RESEARCH ARTICLE

Ameliorative effect of exogenous nitric oxide on oxidative metabolism in NaCl treated chickpea plants

Sunita Sheokand[1*], Vinita Bhankar[2] and Veena Sawhney[2]

[1] Department of Botany & Plant Physiology, CCS Haryana Agricultural University, Hisar 125004, India. sunita_sheokand@hotmail.com

[2] Department of Biochemistry, CCS Haryana Agricultural University, Hisar 125004, India

* Corresponding author:
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ABSTRACT

Accumulation of salts in irrigated soils is one of the primary factors limiting yield in South Asia. We investigated whether exogenous nitric oxide (NO) supplementation as sodium nitroprusside has any ameliorating effect against NaCl induced oxidative damage in chickpea leaves. NaCl treatment (250 mM) alone and in combination with two concentrations of SNP (0.2 and 1 mM) were given to 50 days old chickpea plants for 2, 4 and 6 days. Salt stress adversely affected the relative membrane injury, lipid peroxidation levels, relative water content (RWC) and H_2O_2 content. The effect was time dependent. SNP treatments could ameliorate the toxic effect of short term salt stress of 2 days on relative membrane injury and partial amelioration was observed with 4 and 6 day stress treatment. A partial ameliorative effect of SNP was observed with lipid peroxidation levels, H_2O_2 content and RWC. Salt stress activated the antioxidant system by increasing the activities of SOD, POX, APX and DHAR. However no obvious change was observed in GR activity and CAT activity decreased under salt stress. Both the SNP treatments had a positive effect on antioxidant enzymes SOD, CAT, APX, GR and DHAR under salt stress. NaCl treatment resulted in a decline in the GSH/GSSG and ASC/DHA ratio. SNP treatments increased the reduced form of both the metabolites thus elevating the ratio of GSH/GSSG and ASC/DHA. This study concludes that exogenous application of NO protects chickpea leaves from NaCl induced oxidative stress.

Key words: antioxidant enzymes; chickpea; lipid peroxidation; nitric oxide; salt stress

Abbreviations: *ASC, ascorbate; APX, ascorbate peroxidase; CAT, catalase; DHA, dehydro ascorbate; DHAR, dehydro ascorbate reductase; GR, glutathione reductase; H_2O_2, hydrogen peroxide; NO, nitric oxide; POX, peroxidase; SNP, sodium nitroprusside; SOD, superoxide dismutase*

INTRODUCTION

Nitric oxide is a bioactive, lipophillic free radical gas with well characterized signaling roles in animals (Furchgott 1995). In contrast the role of NO in plants is not so well understood, though there have been many reports on the role of various processes such as growth and development, respiratory metabolism, senescence and maturation as well as response to biotic and abiotic stresses in plants (Kopyra and Gwozdz 2004). Nitric oxide appears to be present in most of the stress reactions (Gould et al. 2003). It has been reported to exert a protective effect in response to drought stress (Wang et al. 2004; Zhao et al. 2008) salt stress (Kopyra and Gwozdz 2003; Chen et al. 2004; Zhao et al. 2004; Shi et al. 2007; Li et al. 2008) heavy metal stress (Kopyra and Gwozdz 2003; Hsu and

Kao 2005; Wang and Yang 2005; Singh et al. 2008) and UV radiation stress (Shi et al. 2005).

Among the abiotic stresses salinity is one of the major stresses in arid and semi arid regions that can severely limit plant productivity (Pitman and LaEuchli 2002). Salt stress like other abiotic stresses leads to oxidative stress through the increase in reactive oxygen species such as superoxide radical, hydrogen peroxide and hydroxyl radicals. These species are highly reactive and cause cellular damage through oxidation of lipids, proteins and nucleic acids (Foyer and Noctor 2000). The antioxidant system is composed of ROS scavenging enzymes such as SOD, CAT, POX, APX, GR, DHAR, MDHAR and metabolites like ascorbate and glutathione (Noctor and Foyer 1998). Many authors have correlated changes in antioxidative system and redox state under salt stress with plant tolerance to salinity (Meneguzzo et al. 1999). The balance between free radical generation and free radical defense determines the survival of the system under stress conditions. Increasing the concentration of free radical scavengers by exogenous application could help in the detoxification of stress induced free radical production. Nitric oxide is a highly reactive molecule and being a free radical allows it to scavenge other reactive intermediates. Nitric oxide has been reported to alleviate the oxidative stress in plants generated by abiotic stresses (Beligni and Lamattina 1999; Hung et al. 2002; Kopyra and Gwozdz 2003; Neill et al. 2003; Kopyra and Gwozdz 2004; Crawford and Guo 2005).

The present investigations were conducted to assess the degree of oxidative stress evoked by NaCl treatments in chickpea plants and whether an additional supply of free radical scavenger could ameliorate the toxic effects of NaCl stress on oxidative metabolism.

