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Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*

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Abstract

Seeds of *Cassia angustifolia* Vahl. were subjected to 0, 20, 50, 100 mM NaCl for 7 d in order to study the effect of salt stress on growth parameters, endogenous Na^+ and Cl^- concentrations, antioxidant system, lipid peroxidation, hydrogen peroxide, and proline contents. Salinity affected all of the considered parameters and caused a great reduction in plant biomass. The root and shoot length, fresh and dry mass and germination percentage were inhibited by NaCl treatments. These changes were associated with an increase in the Na^+ and Cl^- contents in the seedlings and increased activities of superoxide dismutase, catalase, peroxidase, and polyphenol oxidase. The increased enzyme activity coincided with decreased ascorbate content and enhanced H_2O_2 and proline content.

Additional key words: ascorbate, catalase, hydrogen peroxide, oxidative stress, peroxidase, polyphenol oxidase, reactive oxygen species, salinity stress, senna, superoxide dismutase.

Introduction

High salinity in the soil is a common environmental problem, and affects almost all plant functions (Greenway and Munns 1980). Under salt stress, plants have adapted to osmotic and ionic stresses. These mechanisms include osmotic adjustment by accumulation of compatible solutes such as proline, glycine betaine and polyols and lowering the toxic concentration of ions in the cytoplasm by restriction of Na^+ influx or its sequestration into the vacuole and/or its extrusion (Ghoulam *et al*. 2002).

Plants that are subjected to environmental stress often suffer oxidative damage as the balance between the production of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and hydroxyl radical and the quenching activity of antioxidants are upset (Scandalios 1993). Plants have evolved mechanisms to protect cell and subcellular systems from the effects of these reactive oxygen radicals by using enzymes such as superoxide dismutase, catalase, peroxidase, glutathione reductase, polyphenol oxidase and non-enzymic ascorbate and glutathione. It has been suggested that H_2O_2 and $O_2^{\cdot -}$ might play an important role in the mechanism of NaCl injury in *Vigna catjand* and *Oryza sativa* (Singha and Choudhuri 1990). Recently, Gossett *et al*. (1996), using leaves and callus from cotton (*Gossypium hirsutum*) demonstrated that there is a strong relationship between antioxidant capacity and NaCl tolerance.

The objective of the present investigation was to study the effect of salinity stress on the plant antioxidant systems in senna in order to evaluate the relative significance of these antioxidant systems in imparting tolerance to NaCl oxidative stress.

Materials and methods

Seeds of senna (*Cassia angustifolia* Vahl.) were surface sterilized by treating with 0.1 % mercuric chloride solution and then thoroughly washed with double distilled water and germinated in Petri dishes lined with

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Abbreviations: ASA - ascorbate; CAT - catalase; DAS - days after sowing; POX - peroxidase; PPO - polyphenol oxidase; SOD - superoxide dismutase; TBARS - thiobarbituric acid reactive substances.

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double layer of *Whatman No. 1* filter paper. The Petri dishes were moistened with distilled water (control), 20, 50, and 100 mM NaCl solutions daily and were maintained at 28 ± 2 °C in a BOD incubator under a cool fluorescent light of 34.1 μmol $m^{-2}s^{-1}$ (PAR).

Five seedlings from each treatment were sampled to measure the length of primary root and shoot, and their fresh mass were directly determined 5 and 7 DAS. For dry mass determination, the seedlings were dried at 70 °C for 48 h and weighed. Sodium concentrations were determined as described by Allen *et al*. (1976). Chloride content was estimated argentometrically.

