Effect of NaCl on leaf salt secretion and antioxidative enzyme level in roots of a mangrove, *Aegiceras corniculatum*

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Short-term salt (NaCl) treatment on *Aegiceras corniculatum* in roots and leaves showed no change in fresh and dry weight of leaves, roots and leaf area. There was no significant change in total soluble root protein, photosynthetic pigments of leaves and spectral characteristics of thylakoids. However, the activity of antioxidative enzymes (catalase, ascorbate peroxidase and guaiacol peroxidase) in roots decreased by 72, 58 and 80% respectively after 96 hr of treatment (300 mM of NaCl). Secretion of salts from the leaf salt glands and salt accumulation on upper surface of the leaves were quantified that revealed linear increase of salt secretion of leaf with increase in period of salt treatment. It was concluded that loss of activities of antioxidative enzymes at high salt treatment, caused leaf senescence in spite of high rates of salt secretion by *Aegiceras corniculatum*.

Plant growth is limited by salt stress. Salt stress reduces leaf water potential, causes ion imbalance or disturbances in ion homeostasis and reactive stress and hence, growth suppression is directly related to total concentration of soluble salts or osmotic potential of the medium in which the plant grows. Short-term effect is mainly after a few hour or within day and long-term effect is after several days of exposure of plant roots to salt. To cope with salt stress, plants respond with physiological and biochemical changes. These changes aim at retention of water in spite of high external osmoticum and maintenance of photosynthetic activity. Accumulation of low molecular weight compounds such as glycine betaine, sugars and proline at balancing water potential when plant exposed to high salinity. In addition to these, salt stress triggers various antioxidative defenses in plants invariably including superoxide dismutase, ascorbate peroxidase and catalase.

Mangroves grow in sheltered intertidal zones at interface between land and sea in tropical and subtropical regions and exposed to varying degree of salinity. Mangrove forest forms unique zones according to gradient salinity in the mangrove ecosystems. Mangroves are divided into two distinct categories on the basis of their salt management strategies. One is secretors which have salt glands or salt hair to secrete excess salt and the other is non-secretors which lack such features to exclude the salt. *Aegiceras corniculatum* of the family Myrsinaceae is a secretor as well as excluder species and exclude 90-97% of salt through ultrafiltration.

Mangroves of India constitute 7% of world’s mangrove vegetation (spreads over 6740 sq km). In Orissa, though mangrove areas are small (215 sq km), but the species density is very high and maximum number of species (62 out of 64) reported from India occurs in Bhitarkanika, Orissa. These mangroves of coastal Orissa have been extensively damaged during 1999 due to cyclone, but their conservation is quite imminent. *Aegiceras corniculatum* is a characteristic element of minor mangroves occurring in Mahanadi delta and they often found in association with other major mangroves like *Rhizophora* and *Ceriops decandra*. *A. corniculatum* has no aerial roots like *Bruguiera parviflora*, only has underground spreading horizontal roots. These roots pass through shallow depth of substratum. This horizontal root system often exposed to the air. All these exposed surface roots serve the respiratory function, but their soil holding capacity and function are much more significant in the frequent tidal inundated deltaic regions.

High salinity intertidal mangrove-habitat imposes restriction on photosynthetic rate of mangrove leaves by high water deficit and low stomatal conductance. *A. corniculatum* is a sensitive species to higher salinity.

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compared to sympatric species *Avicennia marina*, also a secretor has lower water use efficiency and higher leaf area/plant mass ratio\(^3\) and show decline in photosynthetic rate\(^4\). The salt secretion through salt glands increases with increasing salinity. This plant also shows no change in growth even at highest level of heavy metals\(^6\). *Aegiceras* can however, survive and grow under fairly non-saline conditions. The observations on *A. corniculatum* are the results from long-term studies\(^3,4\), but short-term studies are rather scanty, although studies on a sympatric species *A. marina* are there\(^5\). Here, we have examined the effect of short-term exposure to high salt (salt shock) on biomass, leaf salt secretion, photosynthetic pigments of leaves and thylakoids, spectral characteristics of thylakoids and antioxidative enzymes like catalase (CAT), guaiacol peroxidase (POX), ascorbate peroxidase (APX) activity of roots.

