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Salt stress injury induces oxidative alterations and antioxidative defence in the roots of *Lemna minor*

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Abstract

Lemna minor L. roots were treated with different concentrations of NaCl. Lipid peroxidation was investigated histochemically and biochemically. At higher NaCl concentrations an increase in staining was observed in the root apices as compared to control for lipid peroxidation and loss of membrane integrity as well as an increase in contents of thiobarbituric acid reactive substance and peroxide. Both the non-enzymic antioxidants, ascorbate and glutathione increased with the NaCl concentration in the roots. Whereas an increase in superoxide dismutase, guaiacol peroxidase, and glutathione reductase activities were marked, catalase activity decreased in the roots under NaCl stress.

Additional key words: catalase, guaiacol peroxidase, glutathione reductase, lipid peroxidation, NaCl, superoxide dismutase.

Introduction

Lemna minor L., an aquatic duckweed is a fast growing microphyte and is one of the important component of the natural aquatic ecosystem, which is badly affected with the ever-changing anthropogenic activities in the form of various abiotic stresses (Arber 1963). Salt stress, one of the major environmental stress affecting tremendously the living plants growing in both soil and aquatic environment.

Salt stress is known to cause several physiological changes including oxidative stress (Bowler *et al*. 1992, Shalata and Tal 1998, Hernandez *et al*. 2000, Lee *et al*. 2001). Production of several reactive oxygen species (ROS) like superoxide radicals ($O_2^{\bullet -}$), alkoxyl radicals ($RO^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), perhydroxyl radical ($^{\bullet}HO_2$), *etc*., increases in the presence of NaCl (Hernandez *et al*. 1994, Alia *et al*. 1997, Khan and Panda 2002). They can damage almost every macromolecules (Hendry and Crawford 1994, Alscher *et al*. 1997, Khan *et al*. 2002, Panda 2002). In plant cells both enzymic (superoxide dismutase, SOD, catalase, CAT, ascorbate peroxidase, APX, guaiacol peroxidase, GPX, glutathione reductase, GR) and non-enzymic (ascorbate, glutathione and α-tocopherol) antioxidant defence systems exist, which help in detoxifying the ROS.

As roots are the first affected parts of a plant directly faced to salt stress, the present experiment has been undertaken to study the NaCl-salinity injury and the possible oxidative alterations in the root cells of *Lemna minor* L.

Materials and methods

Floating aquatic microphyte, *Lemna minor* L. was collected from the uncontaminated pond nearby University (90°40′E longitude and 20°04′N latitude) and grown under laboratory conditions under continuous light. Light was provided with white fluorescent tubes (*Philips* 36 W, TLD, India) giving a photon flux density (PFD) of 52 μmol m^{-2} s^{-1}. For salt treatment plants were washed with double distilled water several times and soaked dry and fifty plants were transferred to Petri plates with different concentrations (0, 50, 100, and

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; GPX - guaiacol peroxidase; GR - glutathione reductase; SOD - superoxide dismutase; TBARS - thiobarbituric acid.
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200 mM) of NaCl with three replicates each. The Petri plates were incubated under light at 29 °C for 24, 48 and 72 h. After the treatment minute roots were excised out, soaked dry and sampled for analyses.

The histochemical detection of lipid peroxidation was performed as described by Pompella *et al.* (1987). Root tips were stained with Schiff's Reagent after the roots were rinsed in 0.5 % (m/v) $K_2S_2O_5$ in 0.05 M HCl. It was kept in sulphite solution to retain the staining colour. The localization of the loss of plasma membrane integrity was detected using Evans blue as given by Yamamoto *et al.* (2001). Roots were stained with 10 cm^3 of Evan's Blue solution for 10 min. The roots were washed three times in 100 μM $CaCl_2$ (pH 6.5) after which the dye no longer leaked out the roots. Root tissue (0.2 g) was taken and homogenized with 5 % trichloroacetic acid (TCA) and the homogenate was used for the extraction and estimation of total peroxide content (Sagisaka 1976). Lipid perioxidation was further measured as the amount of thiobarbituric acid reactive substance (TBARS) determined by the thiobarbituric acid (TBA) reaction described by Heath and Packer (1968). 0.2 g of root tissue was homogenized in 2.0 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10 000 *g* of 20 min. To 1 cm^3 of resulting supernatant, 1 cm^3 of TCA (20 %) containing (0.5 %, m/v) of TBA and 10 μM BHT (in 40 % ethanol) were added. The mixture was heated at 95 °C for 30 min and then cooled on ice. The samples were centrifuged at 10 000 *g* for 15 min and absorbance was measured by UV-visible spectrophotometer (*Systronic*, Gujarat, India) at 532 nm and corrected for 600 nm. The concentrations of TBARS was calculated using coefficient of absorbance 155 mmol cm^{-1}. The extraction and estimation of glutathione was done according to Griffith (1980) method. The root tissue was homogenized in 5 % (m/v) sulfosalicylic acid and the homogenate was centrifuged at 10 000 *g* for 10 min. 1 cm^3 supernatant was neutralized with 0.5 cm^3 of potassium phosphate buffer (pH 7.5). Total glutathione content was measured by adding 1 cm^3 neutralized supernatant to a standard solution mixture consisting of 0.5 cm^3 of 0.1 M sodium phosphate buffer (pH 7.5) containing 1 cm^3 EDTA, 0.2 cm^3 of 6 mM 5,5'dithiobis(2-nitrobenzoic acid), 0.1 cm^3 of 2 mM NADPH and 0.1 cm^3 of 1 U cm^{-3} yeast GR Type-III (*Sigma Chemical*, St. Louis, USA). The change in absorbance was measured at 412 nm and followed at 25 ± 2 °C until the absorbance reached 0.5 unit. The ascorbate extraction and estimation was done by the method of Oser (1979). The reaction mixture contained 2 cm^3 2 % Na-molybdate, 2 cm^3 0.15 M H_2SO_4, 1 cm^3 1.5 mM Na_2HPO_4 and 1 cm^3 root tissue extract. It was mixed and incubated at 60 °C in water bath for 40 min, then cooled, centrifuged at 3 000 *g* for 10 min and absorbance was measured at 660 nm.