Enzyme extracts for the determination of superoxide dismutase (SOD), peroxidase (POX), and polyphenol oxidase (PPO), was prepared by homogenizing 0.2 g seedlings with 5 cm^3 of chilled phosphate buffer while catalase (CAT) was prepared by grinding 0.2 g seedlings with 10 cm^3 of chilled phosphate buffer. For SOD the extraction medium was 0.1 M phosphate buffer, pH 7.8, and for CAT, POX and PPO it was 0.1 M phosphate buffer at pH 6.8. The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 10 000 *g* for 20 min. The supernatant was referred to as enzyme extract. All operations were carried out at 4 °C. The superoxide dismutase activity was estimated according to the method of Beauchamp and Fridovich (1971) and modified by Giannopolitis and Ries (1977). The 3.5 cm^3 reaction mixture contained 0.05 M sodium carbonate pH 10.2, 13 mM methionine, 0.1 M EDTA, 63 μM nitroblue tetrazolium chloride (NBT), 13 μM riboflavin and 0.4 cm^3 enzyme extract. Reaction was started by placing tubes below two 15 W fluorescent lamps for 10 min. Reaction was stopped by keeping the tubes in dark for 10 min. Absorbance was recorded at 560 nm. One unit of enzyme activity was defined as the quantity of SOD required to produce a 50 % inhibition of reduction of NBT and the specific enzyme activity was expressed as units per mg protein.

Catalase, peroxidase and polyphenol oxidase activities were assayed according to Chance and Maehly (1955) with modifications. Ascorbate was estimated as described by Mukherjee and Choudhuri (1983). The level of lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS), following the method of Heath and Packer (1968). The seedlings (0.2 g), were homogenized in 10 cm^3 of 0.1 % trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 *g* for 20 min. To 1 cm^3 aliquot of the supernatant 4 cm^3 of 0.5 % thiobarbituric acid (TBA) in 20 % TCA was added. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10 000 *g* for 10 min, the absorbance of the supernatant was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The TBARS content was calculated using its absorption coefficient of 155 $mmol^{-1}$ cm^{-1} and expressed as nmol(TBARS) g^{-1}(f.m.). Hydrogen peroxide was estimated with titanium reagent as described by Teranishi *et al*. (1974). Sample preparation of H_2O_2 estimation was done as described by Mukherjee and Choudhuri (1983). The proline content was determined using the method of Bates *et al*. (1973) and the protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Two-way analysis of variance (*ANOVA*) with three replications per treatment (control, NaCl: 20, 50 and 100 mM), two stages of seedling development (5 and 7 DAS) and interaction between treatments and stages was carried out.

Results

NaCl treatment of seedlings for 5 and 7 DAS resulted in a significant reduction in the root and shoot length (Fig. 1*A*,*B*). Fresh mass of seedling gradually decreased with an increase in NaCl concentration at both stages. The reductions were more pronounced at 100 mM especially for 5 DAS. The dry mass was also affected by NaCl treatment; with a greater reduction as the NaCl concentration increased (Fig. 1*C*,*D*). The germination percentage decreased with increasing salinity at both stages. At the highest NaCl concentration (100 mM) the germination percentage was reduced to less than 50 % in comparison to unstressed seedlings (Fig. 1*E*). The presence of NaCl in the medium induced an important increase in Na^+ and Cl^- in the seedlings at both stages, with the highest concentrations being reached at 100 mM NaCl (Fig. 2).

SOD activity increased significantly at 20 and 50 mM NaCl concentrations at both stages (Fig. 3*A*). However, at 100 mM NaCl the SOD activity decreased at the two stages. Salt-induced SOD activity was significantly higher on 7 DAS than on 5 DAS. Unlike SOD, the CAT, POX and PPO activity (Fig. 3*B*,*C*,*D*) significantly increased with increasing salinity. However, the CAT activity was lower 7 DAS in comparison to 5 DAS while POX activity progressively increased at all NaCl concentrations at both stages of sampling (Fig. *3C*). PPO activity was inhibited at the second stage (Fig. 3*D*). PPO activity was almost similar at 100 mM NaCl at the two stages.

Lowest proline content was observed under control and highest under 100 mM NaCl stress (Fig. 3*E*). Proline content declined at the second stage. Senna showed slightly lower ascorbate content at the two stages at 50 and 100 mM NaCl as compared to control (Fig. 3*F*).

Lipid peroxidation estimated as TBARS content was lower than in control at 20 and 50 mM NaCl at the two stages and leveled to control value at 100 mM NaCl (Fig. 3*G*). Hydrogen peroxide accumulation increased under salinity at both developmental stages (Fig. 3*H*).