MATERIAL AND METHODS

Plant material and treatments: Chickpea (*Cicer arietinm* L. cv HC-3) seeds procured from the Pulses Section, Department of Plant Breeding, CCS Haryana Agricultural University, Hisar, were surface-sterilized with 0.2% $HgCl_2$ and inoculated with a specific *Rhizobium* culture supplied by the Department of Microbiology, CCS Haryana Agricultural University, Hisar. The plants were raised in earthen pots containing 5 Kg of sand under screen house conditions. The plants were supplied with nitrogen free nutrient solution (Wilson and Reisenauer, 1963) at regular intervals. Fifty days after sowing (DAS) the plants were divided into six groups. The first group, irrigated with water alone, served as control. The second and third groups were treated with 0.2 mM and 1 mM SNP (NO donor) respectively as foliar spray. The fourth group was treated with 250 mM NaCl through the rooting medium. The fifth and sixth groups were treated with 250 mM NaCl through the rooting medium and 0.2 mM and 1 mM SNP respectively as foliar spray. The plants were sampled 2, 4 and 6 DAT (days after treatment).

Relative water content (RWC): The first fully expanded leaves of control and treated plants were collected at 10 am and weighed (Wf). The leaves were submerged in water for four hours. They were blotted dry and their saturated weight (Wt) was measured. Leaves were oven dried at 80°C and their dry weights taken (Wd). RWC was calculated using the equation given below and expressed as percentage (Weatherly and Barhs 1962).

$$RWC\ (\%) = (Wf - Wd / Wt - Wd) \times 100.$$

Relative membrane injury (RI): Relative membrane injury was analyzed according to the method of Zhang et al. (2006). Two hundred and fifty mg of fully expanded leaves were rinsed with distilled water and immersed in 10 mL de-ionised water in vials and incubated at 25°C for 4 h. Electrical conductivity (EC) of the bathing medium was measured at 25°C (Xi). The tissue along with leachate was then boiled at 100°C for 30 min to completely disrupt the cell structure. The solution was brought to 25°C and its EC was measured again (Xt). Relative injury was calculated from the equation [(Xi/Xt) $\times$ 100].

Estimation of lipid peroxidation: The levels of lipid peroxidation in the tissues of control and treated plants were quantified by the determination of their MDA content, a breakdown product of lipid peroxidation. MDA content was determined with thiobarbituric acid (TBA) reaction. Leaf tissue (0.3 g) was extracted in 5 mL of 0.1% (w/v) TCA. After centrifugation, 1 mL aliquot of the supernatant was mixed with 20% (w/v) TCA containing 0.5% (w/v) TBA. The mixture was heated at 95°C for 30 min and cooled on ice. After centrifugation at 10,000 r.p.m. for 10 min the absorbance of the supernatant was measured at 532 nm. Correction of non-specific absorbance was made by subtracting the value taken at 600 nm from it. The level of lipid peroxidation is expressed

as mmol of MDA formed using an extinction coefficient of 155 mM cm^{-1} (Heath and Packer 1968).

Preparation of extracts for metabolites: One gm of the plant tissue was ground in 6 mL of chilled 0.8 N $HClO_4$ and centrifuged at 10000 rpm for 30 min. The clear supernatant was decanted and neutralized with 5 M K_2CO_3. It was again centrifuged at 10000 rpm for 30 min. The clear supernatant thus obtained was carefully decanted and the corresponding volume of each preparation was recorded. This supernatant was used for estimation of H_2O_2 and antioxidant metabolites.

Hydrogen peroxide content: Hydrogen peroxide was estimated by the method of Sinha (1972). 200 μL of extract was made to 1 mL with 0.1 M phosphate buffer (pH 7.5). 2 mL of 5% potassium dichromate and glacial acetic acid (1:3 v/v), was added to it. The mixture was then heated in water bath for 10 min and cooled. Its absorbance was read at 570 nm against reagent blank which was without sample extract. The quantity of H_2O_2 was determined from standard curve prepared similarly with 0-100 nmol H_2O_2.

Determination of enzyme activity: Cell free extract for various antioxidant enzymes viz., superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX) glutathione reductase (GR) and dehydroascorbate reductase (DHAR) was prepared by macerating leaf tissue in chilled mortar and pestle with 0.1 M potassium phosphate buffer (pH 7.0), containing 1 mM EDTA, 1% (w/v) PVP and 10% (v/v) glycerol. For the extraction of APX, the extraction media was supplemented with 1 mM ascorbic acid while EDTA was omitted from it (Dalmia and Sawhney 2004). APX (EC 1.11.1.11) activity was assayed according to the procedure of Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 0.1 mM H_2O_2 and 0.5 mM ascorbate. Reduction in ascorbate concentration was recorded at 290 nm as described previously (Dalmia and Sawhney 2004). CAT (EC 1.11.1.6) activity was estimated according to the procedure of Aebi (1984). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 20 mM H_2O_2 and 50 μL enzyme extract. The decrease in H_2O_2 content was monitored by reading the absorbance at 240 nm for 2 min. SOD (EC 1.15.1.1) activity was measured by the method of Bonnett *et al*. (2000). The reaction mixture contained 25 mM Tris-HCl (pH 8.3), 6.2 μM phenazine methosulphate, 30 μM nitroblue tetrazolium (NBT), 52 μM NADH and the enzyme extract. The reaction was started by the addition of NADH and after incubation at 30°C for 90 s it was terminated by the addition of glacial acetic acid. The reaction without NADH gave maximum reduction of NBT. One unit of enzyme activity represented the amount of enzyme required for 50% inhibition of NBT reduction at 560 nm. POX (EC 1.11.1.7) activity was determined by monitoring reduction of o-dianisidine as described by Shannon et al. (1966). The reaction mixture comprised of 0.1 M potassium phosphate buffer (pH 7.8), 0.2% (w/v) o-dianisidine and the enzyme extract. The reaction was initiated by the addition of 0.1 mM H_2O_2. One unit of enzyme activity was defined as the change in one absorbance at 470 nm.

