli, R. K. (2008): Nitric oxide (as sodium nitroprusside) supplementation ameliorates Cd toxicity in hydroponically grown wheat roots. – *Environmental and Experimental Botany* 63: 158-167.
- [70] Smirnoff, N. (1996): The Function and Metabolism of Ascorbic Acid in Plants. – *Annals of Botany* 78: 661-669.
- [71] Smirnoff, N. (2000): Ascorbic acid: metabolism and functions of a multi-faceted molecule. – *current Opinion in Plant Biology* 3: 229-235.
- [72] Sun, Q., Ye, Z. H., Wang, X. R., Wong, M. H. (2007): Cadmium hyperaccumulation leads to an increase of glutathione rather than phytochelatins in the cadmium hyperaccumulator *Sedum alfredii*. – *Journal of Plant Physiology* 164: 1489-1498.
- [73] Talukdar, D. (2012b): Ascorbate deficient semi-dwarf asfL1 mutant of *Lathyrus sativus* exhibits alterations in antioxidant defense. – *Biologia Plantarum* 56: 675-682.
- [74] Thounaojam, T. C., Panda, P., Mazumdar, P., Kumar, D., Sharma, G. D., Sahoo, L. Sanjib, P. (2012): Excess copper induced oxidative stress and response of antioxidants in rice. – *Plant Physiology and Biochemistry* 53: 33-39.
- [75] Tukendorf, A., Rauser, W. E. (1990): Changes in glutathione and phytochelatins in roots of maize seedlings exposed to cadmium. – *Plant Sci* 70: 155-166.
- [76] Uchida, A., Jagendorf, A. T., Hibino, T., Takabe, T., Takabe, T. (2002): Effects of hydrogen peroxide and nitric oxide on both salt and heat stress tolerance in rice. – *Plant Science* 163: 515-523.
- [77] Vijendra, P. D., Huchappa, K. M., Lingappa, R., Basappa, G., Jayanna, S. G., Kumar, V. (2016): Physiological and biochemical changes in moth bean (*Vigna aconitifolia* L.) under cadmium stress. – *Journal of Botany* 2016: 1-13.

- [78] Yadav, S. K., Dhote, M., Kumar, P., Sharma, J., Chakrabarti, T., Juwarkar, A. A. (2010): Differential antioxidative enzyme responses of *Jatropha curcas* L. to chromium stress. – *Journal of Hazardous Materials* 180: 609-615.
- [79] Zulfiqar, A., Paulose, B., Chhikara, S., Dhankher, O. P. (2011): Identifying genes and gene networks involved in chromium metabolism and detoxification in *Crambe abyssinica*. – *Environmental Pollution* 159: 3123-3128.