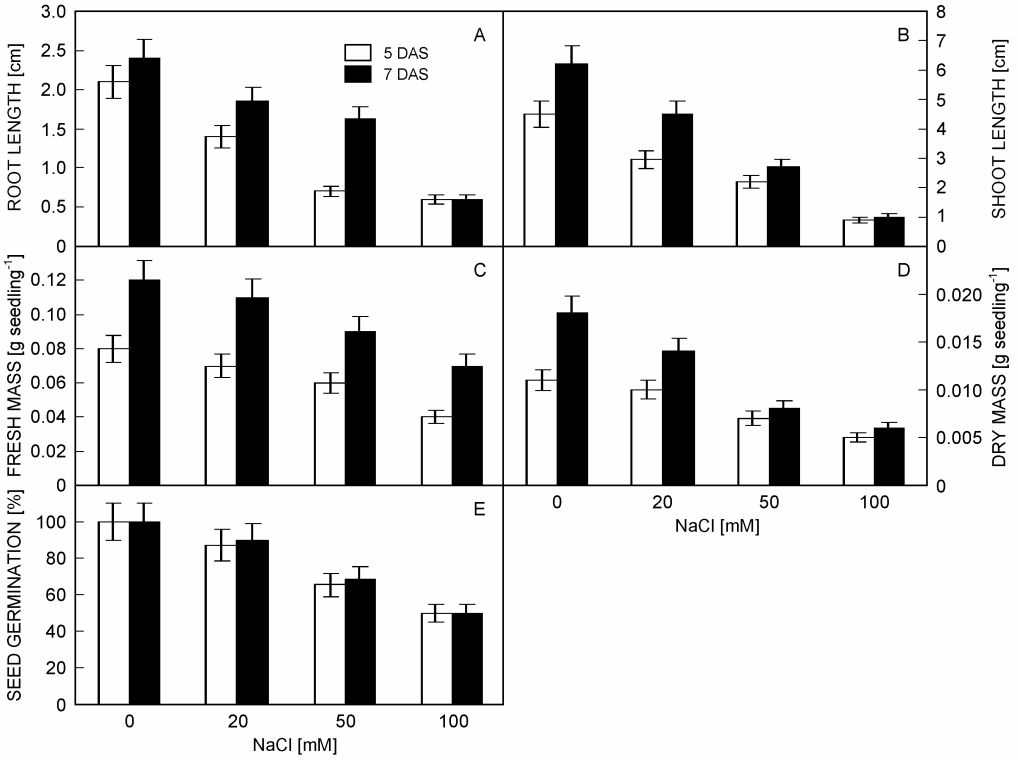

Fig. 1. Effect of NaCl stress on senna seedling root length (*A*), shoot length (*B*), fresh mass (*C*), dry mass (*D*), and on germination percentage (*E*). *Vertical bars* indicate SE of five replicates for each treatment and two dates of sowing.

Discussion

Salinity (NaCl) adversely affected the seedling growth parameters (germination, fresh and dry mass and root and shoot length) of senna seedlings. The results are similar to those of Dash and Panda (2001) in *Phaseolus mungo* and Ghoulam and Fares (2001) in sugar beet. Under salt stress, senna seedlings accumulated more inorganic ions Na^+ and Cl^- (Fig. 2). Similar results were reported in sugar beet cultivars (Ghoulam *et al*. 2002), in rice (Lutts *et al*. 1996) and in *Sorghum bicolor* (Colmer *et al*. 1996). This accumulation of Na^+ and Cl^- ions might be involved in the osmotic adjustment.

