Effect of nitric oxide and putrescine on antioxidative responses under NaCl stress in chickpea plants

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ABSTRACT

Chickpea plants were subjected to salt stress for 48 h with 100 mM NaCl, after 50 days of growth. Other batches of plants were simultaneously treated with 0.2 mM sodium nitroprusside (NO donor) or 0.5 mM putrescine (polyamine) to examine their antioxidant effects. Sodium chloride stress adversely affected the relative water content (RWC), electrolyte leakage and lipid peroxidation in leaves. Sodium nitroprusside and putrescine could completely ameliorate the toxic effects of salt stress on electrolyte leakage and lipid peroxidation and partially on RWC. No significant decline in chlorophyll content under salt stress as well as with other treatments was observed. Sodium chloride stress activated the antioxidant defense system by increasing the activities of peroxidase (POX), catalase (CAT) superoxide dismutase (SOD) and ascorbate peroxidase (APX). However no significant effect was observed on glutathione reductase (GR) and dehydro ascorbate reductase (DHAR) activities. Both putrescine and NO had a positive effect on antioxidant enzymes under salt stress. Putrescine was more effective in scavenging superoxide radical as it increased the SOD activity under salt stress whereas nitric oxide was effective in hydrolyzing H₂O₂ by increasing the activities of CAT, POX and APX under salt stress. [*Physiol. Mol. Biol. Plants* 2008; 14(4) : 355-362] E-mail : sunita_sheokand@hotmail.com

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Abbreviations : APX – Ascorbate peroxidase; DHAR – Dehydro ascorbate reductase; CAT – Catalase; GR – Glutathione reductase; NO – Nitric oxide; POX – Peroxidase; SNP – Sodium nitroprusside; SOD – Superoxide dismutase

Salinity in soil or water is one of the major stresses in arid and semi-arid regions that can severely limit crop production (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). Plants have evolved an antioxidant system to counteract the oxidative stress. The antioxidant system is composed of ROS scavenging enzymes such as SOD, CAT, POX, APX, GR, DHAR and MDHAR (Noctor and Foyer, 1998). Many authors co-related changes in antioxidative enzyme activities and redox state under salt stress with plant tolerance to salinity (Meneguzzo *et al.*, 1999)

The balance between free radical generation and free radical defence 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 (Velikova *et al.*, 2000; Shalata and Neuman, 2001; Verma and Mishra, 2005; Wang *et al.*, 2004a; Wang and Yang, 2005; Zhang *et al.*, 2006). In this study, we have tested two antioxidant compounds, putrescine and nitric oxide donor sodium nitroprusside.

Nitric oxide is a bioactive, lipophilic free radical that acts as a signaling molecule with different kinds of physiological functions. NO has also been reported to act as a signal molecule; mediating responses to biotic and abiotic stresses in the plant kingdom and it 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.*, 2004b), salt stress (Kopyra and Gwozdz, 2003; Zhao *et*

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al., 2004; Chen *et al.*, 2004), heavy metal stress (Hsu and Kao, 2005; Wang and Yang, 2005) and UV radiation stress (Shi *et al.*, 2005). However experimental evidence for NO as a signal molecule for oxidative metabolism under salt stress is still preliminary.

Polyamines have also been suggested to function as free radical scavengers (Drolet *et al.*, 1986; Ye *et al.*, 1997). Their ability to decrease lipid peroxidation, under stress conditions has been attributed to their supposed ability to stabilize membranes by association with negatively charged phospholipids (Besford *et al.*, 1973).

The present investigations were conducted in chickpea leaves to study (a) antioxidative metabolism under NaCl stress (b) whether an additional supply of free radical scavengers putrescine and sodium nitroprusside could ameliorate the toxic effects of NaCl on the oxidative metabolism.