The root tissue was homogenized with phosphate buffer, pH 6.8 (0.1M in pre chilled motor and pestle. The extract was centrifuged at 4 °C for 15 min at 12 000 *g* in a cooled centrifuge. The supernatant was used for the assay of catalase (CAT), guaiacol peroxidase (GPX), superoxide dismutase (SOD) and glutathione reductase (GR). The CAT and GPX activities were assayed as per the method of Chance and Maehly (1955). The 5.0 cm^3 mixture comprised of 3.0 cm^3 phosphate buffer (pH 6.8), 1 cm^3 (30 mM) H_2O_2 and 1 cm^3 enzyme extract. The reaction was stopped by adding 10 cm^3 2 % H_2SO_4 after 1 min incubation at 20 °C. The acidified reaction mixture was titrated against 0.01 M $KMnO_4$ to determine the quantity of H_2O_2 utilized by the enzyme. The CAT activity was expressed as μmol(H_2O_2 destroyed) g^{-1}(f.m.) min^{-1}. The 3.0 cm^3 reaction mixture comprise of 0.1 M phosphate buffer (pH 6.8), guaiacol (30 mM), H_2O_2 (30 mM) and 0.3 cm^3 enzyme extract. The rate of change in absorbance at 420 nm was measured. The GPX activity was expressed as μmol(H_2O_2 destroyed) g^{-1}(f.m.) min^{-1}. The assay of SOD was done as per the method of Giannopolitis and Ries (1977). 3 cm^3 assay mixture for SOD contained 79.2 mM Tris-HCl buffer (pH 6.8), containing 0.12 mM EDTA and 10.8 mM tetraethylene diamine, bovine serum albumin (0.0033 %), 6 mM nitroblue tetrazolium (NBT), 600 μM riboflavin in 5mM KOH and 0.2 cm^3 enzyme extract. Reaction was initiated by placing the glass test tubes in between two fluorescent tubes (*Philips 20 W*). By switching the light on and off, the reaction was started and terminated, respectively. The increase in absorbance due to formazan formation was read at 560 nm. Under the above condition, the increase in absorbance in the absence of enzyme was 100% and 50% initial was taken an equivalent to 1 unit of SOD activity. Glutathione reductase (GR) was assayed by the method of Smith *et al.* (1988). The reaction mixture contained 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM 5,5′-ditiobis-2 nitrobenzoic acid (DTNB) in 0.01 M potassium phosphate buffer (pH 7.5), 2 mM NADPH, 1 cm^3 enzyme extract and distilled water to make up a volume of 2.9 cm^3. Reaction was initiated by adding 2 mM oxidised glutathione or glutathione disulphide (GSSG). The increase in absorbance at 412 nm was recorded at 25 °C over a period of 5 min. The activity is expressed as ΔA_{412} g^{-1} (f.m.) s^{-1}. All the observations were done triplicates and repeated three times and the data represent mean ± SE.

Results

With the increase in concentration of NaCl supplied for 24 h, an uniform increase in staining pattern especially in the root apices and peripheries was visible as compared to control roots (Fig. 1).

The histochemical observations of lipid peroxidation was supported by quantitative biochemical detection of the event. With the increase in concentration of NaCl contents of total peroxide and thiobarbituric acid reactive substance (TBARS) increased. The increase in total peroxide content was maximum (1300 %) in case of 24-h treatment at 200 mM NaCl and a minimum (220 %) at 50 mM. The TBARS content showed maximum increase (174.3 %) in the case of 72-h treatment at 50 mM NaCl and minimum (102.6 %) in the case of 24-h treatment at 50 mM NaCl as compared to the control (Fig. 2*A*,*B*).