Glutathione reductase (EC 1.6.4.2) activity was determined by following the rate of NADPH oxidation as measured by the decrease in the absorbance at 340 nm. About 1.5 mL of the assay mixture contained: 100 mM phosphate buffer (pH 7.8), 2 mM EDTA, 50 μM NADPH, 0.5 mM GSSG and 20 μL of the cell free extract. The assay was initiated by the addition of NADPH at 25°C and the decrease in absorbance was read at 340 nm (Foyer and Halliwell, 1976). DHAR activity was determined by the method of De Tullio et al. (1998). The assay measured the formation of ASC at 265 nm (14 mM^{-1} cm^{-1}) in a reaction mixture containing 0.1 M Na-phosphate buffer (pH 6.2) and 2 mM GSH. The reaction was started by the addition of 1 mM DHA and the cell free extract. Changes in absorption at 265 nm were followed for 1 min. The rate of non-enzymatic DHA reduction was corrected by subtracting the values obtained in the absence of enzyme extract.

Antioxidant metabolites: Ascorbate and DHA were determined by the modified procedure of Law et al. (1983). To measure total ascorbate, the reaction mixture consisted of extract, 10 mM DTT, 0.5% NEM and phosphate buffer (pH 7.4). The rest of the steps were similar for both the estimations. 2% dinitrophenyl hydrazine in 9 N H_2SO_4 and a drop of 10% thiourea in 70% ethanol were added. The tubes were kept in boiling water bath for 15 min. After cooling 80% H_2SO_4 was added to the tubes at 0°C and mixed on a vortex shaker. Absorbance was read at 530 nm. A reference curve was prepared with (0-100 n moles) DHA.

Total (GSH+GSSG) and oxidized (GSSG) glutathione was estimated by the modified method of Griffith (1980). 1 mL of supernatant was neutralized with 360 μL of 1M triethanolamine while 40 μL of 2-vinyl pyridine was added

for the measurement of GSSG to derivatize GSH. The mixture was allowed to stand at room temp for 1 h. Further procedure was same for both total and oxidized glutathione. 1.5 mL of reaction mixture consisted of 125 mM phosphate buffer (pH 7.5) containing 6.3 mM EDTA, 0.5 units of GR enzyme, 0.3 mM NADPH, 6 mM DTNB and 100 μL of each of the above treated extracts. Change in absorbance at 412 nm was recorded for 4 min. A reference curve was prepared with 1-50 μ mol GSSG.

Statistical Analysis: Sample variability of n = 3 is given as standard error of the mean (SE). All assays were run in triplicates.

RESULTS

Chickpea plants were treated with 250 mM NaCl for 2, 4 and 6 days at the vegetative stage to assess the effect of increasing duration of stress on the indices of stress and antioxidant metabolism. The effect of exogenously supplied NO donor SNP (0.2 and 1 mM) was also studied in the control as well as salt treated plants to determine the effective SNP concentration for amelioration of NaCl induced oxidative stress.

Relative membrane injury and lipid peroxidation: Salt stress (NaCl) adversely affected the membrane integrity (Figure 1A and B). Chickpea plants treated with 250 mM NaCl for 2 days showed a 50% increase in relative membrane injury which further increased to 2.5 and 3 fold on increasing the duration of stress to 4 and 6 days respectively. Exogenous application of two concentrations of NO donor SNP (0.2 and 1 mM) resulted in almost complete amelioration of the deleterious effect of short duration NaCl stress of 2 days on membrane injury. However when the duration of stress was increased to 4 and 6 days, the amelioration of stress effect was only partial (Figure1B). The membrane injury could be attributed to increased lipid peroxidation levels. The MDA content increased by 45% with NaCl stress of 2 days. Increasing the duration of stress resulted in no further increase in MDA levels. Both the concentrations of SNP were effective in bringing down the lipid peroxidation levels by 75% (Figure 1A).

Hydrogen peroxide content: Hydrogen peroxide content increased with NaCl treatment (Figure 1C). Increasing the duration of the treatment further increased the H_2O_2 content. The lower SNP treatment of 0.2 mM was more effective with 2 days NaCl treatments whereas the higher SNP treatment was more effective with 4 and 6 days of NaCl treatments. Under normal conditions SNP treatments slightly increased the H_2O_2 content.