MATERIALS AND METHODS

Plant material and treatments

Chickpea (*Cicer arietinum* 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. The plants were raised in earthen pots containing 5 Kg of sand under green house conditions. The plants were supplied with nitrogen free nutrient solution (Wilson and Reisenauer, 1963) at regular intervals. The plants were divided into six groups, 50 days after sowing (DAS). The first group, irrigated with water alone, served as control. The second group was treated with 0.5 mM putrescine as foliar spray and the third group was treated with 0.2 mM Sodium nitroprusside (NO donor) as foliar spray. The fourth group was treated with 100 mM NaCl through the rooting medium. The fifth group was treated with 100 mM NaCl through the rooting medium and 0.5 mM putrescine through foliar spray. The sixth group was treated with 100 mM NaCl through the rooting medium and 0.2 mM SNP through foliar spray. The plants were sampled 2 DAT (days after treatment).

Relative Water Content (RWC)

The first fully expanded leaves of control and treated plants were collected at 10 a.m and weighed (Wf). The leaves were submerged in water for four hours. They were blotted 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 Bahrs, 1962)

$$\text{RWC (\%)} = \frac{(\text{Wf}-\text{Wd})}{(\text{Wt}-\text{Wd})} \times 100$$

Relative Membrane Injury (RI)

Relative membrane injury was analysed according to the method of Zhang *et al.* (2006). 250 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 (X_i). 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 (X_t). Relative injury was calculated from the equation $[(X_i/X_t) \times 100]$.

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 (malondialdehyde) content, a break down 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 minutes and cooled on ice. After centrifugation at 10,000 rpm 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).

Chlorophyll content

Leaf discs (0.03 g) were washed, blotted dry and then dipped in dimethyl sulphoxide overnight as described earlier by Sawhney and Singh (2002). The absorbance of the solution was recorded next day at 645 and 663 nm respectively.

Determination of enzyme activity

Cell free extract for various antioxidant enzymes viz, superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POX) 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).

Ascorbate peroxidase (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). Catalase (EC 1.11.1.6) activity was estimated according to the procedure of Sinha (1972). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM H_2O_2 and diluted enzyme extract. The assay with boiled extract constituted the control. After 5 minutes, the reaction was terminated by the addition of dichromate : acetic acid which formed a complex with residual H_2O_2 and its absorbance was recorded at 570 nm. The change in absorbance of one as compared to the control was considered to correspond as one unit.

Superoxide dismutase (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. Peroxidase (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 (GR, 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 ASA at 265 nm ($\epsilon = 14\text{mM}^{-1}\text{cm}^{-1}$) in a reaction mixture containing 0.1M 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.

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 given short term stress with 100 mM sodium chloride for 48 h at the vegetative stage and relative damage to the indices of stress and antioxidant metabolism was assessed. The role of exogenously supplied antioxidants SNP and putrescine was also studied in the control as well as salt treated plants.

Membrane injury and lipid peroxidation

Salt stress (NaCl) adversely affected the membrane integrity (Fig. 1 A & B). Chickpea plants treated with 100 mM NaCl for 48 h showed a 45 % increase in relative membrane injury. Exogenous application of both the antioxidants putrescine and SNP were effective in partially ameliorating the deleterious effect of NaCl on membrane injury. Putrescine at 0.5 mM concentration was effective in reducing the membrane injury by 25 % and SNP by 20 % under stress conditions. The membrane injury could be attributed to increased lipid peroxidation levels. The MDA content increased by 22 % with NaCl stress (Fig. 1A). Both SNP and putrescine could reverse the harmful effect of salt stress on lipid peroxidation and were effective in bringing down the values to below control levels.