**Materials and Methods**

*Plant material and growth conditions—* Propagules of *Aegiceras corniculatum* (L.) Blanco were collected from mangrove forests of Bhitarkanika (latitude 20°4′ to 20°8′N; longitude 86°45′ to 87°30′E) Orissa, India and was raised in the net house. Plants were watered with non-saline and non-brackish water. Three months old plants were taken and washed thoroughly with tap water to remove soils from root system, washed in distilled water and then transferred to half-strength Hoagland’s medium\(^1\) for hydroponic culture. These cultures were aerated for 24 hr by air bubblers. The experiment was performed under controlled temperature (22±2°C day/night); under a photon flux density of 300 μmole m\(^{-2}\)s\(^{-1}\) with a photoperiod of 14 hr and 80% RH. Plants were harvested, leaves and roots were used for biochemical determination and measurement of biomass. Plants only in Hoagland’s medium were designated as control and those in the aforesaid medium containing NaCl (300mM) as treated plants. Effect of short-term exposure to salinity on biomass was determined by measuring fresh and dry weight of leaves and roots, foliar area. Enzymatic activity was measured at an interval of 24 hr.

*Measurement of biomass—* *Aegiceras corniculatum* is a salt-sensitive species. In a pilot experiment, we grew nursery-raised three month-old plants at different concentrations of NaCl (100, 150, 200, 250 and 300 mM) for 30 days supplemented with Hoagland’s medium (Fig. 1). The plants could survive in NaCl (300 mM) supplemented medium for 10 days, while at lower concentrations, the plants survived for more than 4 weeks. At 300mM NaCl concentration, plants showed sign of senescence after 10 days under present experimental conditions. We, therefore, analyze the effect of short-term exposure to NaCl (300 mM) on *A. corniculatum*. After 96 hr of NaCl (300 mM) treatment, roots and leaves were washed with tap water to remove surface salt and other materials, dried with blotting paper, weighed and then dried in an oven to constant weight at 80°C. Area of leaves was measured graphically.

*Extraction of enzymes—* Roots were cut (2cm) from lower end and washed properly with distilled water to remove surface particles, if any, then blotted and weighed. They were homogenized under ice-cold condition in a chilled pestle and mortar with a grinding medium [(3 ml, buffer g\(^{-1}\) fresh weight of roots) containing 100 mM of phosphate buffer (pH 8); 1 mM, ethylenediaminetetraacetic acid (EDTA); 10mM sodium ascorbate, 0.11%, mercaptoethanol; and 10%, polyvinylpolypyrrolidone. Homogenate was centrifuged at 10,000g for 10 min and supernatant was taken for assay of catalase (CAT), guaiacol peroxidase (POX) and ascorbate peroxidase (APX). All assays were done at room temperature.

*Assay of enzyme activity—* Activity of catalase was determined by monitoring the disappearance of H\(_2\)O\(_2\) at 240 nm (molar absorption coefficient, 39.4 mM\(^{-1}\) cm\(^{-1}\)) following the procedure as described earlier\(^6,10\). The assay mixture contained 50 mM of phosphate buffer (pH 7) and 20 mM of H\(_2\)O\(_2\). The activity of peroxidase and ascorbate peroxidase was determined following the procedure as described\(^25\) and modified earlier\(^28\). The assay mixture for general peroxidase contained 50 mM of phosphate buffer (pH 6), 0.8 mM, EDTA; 1 mM, H\(_2\)O\(_2\); and 0 mM, guaiacol. For ascorbate peroxidase, assay medium was same as that for guaiacol peroxidase except using ascorbate as electron donor. Guaiacol peroxidase was measured by monitoring the increase in absorbance at 470 nm (absorption coefficient 26.6 mM\(^{-1}\)cm\(^{-1}\)) due to tetraguaiacol formation. Oxidation of ascorbate was followed by decrease in absorbance at 290 nm (absorption coefficient 2.8 mM\(^{-1}\)cm\(^{-1}\)).