Relative water content: Sodium chloride treatment adversely affected the relative water content (RWC) (Figure 1D). The RWC decreased with increasing duration of stress. A 25% decline was observed 2 DAT which further increased to 35%, 6 DAT. Sodium nitroprusside treatments had a positive effect on RWC. The higher SNP treatment was more effective and resulted in more than 50% amelioration.

Antioxidative enzymes: An increase in SOD activity was observed with NaCl stress. Increasing the duration of stress further increased the SOD activity indicating an induction of the defense system (Figure 2A). SNP treatments had a positive effect in the control as well as NaCl treated plants. The effect of SNP increased as the duration was increased from 2 to 4 and 6 days. The H_2O_2 scavenging enzyme POX also showed an increased activity under salt treatment (Figure 2B). A 20% increase was observed 2 DAT which further increased to 25% 4 and 6 DAT. SNP treatments caused no further increase in activity under stress conditions. The other H_2O_2 scavenging enzyme, CAT, however decreased under salt stress conditions (Figure 2C). A 30% decline was observed 2 DAT which further decreased to 32%, 4 DAT and 42%, 6 DAT. SNP treatments could partially ameliorate the toxic effect of NaCl treatments on CAT activity. Under normal conditions also SNP treatments increased the CAT activity. The lower concentration of 0.2 mM SNP for 2 days was more effective in increasing the CAT activity in control.

The enzymes of the Halliwell and Asada cycle were also studied. APX (Figure 2D), DHAR (Figure 2E) and GR (Figure 2F) activity increased under stress conditions. A 11% increase in APX activity was recorded 2 DAT which further increased to 21% 4 DAT and to 31% 6 DAT. SNP treatments further increased the APX activity by 20% under salt stress conditions 2 DAT. An initial increase of 26% in DHAR activity was observed 2 DAT however 4 and 6 DAT the activity decreased and was observed to be 7 and 5% above control levels. SNP treatments resulted in a small further increase in DHAR activity. A small increase of 5% was observed in GR activity 2 DAT which further increased to 8%, 4 DAT. SNP treatments however further increased the GR activity. The lower SNP treatment of 0.2 mM was more effective and

further increased the GR activity by 31%, 2 DAT. No further increase was observed 4 and 6 DAT.

Ascorbate content, ASC/DHA ratio, glutathione content and GSH/GSSG ratio: The ratio of ASC/DHA decreased with salt stress treatments. The decline was time dependent and decreased further as the duration of stress was increased (Figure 3B). SNP treatments had a positive effect in control as well as salt treated plants on ASC redox ratio. In the salt stressed plants the ameliorative effect of SNP decreased with time. An increase in glutathione content was observed with 2 days of NaCl treatment (Figure 3C). No further increase was observed on increasing the duration of stress to 4 and 6 days. SNP treatments further increased the total glutathione content. The effect of NO donor increased with time. A significant increase in GSSG content was observed with salt stress. SNP treatments were effective in decreasing the GSSG content. The GSH content decreased with salt stress. Increasing the duration of stress had an adverse effect on GSH content. SNP treatments had a positive effect and an increase in GSH content was observed with increasing duration of SNP treatments. Under normal conditions also SNP treatments resulted in a small increase in total glutathione content.