The activity of antioxidant enzymes was reported to increase under salinity in wheat shoot (Meneguzzo *et al*. 1999, Sairam and Srivastava 2002) and pea (Hernandez *et al*. 1999). Most of the results of the study conducted here show a correlation between the resistance to NaCl stress and more effective antioxidative system. The observed increase in SOD activity (Fig. 3*A*) could increase the ability of the seedlings to scavenge O_2^- radicals, which could cause membrane damage. At higher NaCl concentration (100 mM) it seems that such resistance to oxidative stress may be overcome leading to

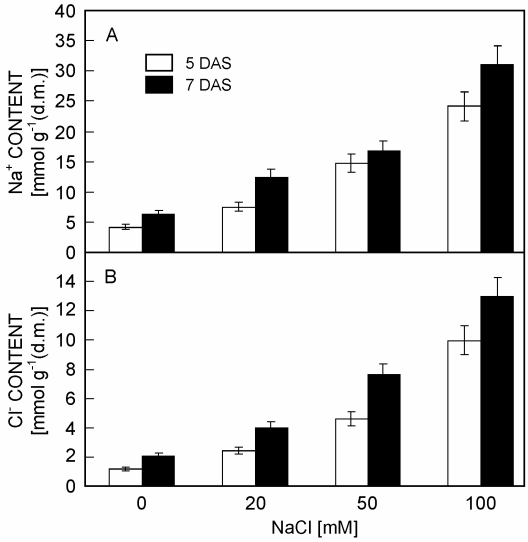

Fig. 2. Na^+ (*A*) and Cl^- (*B*) accumulation in 5- and 7-d-old seedlings of senna subjected to salt treatments. *Vertical bars* indicate SE of five replicates for each treatment and two dates of sowing.

growth reductions (Fig. 1) (Agarwal and Joshi 2002, Pandey and Agarwal 2002). Increase in CAT and POX activity is supposed to be an adaptive trait possibly helping to overcome the damage to the tissue metabolism by reducing toxic levels of H_2O_2 produced during cell metabolism and protection against oxidative stress (Rasheed and Mukerji 1991, Scalet *et al*. 1995, Gossett *et al.* 1996, Dionisio-Sese and Tobita 1998, Sudhakar *et al*. 2001, Bor *et al*. 2003). Similarly in the present study, the salt induced enhancement of CAT and POX activity (Fig. 3*B*,*C*) may suggest its effective scavenging mechanism to remove H_2O_2 (Fig. 3*H*) and imparting tolerance against NaCl oxidative stress. The POX and PPO are the two major enzymes responsible for oxidation of phenolic compounds (Sheen and Calvert 1969). The increased PPO activity in senna might reduce the phenol accumulation in the seedlings under stress (Fig. 3*D*). Increased PPO activity was also reported by Demir and Koçalişkan (2001) in bean seedlings. It seems possible that oxido-reductases POX and PPO may play an important role as defense against salt stress.

