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Research Article

Antioxidative Potentials as a Protective Mechanism in *Catharanthus roseus* (L.) G.Don. Plants under Salinity Stress

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Abstract: Antioxidant responses were analysed in *Catharanthus roseus* (L.) G.Don. under 0, 50 and 100 mM NaCl in order to investigate the plant's protective mechanisms against long-term salt-induced oxidative stress. The NaCl treatments were repeated in 4 different stages of growth, i.e. 30, 45, 60 and 75 days after sowing (DAS). The plants were uprooted randomly 90 DAS and the non-enzymatic and enzymatic antioxidant potentials were analysed. High salinity caused a decrease in reduced glutathione (GSH) and an enhancement in total ascorbate (AA) content and the antioxidant enzyme and ascorbate peroxidase (APX, EC 1.11.1.11) activities. Moreover, salinity induced a significant decline in superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POX, EC 1.11.1.7) activities. The changes found in catalase (CAT, EC 1.11.1.6) activities may be of great importance in the H_2O_2 detoxification mechanism under oxidative stress.

Key Words: Antioxidants, antioxidant enzyme, salinity, *Catharanthus roseus*

Introduction

Environmental factors influence the characters, composition, growth and development of individual plants and plant communities. When any of these environmental factors exceed the optimum tolerance of a plant, the result is stress to the plant, which in turn influences its developmental, structural, physiological and biochemical processes. Soil salinity is one of these environmental stresses (Lawlor, 2002). Every year more and more land becomes non-productive owing to salt accumulation. The major effect of salinity is the inhibition of crop growth by the reduced hormone delivery from roots to leaves (Azooz et al., 2004). Salt alters a wide array of metabolic processes, culminating in stunted growth, and reduced enzyme activities and biochemical constituents (Muthukumarasamy & Panneerselvam, 1997). High salinity is known to cause both hyperionic and hyperosmotic effects in plants, leading to membrane disorganisation and metabolic toxicity (Hasegawa et al., 2000). In addition, an important consequence of salinity stress in plants is the excessive generation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), H_2O_2 and the hydroxyl radicals particularly in chloroplast and mitochondria (Mittler, 2002; Neill et al., 2002). Generation of ROS such as superoxide, H_2O_2 and hydroxyl molecules causes rapid cell damage by triggering off a chain reaction (Imlay, 2003). Plants under stress produce some defence mechanisms to protect themselves from the harmful effect of oxidative stress. ROS scavenging is one of the common defence responses against abiotic stresses (Vranova et al., 2002). ROS scavenging depends on the detoxification mechanism provided by an integrated system of non-enzymatic reduced molecules like ascorbate and glutathione and enzymatic antioxidants (Srivalli et al., 2003). The major ROS scavenging activities include complex non-enzymatic (ascorbate, glutathione, a-tocopherol) and enzymatic (CAT, APX, GR, SOD etc.) responses (Prochazkova et al., 2001). The pathways include the water-water cycle in chloroplasts and the ascorbate-glutathione cycle (Asada, 1999). Antioxidant mechanisms may provide a strategy to enhance salt tolerance in plants.

Among the several approaches to solve the problem of saline soils, the biological approach to identify and grow

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salt-tolerant plants in such soils to enable soil reclamation is promising. It is essential to test important medicinal plants for their salinity tolerance and the economic exploitation of saline soils (Karadge & Gaikwad, 2003). *Catharanthus roseus* (L.) G.Don. is an important medicinal plant of the family Apocynaceae used for treating many fatal diseases, and has good antioxidant potential (Jaleel et al., 2006). Considerable work has already been carried out on this plant with regard to its medicinal importance but the salinity effects and antioxidant potential have attracted little attention. Therefore, in the present investigation, an attempt was made to understand the antioxidant potentials of periwinkle by evaluating both enzymatic and non-enzymatic antioxidants to salinity stress.

Materials and Methods

Plant Culture and Salt Stress Induction

Seeds of *Catharanthus roseus* (L.) G.Don. were surface sterilised with 0.2% mercuric chloride ($HgCl_2$) solution for 5 min with frequent shaking and then thoroughly washed with deionised water. The seeds were pre-soaked in 500 ml of deionised water (control), 50 and 100 mM NaCl solutions for 12 h. Seeds were sown in plastic pots (300 mm diameter) filled with 3 kg of soil mixture containing red soil, sand and farmyard manure at 1:1:1 ratio. Before sowing the seeds, the pots were irrigated with the respective treatment solutions and the electrical conductivity (EC) of the soil mixture was measured. Four seeds were sown per pot and the pots were watered to the field capacity with deionised water up to 90 DAS and great care was taken to avoid leaching. The initial EC level of the soil was maintained by flushing each pot with the required volume of corresponding treatment solution 30, 45, 60 and 75 DAS.