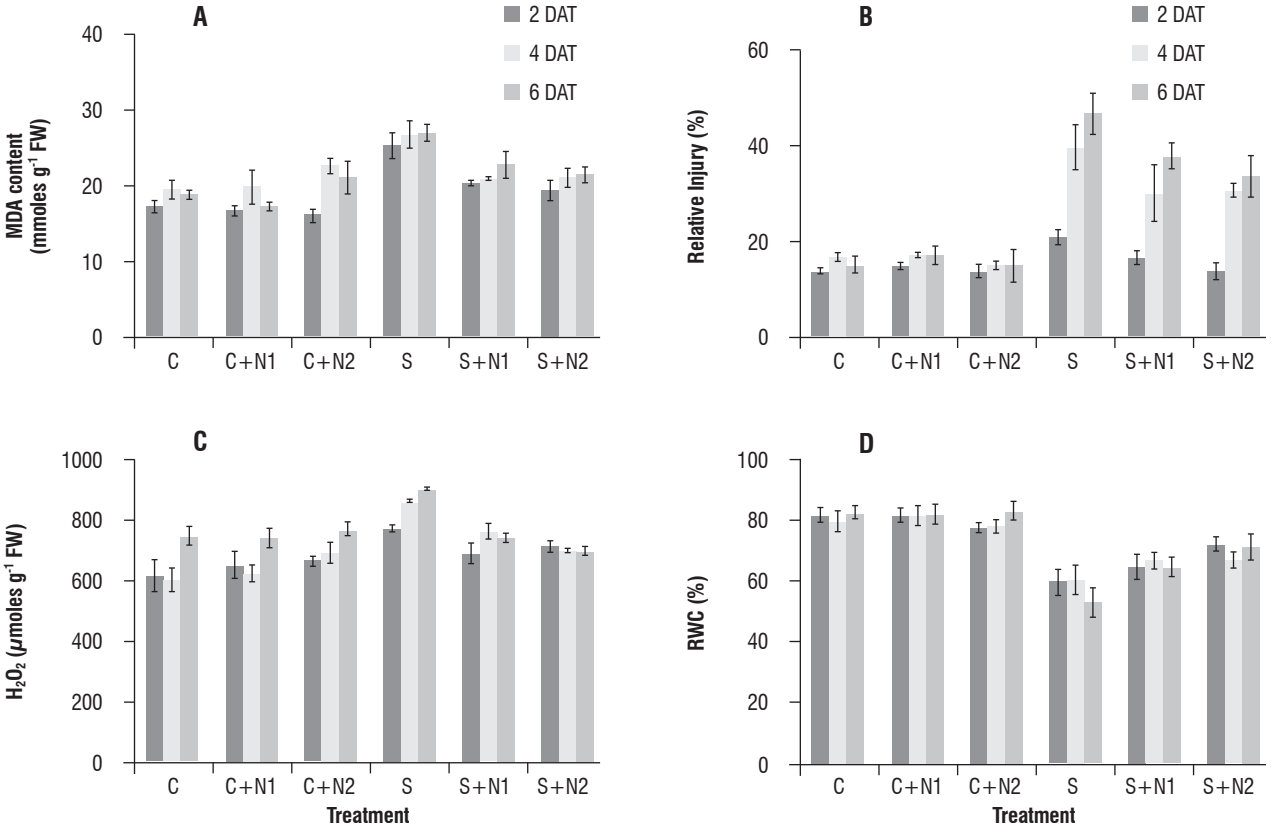

Figure 1. Effect of NaCl and SNP treatments on lipid peroxidation level (A), relative membrane injury (B), H_2O_2 content (C) and relative water content (D) in chickpea leaves. Abbreviations used: C, control; C+N1, control+0.2 mM SNP; C+N2, control+1mM SNP; S, 250 mM NaCl; S+N1, 250 mM NaCl+0.2 mM SNP; S+N2, 250 mM NaCl+1 mM SNP). The data are mean values ±SE (n=5).