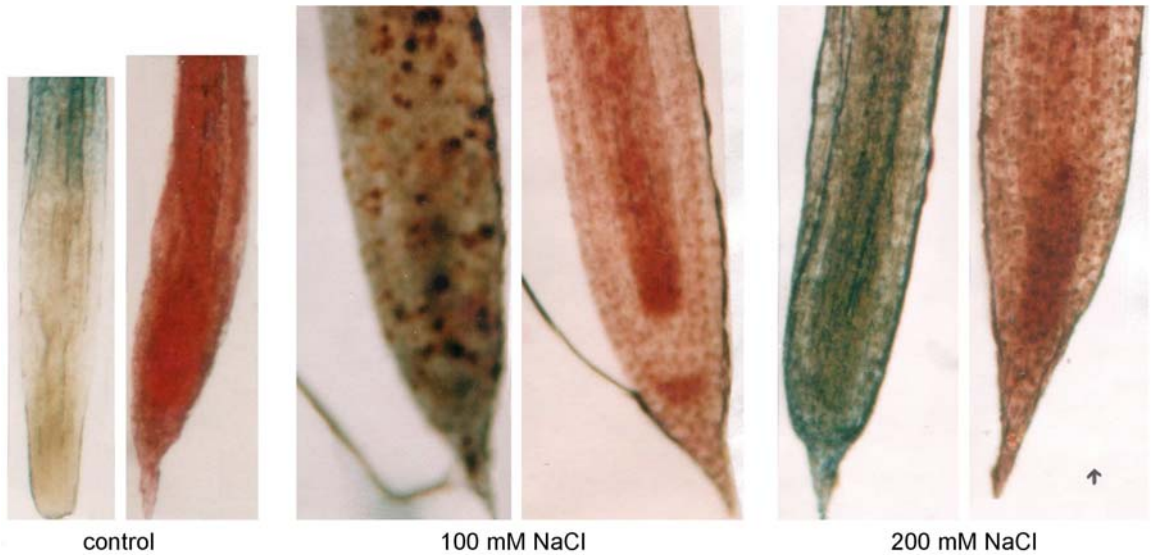

Fig. 1. Histochemical detection of loss of membrane integrity (*left*) and lipid peroxidation (*right*) caused by different concentrations of NaCl in the roots of *Lemna minor* L. *Arrow* indicates 1 mm.

Similarly, the ascorbate content increased with the increase in NaCl concentration and duration of salt treatment. The highest ascorbate content (160.8 %) was found at 200 mM NaCl after 72-h salt treatment, whereas, it was minimum (104.47 %) at 50 mM NaCl after 24-h salt treatment as compared to the control. The glutathione content increased to 112.9 % at 200 mM NaCl after 72-h treatment and only to 105.14 % at 50 mM NaCl (Fig. 2*C*,*D*).

The increase in superoxide dismutase (SOD) activity was 149.5 % at 200 mM NaCl after 72-h treatment and 138.09 % at 50 mM NaCl as compared to the control. The increase in glutathione reductase (GR) activity was the highest (468.9 %) in 200 mM NaCl after 24-h treatment and 129.3 % at 50 mM NaCl as compared to the control (Fig. 3*A*,*B*). Catalase (CAT) activity showed a decrease in control and 50 mM NaCl treatments, whereas an increase in CAT activity was observed at 100 (125 %) and 200 mM (141.03 %) NaCl. The guaiacol peroxidase (GPX) activity markedly increased (454.4 %) at 200 mM concentration after 72-h treatment and slightly (66.4 %) at 50 mM NaCl after 24-h treatment (Fig. 3*C*,*D*). For each NaCl concentration this activity increased with duration of incubation.

Discussion

Using the histochemical staining, lipid peroxidation and loss of membrane integrity was observed on the root surface, but not within any broken lines in the roots. The loss of membrane integrity was observed clearly at the outer limits of broken lines in the roots. Our results showed that the lipid peroxidation was caused due to interaction of NaCl with the root surface, in contrast with the fact that the loss of membrane integrity was caused by the formation of broken lines in the root after a long-term treatment with NaCl (Yamamoto *et al.* 2001, Khan and Panda 2002, Malenčić *et al.* 2003). With the increase in SOD activity more of hydrogen peroxide would be expected to be produced in cell which was substantiated by an accumulation of H_2O_2 in *Lemna* under salt stress (Foyer *et al.* 1994, Qin *et al.* 1998).