The position of each pot was randomised at 4-day intervals to minimise spatial effects in the greenhouse, where the temperature was 28 °C during the day and 22 °C at night and the relative humidity varied between 60% and 70%. The seedlings were thinned to one per pot 10 DAS. Plants were harvested randomly 90 DAS and analysed for non-enzymatic and enzymatic antioxidant responses. Three plants were taken from each group for analysis.

Non-Enzymatic Antioxidants

Ascorbic acid content was assayed as described by Omaye et al. (1979). The extract was prepared by grinding 1 g of fresh material with 5 ml of 10% TCA, centrifuged at 3500 rpm for 20 min, re-extracted twice and supernatant made up to 10 ml and used for assay. To 0.5 ml of extract was added 1 ml of DTC reagent (2,4-dinitrophenyl hydrazine-thiourea-$CuSO_4$ reagent) followed by incubation at 37 °C for 3 h. Then 0.75 ml of ice-cold 65% H_2SO_4 was added and the mixture obtained was allowed to stand at 30 °C for 30 min. The resulting colour was read at 520 nm in a spectrophotometer (U-2001-Hitachi). The AA content was determined using a standard curve prepared with AA and the results were expressed in mg g^{-1} dry weight (DW).

The reduced glutathione content was assayed as described by Griffith & Meister (1979). Some 200 mg of fresh material was ground with 2 ml of 2% metaphosphoric acid and centrifuged at 17,000 rpm for 10 min. The addition of 0.6 ml of 10% sodium citrate neutralised the supernatant. Then 1 ml of assay mixture was prepared by adding 100 µl of extract, 100 µl of distilled water, 100 µl of 5,5-dithio-bis-(2-nitrobenzoic acid) and 700 µl of NADPH. The mixture was stabilised at 25 °C for 3-4 min. Then 10 µl of glutathione reductase was added, the absorbance was read at 412 nm in the spectrophotometer and the GSH contents were expressed in µg g^{-1} fresh weight (FW).

Antioxidant Enzyme Assays

Crude enzyme extract was prepared for assay of SOD and POX according to Joshi et al. (1984). The enzyme protein was determined by Bradford's (1976) method for expressing the specific activity of enzymes. The procedure and conditions of the assay of these 2 enzymes were as follows.

Superoxide dismutase (EC 1.15.1.1) activity was assayed as described by Beauchamp & Fridovich (1971). The reaction mixture contained 1.17×10^{-6} M riboflavin, 0.1 M methionine, 2×10^{-5} M potassium cyanide (KCN) and 5.6×10^{-5} M nitroblue tetrazolium salt (NBT) dissolved in 3 ml of 0.05 M sodium phosphate buffer (pH 7.8). A 3 ml portion of the reaction medium was added to 1 ml of enzyme extract. The mixtures were illuminated in glass test tubes by 2 sets of Philips 40 W fluorescent tubes in a single row. Illumination was started to initiate

the reaction at 30 °C for 1 h. Identical solutions that were kept in darkness served as blanks. The absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity was expressed in units. One unit (U) is defined as the amount of change in the absorbance by 0.1 h^{-1} mg^{-1} protein.

Peroxidase (EC 1.11.1.7) was assayed as described by Kumar & Khan (1982). The assay mixture of POX contained 2 ml of 0.1 M phosphate buffer (pH 6.8), 1 ml of 0.01 M pyrogallol, 1 ml of 0.005 M H_2O_2 and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C, after which the reaction was terminated by adding 1 ml of 2.5 N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at zero time. The activity was expressed in U mg^{-1} protein. One U is defined as the change in the absorbance by 0.1 min^{-1} mg^{-1} protein.

Ascorbate peroxidase (EC 1.11.1.11) activity was determined as described by Asada & Takahashi (1987). The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 200 µl of enzyme extract. The absorbance was read as the decrease at 290 nm against the blank, and correction was done for the low, non-enzymatic oxidation of ascorbic acid by H_2O_2 (extinction coefficient 2.9 mM^{-1} cm^{-1}). The enzyme activity was expressed in U mg^{-1} protein (U = change in 0.1 absorbance min^{-1} mg^{-1} protein).