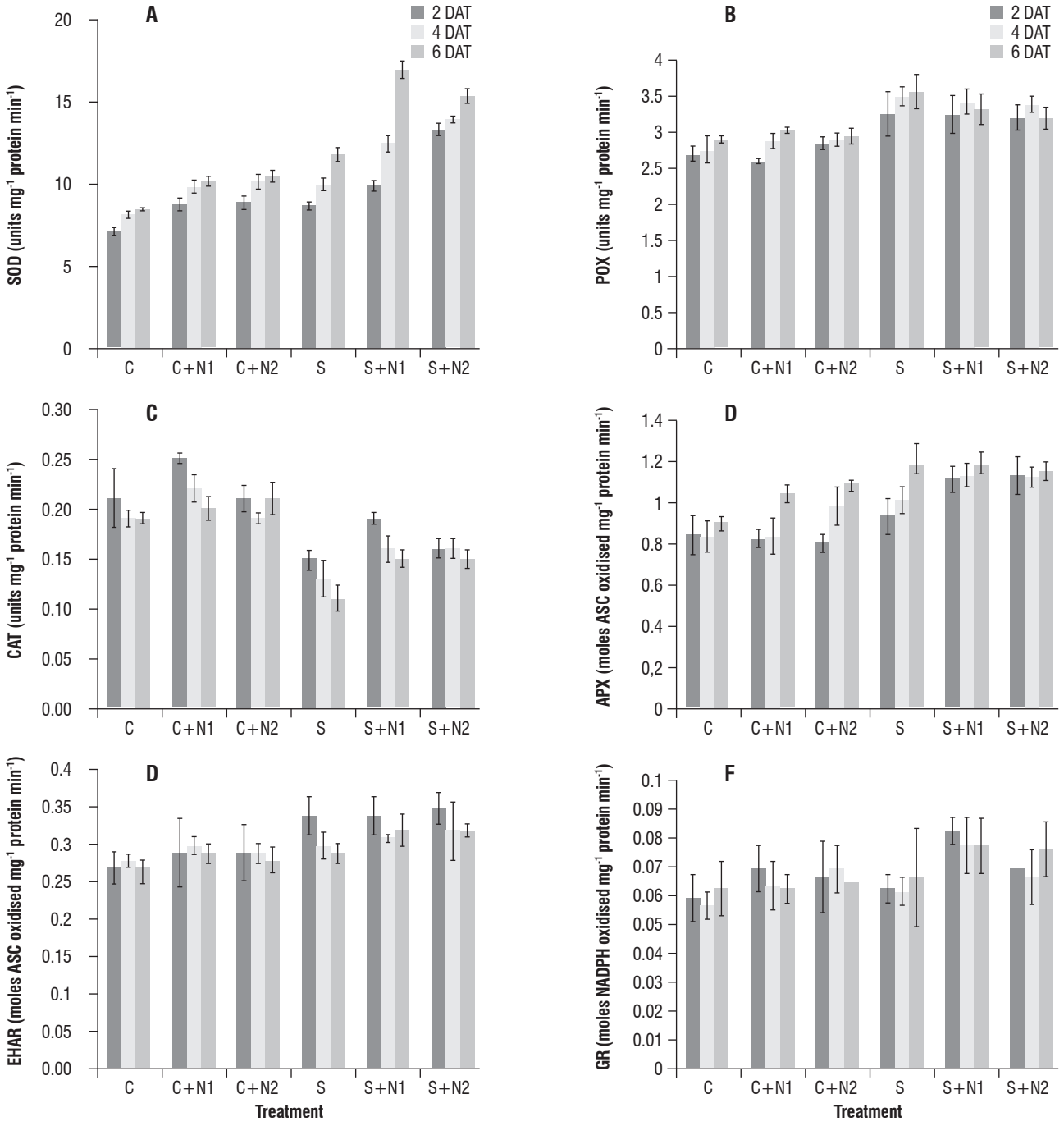

Figure 2. Effect of NaCl and SNP treatments on the activity of antioxidant enzymes SOD (A), POX (B), CAT (C), APX (D), DHAR (E) and GR (F) in chickpea leaves. Abbreviations used: C, control; C+N1, control+0.2 mM SNP; C+N2, control+1mM SNP; S, 250 mM NaCl; S+N1, 250 mM NaCl+0.2 mM SNP; S+N2, 250 mM NaCl+1 mM SNP). The data are mean values ±SE (n=5).