Relative water content and chlorophyll content

Short term NaCl treatments adversely affected the relative water content (RWC). A 20 % decline in RWC was observed with 100 mM NaCl treatments. Putrescine was effective in partially increasing the relative water content. SNP however had no effect (Fig. 1C). A small decline of 10 % was recorded for the chlorophyll content in the salt-stressed leaves (Fig. 1D). Both sodium

Fig. 1. Effect of NaCl (100 mM), NO (0.2 mM) and putrescine (0.5 mM) treatments on (A) Lipid Peroxidation level (B) Relative Injury (C) Relative water content and (D) Chlorophyll content in leaves of chickpea.

nitroprusside and putrescine resulted in a 3 to 6 % increase in chlorophyll content.

Antioxidative Enzymes

Superoxide dismutase activity recorded a steep rise (2.9 fold) with salt treatments, indicating an induction of the defense system (Fig. 2C). Putrescine treatment further increased the SOD activity to 3.6 fold under stress conditions. The activities of hydrogen peroxide scavenging enzymes catalase and peroxidase also increased under salt stress but to a lesser extent as compared to SOD. Catalase and peroxidase activities recorded a 23 % and 15 % increase in their activities under salt stress (Fig. 2A & B). SNP treatments could further increase the activities to 30 % and 34 % respectively. Putrescine treatments however had no significant effect on the enzyme activities under stress conditions. The ascorbate peroxidase activity that increased by 13 % under salt stress was further increased to 18 % with SNP treatments (Fig. 2D). Putrescine again was ineffective in further increasing the enzyme activity,

Glutathione reductase and dehydroascorbate reductase activity showed no significant change under stress as well with SNP and putrescine treatments (Fig. 2E & F).

DISCUSSION

Chickpea is an important pulse crop grown in the arid and semi-arid 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 such areas, saline ground water is the only source of supplementary irrigation. Therefore, in the present investigations, short term effects of NaCl treatments on antioxidant metabolism were studied.

NaCl treatments of 100 mM for 48 h, damaged the cellular membranes. This damage was reflected in terms of increased electrolyte leakage (Fig. 1B) and increased level of lipid peroxidation (Fig. 1A). Salt stress induces lipid peroxidation through reactive oxygen species

Fig. 2. Effect of NaCl (100 mM), SNP (0.2 mM) and putrescine (0.5 mM) treatments on activity of antioxidant enzymes (A) CAT (B) POX (C) SOD (D) APX (E) GR (F) DHAR in leaves of chickpea.

production (Liang *et al.*, 2003; Verma and Mishra, 2005), thus making the membrane leaky as evinced by increased electrolyte leakage. Exogenously applied antioxidants, SNP (NO donor) and polyamine (putrescine) 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 peroxy (LOO⁻) radicals is rapid, and could, thus stop the propagation of radical mediated lipid oxidation in a direct fashion (Beligini and Lamattina, 1999). In the present investigations also, SNP could completely ameliorate the adverse effect of NaCl treatment on membrane injury and lipid peroxidation. A protective effect of NO on relative membrane injury has been reported under drought stress (Garcia-Mata and Lamattina, 2001, Wang *et al.*, 2004a), salt stress (Zhao *et al.*, 2004, Zhang *et al.* 2006), ageing and senescence (Tu *et al.*, 2003, Hung and Kao, 2005) and heavy metal stress (Hsu and Kao, 2005). Lower concentrations of NO might act as an antioxidant (Zhang *et al.*, 2006). Polyamines are also known for their anti-stress and anti-senescence effects due to their membrane stabilizing and antioxidant properties (Kaur-Sawhney and Galston, 1991, Borell *et al.*, 1997). According to Tadolini (1988), polyamines act as antioxidants by inhibiting lipid peroxidation. This is accounted by the ability of polyamines to form a ternary complex with iron and the phospholipid polar heads that may change the susceptibility of Fe²⁺ to auto-oxidation and thus its ability to generate free oxygen radicals. Moreover, PAs are shown to stabilize membranes by associating (as organic polycations) with negatively charged phospholipids. In this study also, putrescine at 0.5 mM concentration was effective in bringing down the MDA levels and electrolyte leakage to control values. Polyamine's general nature to reduce membrane leakage and lipid peroxidation has been reported earlier also (Tiburcio *et al.*, 1994, Borell *et al.*, 1997, Velikova *et al.*, 2000, Verma and Mishra, 2005).