Catalase (EC 1.11.1.6) was measured according the method given by Chandlee & Scandalios (1984) with a small modification. The assay mixture contained 2.6 ml of 50 mM potassium phosphate buffer (pH 7.0), 0.4 ml of 15 mM H_2O_2 and 0.04 ml of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in U mg^{-1} protein (U = 1 mM of H_2O_2 reduction min^{-1} mg^{-1} protein).

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). The values are mean ± SD for 7 samples in each group. P values ≤ 0.05 were considered significant.

Results

Effect of Salinity on Non-Enzymatic Antioxidants

There was a significant ($P \leq 0.05$) alteration in the non-enzymatic antioxidant potentials like AA and GSH under salinity stress when compared to control plants. AA showed an increase in all parts of the plant under salinity conditions as the GSH content varied in different parts of the plant and decreased up to 41% in the roots and 42% in leaves in 100 mM NaCl (Figure 1) when compared to untreated plants.

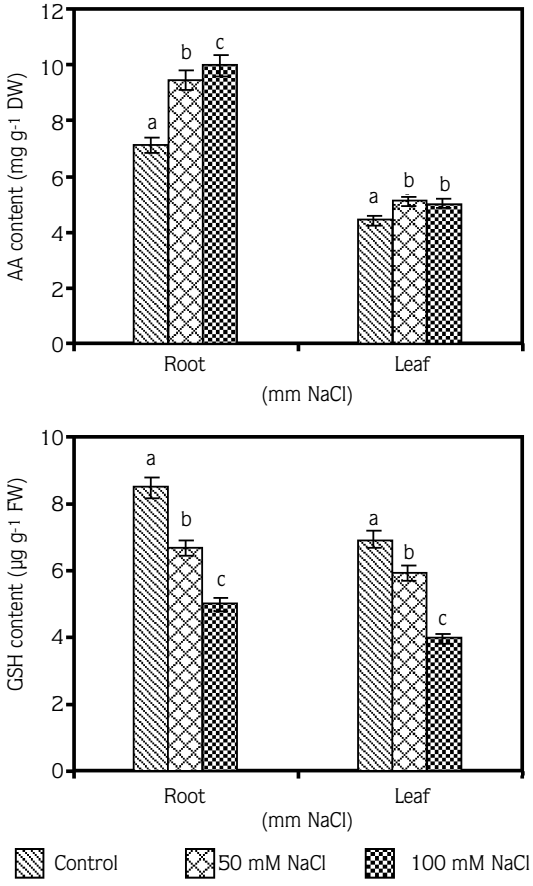

Figure 1. Effect of increasing NaCl concentration on (a) ascorbic acid (mg g^{-1} DW) and (b) reduced glutathione (µg g^{-1} FW) content of *Catharanthus roseus* (L.) G.Don. Values are given as mean ± SD of 7 experiments in each group. Bar values not sharing a common superscript (a,b,c) differ significantly at $P \leq 0.05$ (DMRT).

Effect of Salinity on Antioxidant Enzymes

The antioxidant enzymes such as SOD, POX, APX and CAT showed deviations in their activities under salinity

conditions. In different parts of the plants, the enzyme activities varied greatly. High salinity reduced SOD activity in both roots (28% in high salinity) and in leaves (16% at low and 38% at high salinity), but low salinity led to an increase in root SOD activity up to 2.85% (Figure 2a). In leaves, the POX activity decreased under salinity and in roots it showed a slight increase of up to 10% in low salinity and a 29% decrease in high salinity (Figure 2b) when compared to control plants. Moreover, 100 mM salinity caused an increase in APX in roots (12%) when compared to untreated plants. Similarly, the treatment with 100 mM NaCl induced a significant increase in APX content of about 11% in leaves in comparison with the control (Figure 2c). In contrast to other enzymes, CAT showed minimum activity in leaves but high activity in roots. The increase in CAT activity was not significant in the leaves (at 50 mM NaCl). In the roots, the CAT activity decreased up to 55% at the highest concentration of NaCl (Figure 2d).