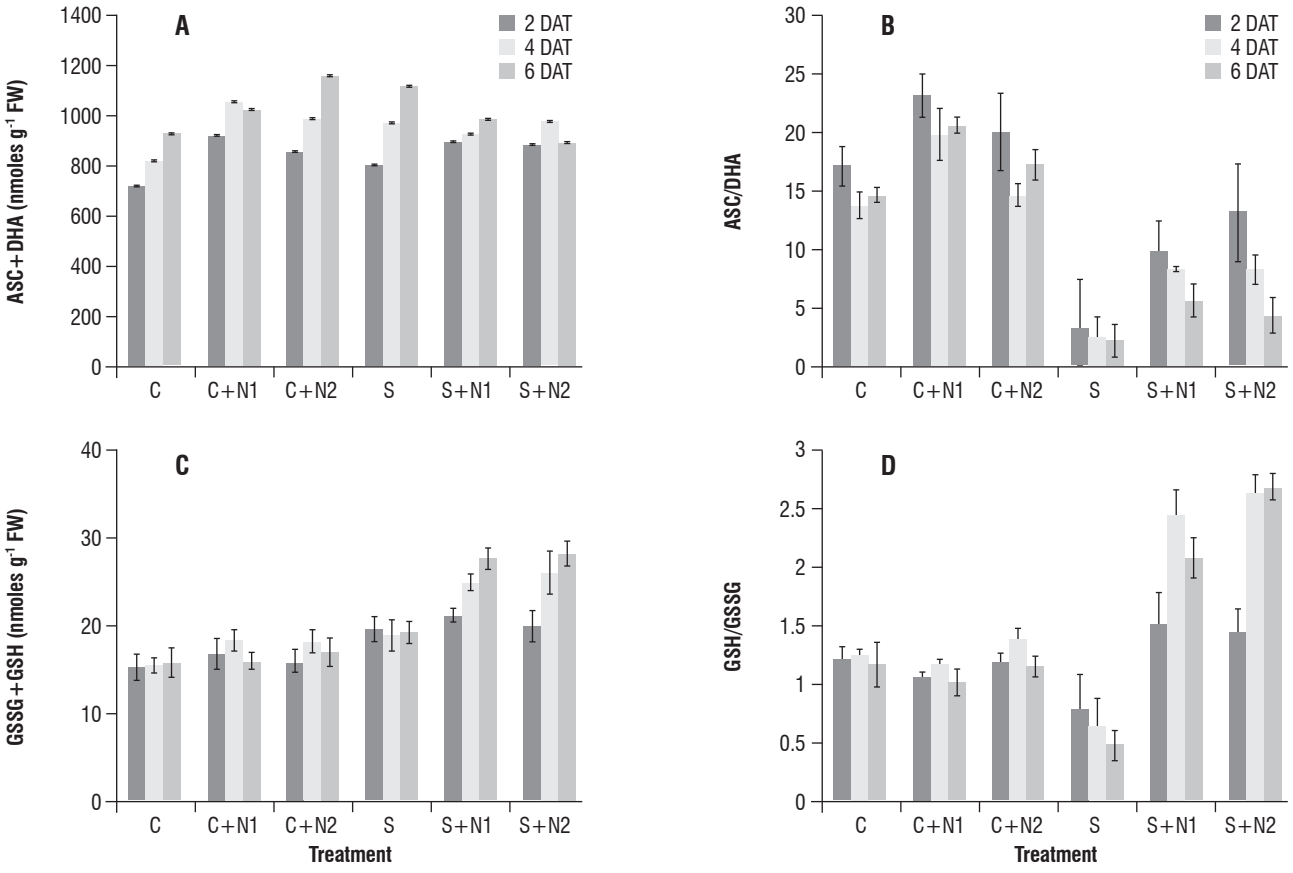

Figure 3. Effect of NaCl and SNP treatments on total ascorbate content (A), ascorbate redox ratio (B), total glutathione content (C) and glutathione redox ratio (D) in chickpea leaves. Abbreviations used: C, control; C+N1, control+0.2 mM SNP; C+N2, control+1mM SNP; S, 250 mM NaCl; S+N1, 250 mM NaCl+0.2 mM SNP; S+N2, 250 mM NaCl+1 mM SNP). The data are mean values ±SE (n=5).

DISCUSSION

Chickpea is an important pulse crop grown in the arid and semiarid regions. In India it is cultivated during winter depending on soil moisture stored from the preceding summer rain, which is often inadequate to ensure a satisfactory crop. In most such areas saline ground water is the only source of supplementary irrigation. Therefore in the present investigations effect of different durations of NaCl treatment on antioxidant metabolism were studied.

NaCl treatments of 250 mM for different durations damaged the cellular membranes. This damage was reflected in terms of increased electrolyte leakage (Figure 1B) and increased level of lipid peroxidation (Figure 1A). Salt stress induces lipid peroxidation through reactive oxygen species production (Liang et al. 2003; Zhang et al. 2006; Shi et al. 2007; Li et al. 2008), thus making the membranes leaky as evinced by increased electrolyte leakage. The membrane injury was time dependent and increased with duration of stress. Exogenously applied antioxidant SNP had a protective effect on salt induced membrane damage. The reaction of NO with ROS could prevent the injury to membranes. It has been reported that the reaction of NO with lipid alcoxyl (LO) and peroxyl (LOO^-) radicals is rapid, and could stop the propagation

of radical mediated lipid oxidation in a direct fashion (Beligni and Lamattina 1999). In the present investigations also, SNP could completely alleviate the adverse effect of NaCl on short duration stress of 2 days, but as the duration of stress was increased to 4 and 6 days alleviation was partial. A protective effect of NO on relative membrane injury has been reported under drought stress (Garcia-Mata and Lamattina 2001; Wang et al. 2004; Zhao et al. 2008) salt stress (Zhao et al. 2004; Zhang et al. 2006; Shi et al. 2007; Li et al. 2008) heavy metal stress (Hsu and Kao 2005; Singh et al. 2008) UV stress (Shi et al. 2005) wounding (Grun et al. 2006) and in ageing and senescence (Tu et al. 2003; Hung and Kao 2005).

Sodium chloride treatment adversely affected the RWC as salinity is known to cause both osmotic and ionic effects. The RWC decreased with increasing duration of stress. SNP treatments had a positive effect on RWC (Figure 1D). The higher SNP treatment was more effective and resulted in more than 50% amelioration. A protective effect of NO on RWC under salt stress has been reported in maize leaves (Zhang et al. 2006) and *Phragmites communis* calluses (Zhao et al. 2004). Garcia-Mata and Lamattina (2001) reported that SNP treated water stressed wheat seedlings tended to retain more water content.

Hydrogen peroxide content is an index of oxidative damage. The H_2O_2 content recorded a progressive increase as the duration of NaCl stress increased (Figure 1C). Increased H_2O_2 production was reported to occur in response to salt stress lasting for days in rice plants (Uchida et al. 2002). An increase in H_2O_2 content under salt stress has also been reported in barley leaves (Li et al. 2008) cucumber roots (Shi et al. 2007) and correlated with oxidative stress. These concentrations of NO decreased the H_2O_2 levels and partially alleviated the NaCl induced oxidative damage. A protective role of NO on H_2O_2 content has been reported under water stress (Zhao et al. 2008) salt stress (Shi et al. 2007; Li et al. 2008) heavy metal stress (Singh et al. 2008) and ABA induced stress (Hung and Kao 2003).

Mechanisms of ROS detoxification exist in all plants and can be categorized as enzymatic (SOD, CAT, APX *etc*) and non enzymatic (Glutathione, ascorbic acid *etc*). The level of response depends on the species, the development and metabolic state of plant, as well duration and intensity of stress. Plants resist the stress induced production of ROS by increasing components of antioxidant defense system.

The protective role of NO against oxidative damage could be explained by its role as a signaling molecule, which activates antioxidant enzymes (Huang et al. 2002; Shi et al. 2005). To assess whether the reduction of salt stress induced oxidative damage as evinced by decreased membrane injury, lipid peroxidation and H_2O_2 content may be due to antioxidant properties of NO, the activity of antioxidant enzymes such as SOD, POX, CAT, APX, DHAR and antioxidant metabolites glutathione and ascorbic acid were studied.