Salt stress for 48 h decreased the RWC by 20%. Sodium nitroprusside had no significant effect on RWC. Garcia-Mata and Lamattina (2001) reported that SNP-treated water-stressed wheat seedlings tended to retain more water than water stressed seedlings. Putrescine was partially effective in increasing the RWC. The protective effect of putrescine on RWC under PEG treatments has been reported earlier (Vakharia *et al.*, 2003).

Short term salt treatment had little effect on the chlorophyll content; only a small decline of 11 % was observed. Krishnamurthy (1991) reported a 63 %

decrease in chlorophyll content, with long term salinization treatments in wheat, and putrescine was effective in partially overcoming the reduction of chlorophyll. NO has also been reported to overcome the age induced decline in chlorophyll content in wheat seedlings (Tu *et al.*, 2003) under oxidative stress (Belgini and Lamattina, 1999) and salt stress (Zhang *et al.*, 2006). Lipid peroxidation and electrolyte leakage can be considered as indices of stress under short term NaCl treatments and both NO and putrescine were effective in ameliorating the salt induced negative effects on these parameters.

To assess whether the reduction of salt stress induced toxicity on lipid peroxidation and membrane permeability may be due to the antioxidant properties of NO and putrescine, the activity of antioxidant enzymes such as SOD, POX, CAT, APX and DHAR were studied.

A significant increase in SOD activity (Fig. 2C), indicates the activation of defense system under short term salt stress. Putrescine treatments further increased the activity of SOD under salinity. It has been suggested that putrescine could bind to antioxidant enzymes, such as superoxide dismutase or be conjugated to small antioxidant molecules and allow them to permeate to the sites of oxidative stress within the cells. A covalent put-superoxide (Put-O₂) dismutase complex was 20 fold more membrane permeable than superoxide dismutase alone, thus facilitating oxidant protection in a mammalian system (Poduslo and Curran, 1996, Wengenack *et al.*, 1997). Nagele *et al.* (1994) reported that the superoxide dismutase-copper complex of copper-put-pyridine has many functions in mammalian cells. The complex can dismutate superoxide with high efficiency and enhance oxidation of glutathione. Verma and Mishra (2005) have also proposed that the increased activity of SOD due to putrescine, under salinity, might be in order to protect the biomolecules from attack of superoxide radicals (O₂⁻). NO, however caused no further elevation in SOD activity.

The H₂O₂ scavenging enzymes CAT and POX (Fig. 2A & B) recorded a relatively smaller increase of 23 % and 15 % respectively under salt stress. Nitric oxide was effective in further increasing the activities by 7 % to 20 % respectively. Tu *et al.* (2003) reported that wheat leaves treated with 0.10 m mol/L SNP reduced H₂O₂ by activating catalase in ageing wheat leaves. Belgini *et al.* (2002) found that SNAP (Snitroso-N-acetyl-DL-pericillamine), another NO donor, could delay the GA-induced loss of Cat2 mRNA and protein in barley aleurone. Wang *et al.* (2005) also reported that SNP

increased the activity of peroxidase but had little effect on SOD in drought stressed poplar leaves. Putrescine treatments had no further effect on salt induced activation of CAT and POX. Short term NaCl treatment induced no change in GR and DHAR activities. APX also recorded a small increase of 13 %, which increased to 18 % with NO treatments. Putrescine had no effect and maintained the enzyme activities at control levels. Thus, in the present investigations, both putrescine and NO were found to be effective in alleviating the toxic effects of salt stress through their antioxidant properties. Putrescine was more effective in scavenging superoxide radical, as it increased the SOD activity under salt stress while nitric oxide was effective in scavenging H₂O₂ by increasing the activities of CAT, POX and APX under salt stress.

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