Discussion

The non-enzymatic antioxidative mechanisms like GSH and AA responded differently to NaCl treatment. In this investigation, we observed a significant ($P \leq 0.05$) increase in AA content in all the parts of *Catharanthus* plants under NaCl treatment. The increased AA content is correlated with the stress-protecting mechanism of the plant under salinity conditions (Shalata et al., 2001). AA is an important antioxidant, which reacts not only with H_2O_2 but also with O_2^-, OH and lipid hydroperoxidases (Reddy et al., 2004). AA can function as the "terminal

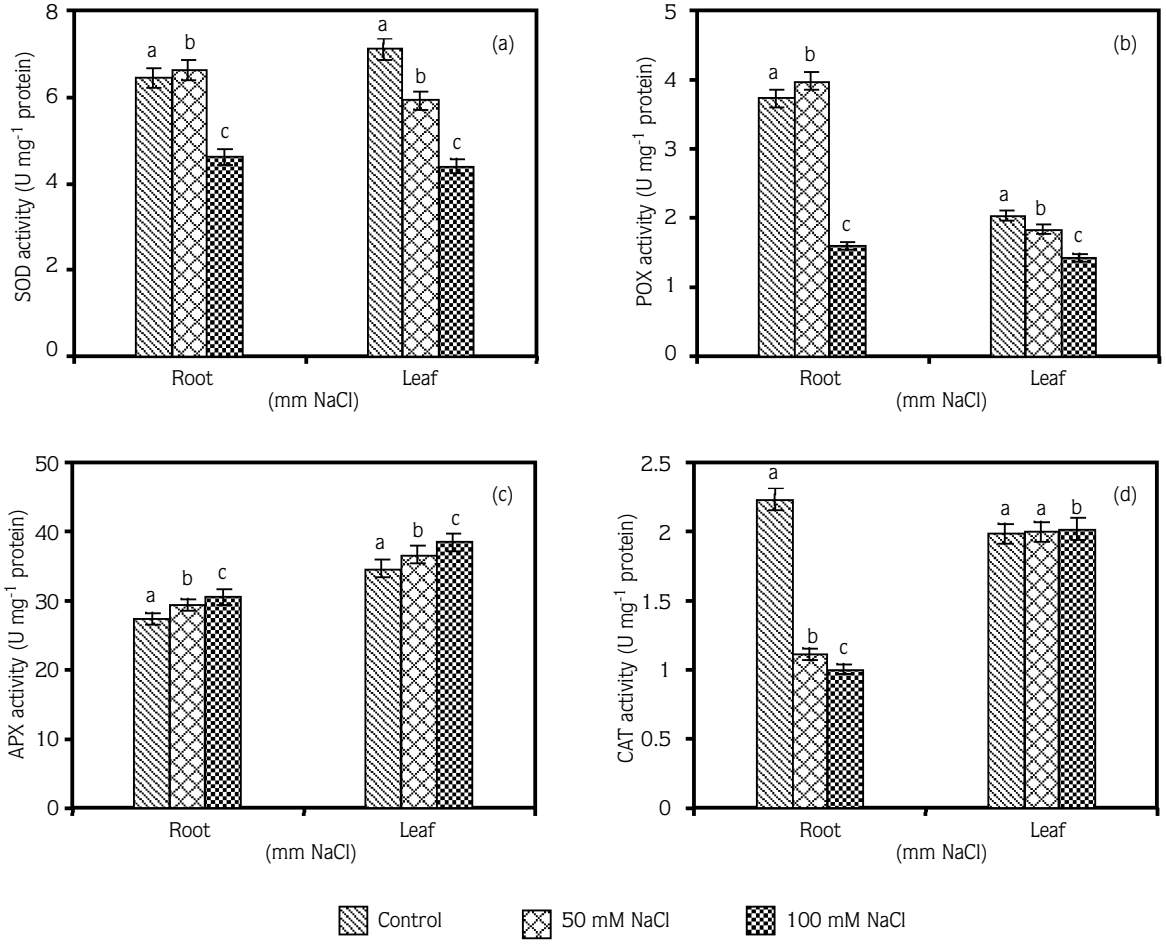

Figure 2. Effect of increasing NaCl concentration on activities of (a) superoxide dismutase (U mg^{-1} protein), (b) peroxidase (U mg^{-1} protein), (c) ascorbate peroxidase (U mg^{-1} protein) and (d) catalase (U mg^{-1} protein) of *Catharanthus roseus* (L.) G.Don. Values are given as mean ± SD of 7 experiments in each group. Bar values not sharing a common superscript (a,b,c) differ significantly at $P \leq 0.05$ (DMRT).

antioxidant" because the redox potential of the AA/monodehydro ascorbate (MDA) pair (+ 280 nm) is lower than that of most of the bioradicals (Scandalios et al., 1997). However, very little is known about the regulation of AA biosynthesis in higher plants. It is conceivable that adaptations of intracellular AA to oxidative stress might clearly depend on the balance between the rates and capacity of AA biosynthesis and turnover related to antioxidant demand (Chaves et al., 2002). A high level of endogenous ascorbate is essential effectively to maintain the antioxidant system that protects plants from oxidative damage due to biotic and abiotic stress (Shigeoka et al., 2002).