The ASA content (Fig. 3*F*) decreased at both stages,

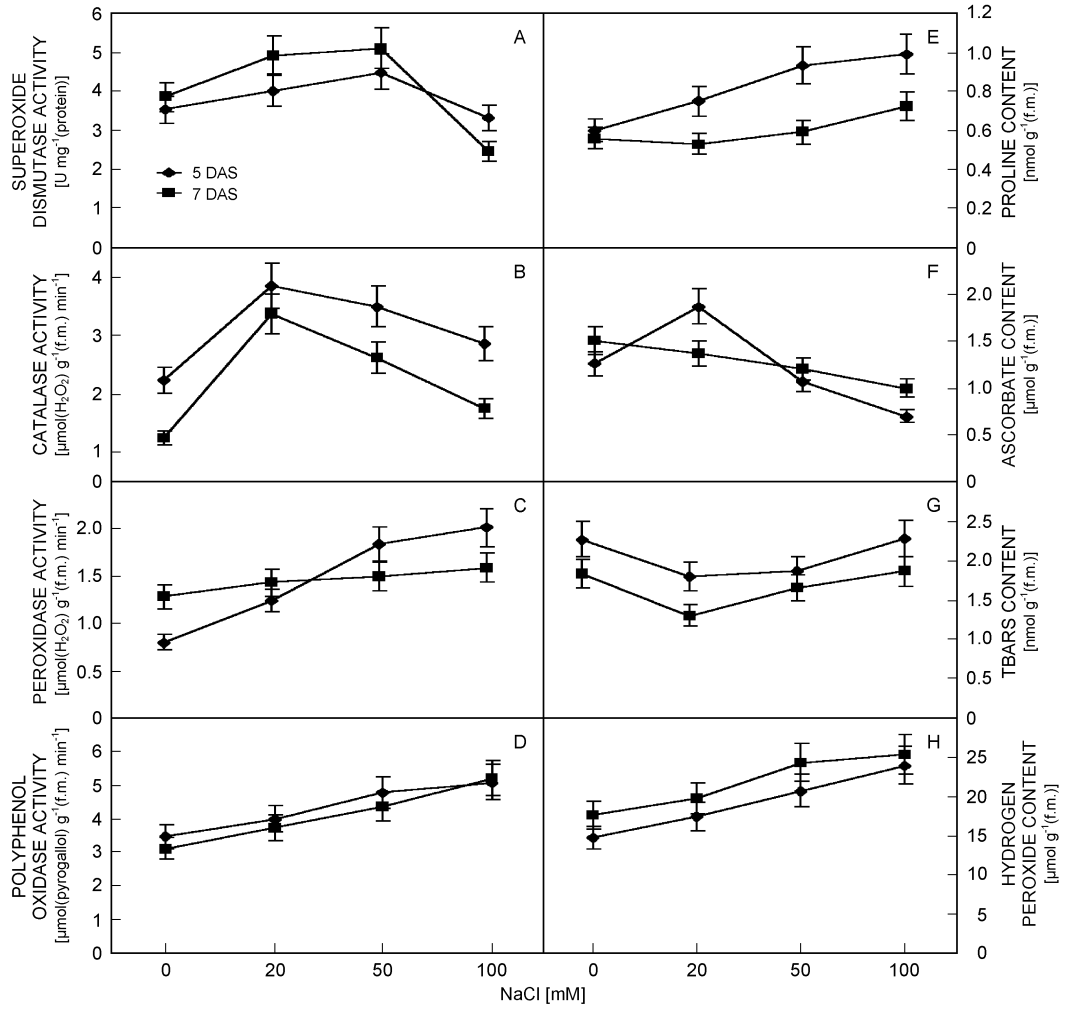

Fig. 3. Effect of NaCl stress on SOD (*A*), CAT (*B*), POX (*C*), PPO (*D*) activities and proline (*E*), ASA (*F*), TBARS (*G*) and H_2O_2 (*H*) contents of senna seedlings. *Vertical bars* indicate SE of three replicates for each treatment and two dates of sowing.

which may be due to increased activities of dehydro-ascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) (not measured). It has been demonstrated that salt treatment increases lipid peroxidation or induce oxidative stress in plant tissues (Mittal and Dubey 1991,Hernandez *et al*. 1994). The results reported here show that the degree of accumulation of TBARS was low indicating a decreased lipid peroxidation due to salt stress. The elevated activities of SOD, CAT, POX and PPO and decreased lipid peroxidation under NaCl stress, which probably come from an increased capacity for oxygen radical scavenging and maintenance of cellular membranes indicate the relationship between salt tolerance and antioxidant defense system. A lower lipid peroxidation resulted from elevated activities of antioxidants under salt stress was also reported in rice (Dionisio-Sese and Tobita 1998), tomato (Shalata *et al*. 2001), sugar-beet (Bor *et al*.

2003) and cotton (Meloni *et al.* 2003). With increase in NaCl concentration and duration of stress, proline content increased in *Phaseolus mungo* (Dash and Panda 2001), wheat seedlings (El-Shintinawy and El-Showbagy 2001) and *Ceriops roxburghiana* (Rajesh *et al*. 1999). The increase in proline content on both days (Fig. 3*E*) was positively correlated to the level of salt tolerance. Many functions have been postulated for proline accumulation in high tissues. Proline could be involved in the osmotic adjustment of plants (Gzik 1996) and could also be a protective agent of enzymes and membranes (Bandurska 1993). Such a function coincided with our results associating proline content and antioxidative enzymes in senna seedlings.

A perusal of the results show that the lesser degree of membrane damage based on the low rate of lipid peroxidation (TBARS), and salt induced enhancement of antioxidative enzymes indicate that senna seedlings had a higher capacity for the scavenging of ROS generated by salt stress. Thus, the present study confirms a correlation between antioxidant defense system and tolerance to NaCl induced oxidative stress.

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