Increased activity of SOD (Figure 2A) observed in the present studies points towards its induction to quench higher levels of superoxide radical generated due to NaCl stress. The SOD activity increased further as the duration of stress was increased. SNP treatments further increased the SOD activity thus promoting the conversion of superoxide radical to hydrogen peroxide, which is an important step in protecting cells. Similar positive effects of NO on SOD activity under salt stress conditions have been reported (Kopyra and Gwozdz 2003; Shi et al. 2007; Li et al. 2008). The H_2O_2 produced is also toxic and must be scavenged. The H_2O_2 scavenging enzymes POX and CAT were also studied. NaCl stress resulted in increased POX activity (Figure 2B), however a decline in CAT activity (Figure 2C) was observed. Variable results have been observed regarding CAT activity under salt stress conditions. Increased CAT activity under salt stress has been reported by Shi et al. (2007) and decreased activity has been reported (Hai-Hua et al. 2002; Eyidogan and Oz 2007; Li et al. 2008). CAT deactivation by salt stress may be due to the prevention of new enzyme synthesis (Feierabend and Dehne 1996) or CAT photo-inactivation (Polle et al. 1997). NO treatments had no obvious effect on POX activity. Similar result has been reported by Hai-Hua et al. (2002) in salt stressed wheat leaves. However NO could partially alleviate the toxic effect of NaCl on CAT activity (Figure 2C). Stimulation of CAT activity by NO has been reported earlier under salt stress (Shi et al. 2007; Li et al. 2008) heavy metal stress (Kopyra and Gowzdz 2003; Singh et al. 2008) and osmotic stress (Zhao et al. 2008)

Among the enzymes of the ascorbate and glutathione pathway (Halliwell and Asada cycle) the activities of APX (Figure 2D) and DHAR (Figure 2E) increased under salt stress whereas GR (Figure 2F) activity was maintained at control levels. Shi et al. (2007) also reported no significant differences in GR activity between control and salt stressed plants. Eyidogan and Oz (2007) suggested that the induction

of APX in the leaves of chickpea plants subjected to salt stress may be mediated by the overproduction of H_2O_2 under catalase deactivation. NO promoted APX, GR and DHAR activities under salt stress which is important for the efficient scavenging of H_2O_2. NO induced increase in APX, GR and DHAR under salt stress in cucumber roots (Shi et al. 2007), APX and GR activities under salt treatments in wheat seedling leaves (Hai-Hua et al. 2005), APX induction under salt stress in barley leaves (Li et al. 2008) and induction of APX activity in PEG treated suspension cultures (Zhao et al. 2008) have been reported earlier.

Ascorbate peroxidase reduction of H_2O_2 requires ascorbate as substrate and DHAR conversion back to ascorbate needs glutathione as substrate therefore both glutathione and ascorbate are important. The ratio of GSH/GSSG and ASC/DHA are considered as markers of oxidative stress. The antioxidant metabolite glutathione content (Figure 3C) increased under salt stress, however differences in redox state (Figure 3D) were observed. Salt stress conditions resulted in a decrease in the GSH/GSSG ratio. The ratio decreased with the increase in duration of stress. SNP treatments had a positive effect and maintained the ratios much above control levels. Hai-Hua et al. (2005) have also reported an increased GSH/GSSG ratio with exogenous application of NO in wheat seedlings subjected to 150 mmol NaCl stress. An increase in GSH to GSSG ratios indicate a compensatory mechanism in which there is increased recycling of glutathione to keep it in its active reduced form. The ASC/DHA ratio also decreased significantly with NaCl treatments (Figure 3B). The decline was time dependent. SNP treatments increased the ratio under normal conditions and partially alleviated the effect of NaCl stress. The positive effect of SNP under salt stress conditions decreased with time. The fact that NO maintains the ASC and GSH in reduced state under salt stress may be because of increased activities of the enzymes GR, APX and DHAR. This results in the increased capacity of the antioxidant system to scavenge H_2O_2. Hung and Kao (2003) have reported that NO counteracts ABA induced decrease in ASC and GSH and this might account in part for the decreased H_2O_2 content in rice leaves treated with ABA and NO as compared to ABA alone.

The protective role of NO is mainly based on its ability to maintain the cellular redox homeostasis and regulate the level and toxicity of ROS induced by NaCl stress. This ability of NO to exert a protective function against oxidative stress caused by many factors might be due to: 1) reaction with lipid radicals, which stops the propagation of lipid oxidation; 2) scavenging the superoxide anion and formation of $ONOO^-$, which in plants is not as toxic as in animals and can be neutralized by ascorbate and glutathione; 3) activation of antioxidant enzymes (SOD, CAT, POX). In conclusion both the concentrations of SNP tested were effective in decreasing the RWC, H_2O_2 content, increasing the activity of antioxidant enzymes POX, APX and DHAR and the GSH/GSSG and ASC/DHA ratio under salt stress conditions. The lower concentration of 0.2 mM SNP was more effective in increasing the CAT and GR activity whereas the higher concentration of 1 mM SNP was more effective in increasing the SOD activity and decreasing the relative membrane injury and lipid peroxidation levels under salt stress conditions.

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