We observed a great reduction in GSH content in different parts of *Catharanthus* plants under different concentrations of NaCl treatments. The main functions of GSH in the protection against oxidative stress are its involvement in the ascorbate-glutathione cycle and in the regulation of protein thiol-disulphide redox status (Alscher et al., 1997). GSH also plays a protective role in salinity tolerance by the maintenance of the redox status (Shalata et al., 2001). The decrease in GSH found in this study might be due to the predominant oxidation under salinity conditions and it is necessary for increasing the stress protectant antioxidant enzyme glutathione reductase.

SOD activity directly modulates the amount of ROS. In the present study, a reduced level in SOD activity was found in leaves under high salinity, but a slight increase was found under low salinity in roots. According to Pastori et al. (2000), many stress situations caused an increase in the total foliar antioxidant activity, but in our results we noted only a reduction in foliar antioxidant activity. Reduced foliar SOD activity was reported in rice under salt stress (Dioniso-Sese & Tobita, 1998). In some previous studies the SOD showed an increase and in some a reduction in SOD activity was also noted (Muthukumarasamy et al., 2000; Sreenivasulu et al., 2000; Rout & Shaw, 2001; Sairam & Srivastava, 2002; Pal et al., 2004). The reduction in foliar SOD activity under high salinity can be also a consequence of an altered synthesis and accumulation of less active enzymes (Dioniso-Sese & Tobita, 1998) and/or of a higher turnover of SODs (Chaparzadeh et al., 2004). There was a significant ($P \leq 0.05$) increase in SOD, POX and APX activities of roots under low salinity than high salinity, suggesting the existence of an effective scavenging mechanism to remove ROS, because roots are the first organs that come in contact with salt and are thought to play a critical role in plant salt tolerance. Many variations are shown in previous works like in the pea (Hernandez et al., 2000), wheat (Sairam & Srivastava, 2002; Sairam et al., 2002) and mulberry (Sudhakar et al., 2001). One of the earlier studies carried out in our lab by Muthukumarasamy et al. (2000) in radish showed a reduction in POX activity. The low basal rate and reduction in POX activity of leaves seem to indicate that this enzyme does not take a crucial part in defence mechanisms against oxidative stress or that, suffering POX for salt toxicity, cooperation is activated between different antioxidant enzymes for establishing a proper H_2O_2 homeostasis (Chaparzadeh et al., 2004). In our results, we did not observe any significant ($P \leq 0.05$) increase in CAT activity in leaves under high salinity injury. This result coincides with previous work in rice leaves under salinity stress (Lin & Kao, 2000). The level of CAT activity in roots was reduced under 100 mM salinity in *Catharanthus* plants. Similar observations were made in the case of rice cultivars by Pal et al. (2004). The changes in CAT may vary according to the intensity of stress, time of assay after the stress and induction of new isozyme(s) (Shim et al., 2003). The level of antioxidative response depends on the species, the development and the metabolic state of the plant, as well as the duration and intensity of the stress (Reddy et al., 2004). Our results showed that the activity of antioxidant enzymes under salinity depends on kind, age and organs of plants as well as on the salinity level.

Here, it can be concluded that, at high concentrations, NaCl leads to oxidative stress and in turn causes a significant increase in antioxidative responses. Salinity stress increased the antioxidative mechanisms in roots, which are the immediate organs to suffer from salinity, more efficiently than in leaves. Several defence strategies function in the roots and shoots against oxidative stress. The enhancement in non-enzymatic as well as enzymatic antioxidant responses plays an important role in the regulation of growth for the positive adaptation of plants to salt stress. Our data suggest that antioxidant potential operates in the defence mechanism of *Catharanthus* plants against salinity stress and that the different effectiveness levels of the NaCl treatments in enhancing this potential may be related to the induction of non-enzymatic and enzymatic antioxidants for better mitigation under stress conditions.

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