Both the non-enzymic antioxidants, ascorbate and

glutathione showed an increase with the increasing salt stress indicating a cellular capacity to overcome the stress (Rataczak and Garnczarska 2000, Lee *et al.* 2001). Though there was increase in SOD, GR and GPX activities, a decrease in CAT activity was marked, implicating a concerted action of antioxidantive enzymes to overcome the oxidative stress resulted by salt stress in the root (Shim *et al.* 1999, Sreenivasulu *et al.* 2000, Hernandez *et al.* 2001, Almansa *et al.* 2002, Malenčić *et al.* 2003). In conclusion, *Lemna* root apex showed tobe a sensitive site for the NaCl-salinity induced oxidative damage and a coordinated antioxidant defence mechanism was involved as a response to salt stress tolerance.

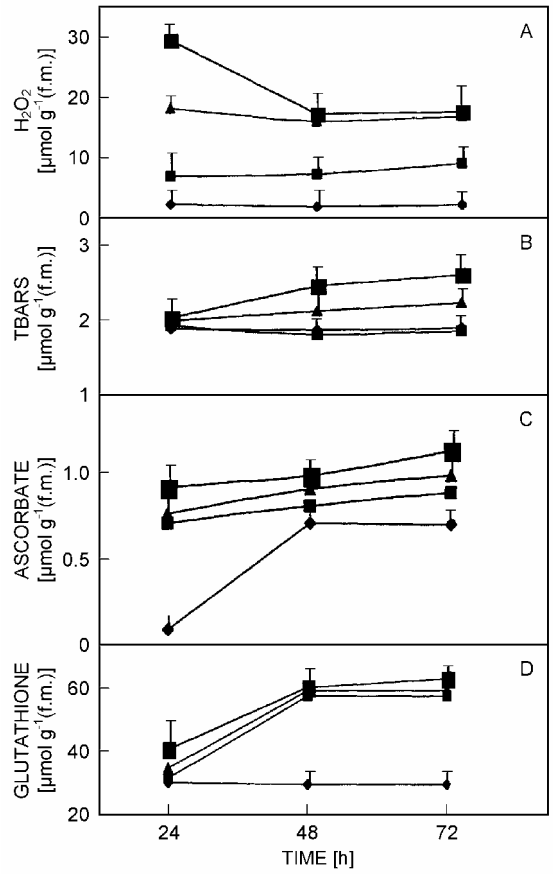

Fig. 2. Changes in total peroxide (*A*), TBARS (*B*), ascorbate (*C*), and glutathione (*D*) contents in roots of *Lemna minor* L. under NaCl of different concentrations (*rhombs* - control, *small squares* - 50 mM, *triangles* - 100 mM, *large squares* - 200 mM NaCl). Means of three separate experiments ± SE.

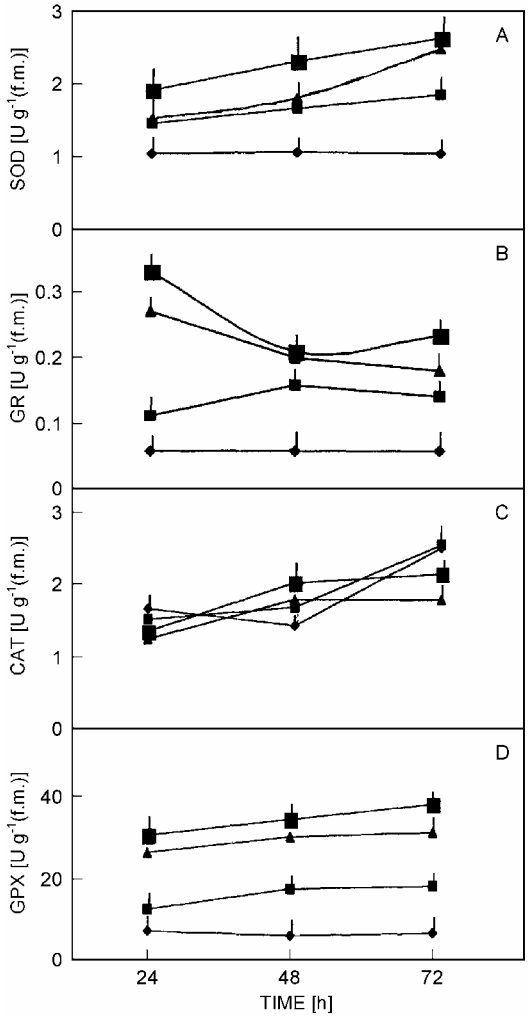

Fig. 3. Changes in SOD (*A*), GR (*B*), CAT (*C*), and GPX (*D*) activities in roots of *Lemna minor* L. under NaCl of different concentrations (*rhombs* - control, *small squares* - 50 mM, *triangles* - 100 mM, *large squares* - 200 mM NaCl). Means of three separate experiments ± SE.

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