*Protein extraction and analysis—* Root proteins were extracted following TCA-acetone method of Damerval\(^7\) and total soluble root proteins were resolved in SDS-PAGE following the method of Laemmli\(^16\).

*Protein estimation—* Protein concentration was evaluated by the method as described earlier\(^7\) using bovine serum albumin as standard.
Native PAGE and activity staining of antioxidant enzymes — Native polyacrylamide gel electrophoresis (PAGE) was performed at 4°C for catalase (CAT), and peroxidase (POX) using Laemmli\textsuperscript{18} buffer systems with an exception that SDS was absent from all buffers. Samples were mixed with 10% glycerol (v/v) and 0.25%, bromphenol blue before loading onto the gels. For each lane, 5 µg of protein extract was applied. The gels were run at constant voltage of 200 V at 4°C in Bio-Rad (USA) Mini protein II electrophoresis apparatus until bromphenol blue marker dye had swept through most of the gel.

Polyacrylamide gel (7.5%) containing 0.5% soluble starch was prepared for CAT activity staining, the gels were stained\textsuperscript{24} and visualized for CAT activity. Immediately after electrophoresis, the gel was incubated in a solution containing sodium thiosulphate (18 mM) and 0.5% H$_2$O$_2$ (w/v) for 30 sec at room temperature. The gel was rinsed with distilled water and flooded with potassium iodide solution (90 mM) acidified with 0.5% glacial acetic acid. Negative bands of CAT enzymes were appeared in blue back ground of the gel.

Activity of POX in the gels was visualized on PAGE (7.5%) according to staining procedure of Scandalios\textsuperscript{30}. The gels were incubated at room temperature in the staining solution containing 49 mg O-dianisidine, 29 mg β-naphthol, 20 ml acetone, 10 ml of 0.1 M Tris-acetate buffer (pH 4), 0.3 ml of 30% H$_2$O$_2$ and 70 ml water. Brick red colour bands appeared on gel, the reaction was arrested by immersing the gel into a large volume of acetic acid (7%) for 10 min after staining of bands sufficiently.

Thylakoids isolation — Thylakoids were isolated following the procedure of Nakatani and Barber\textsuperscript{36}. Leaves were homogenized in chilled mortar and pestle using ice-cold grinding buffer (leaf to buffer ratio 1:8) containing 15 mM tricine (pH 7.5), 5 mM MgCl$_2$, 400 mM sorbitol and homogenate was filtered through 4 layers of nylon and 4 layers of cheese cloth, and the resulting slurry was centrifuged at 2000 g for 5 min. The pellet was washed twice in a medium containing 10 mM tricine (pH 7.5), 10 mM NaCl, and 5 mM MgCl$_2$ at 2000 g for 5 min and finally the pellet was suspended in a buffer containing 20 mM Hepes (pH 7), 10 mM, NaCl, 5 mM, MgCl$_2$; and 100 mM, sorbitol. Chlorophyll and carotenoids were determined as mentioned in following para.

Chlorophyll and carotenoids determination — Chlorophyll and carotenoids were extracted by homogenizing fresh leaves (0.1 g) in 100% methanol (2 ml). After centrifugation for 10 min at 2000 g, chlorophylls and carotenoids content were determined spectrophotometrically following the procedure described earlier\textsuperscript{29} (Chl a (µg/ml) = 15.65 × A$_{665}$ - 7.34 × A$_{653}$; Chl b (µg/ml) = 27.05 × A$_{663}$ - 11.21 × A$_{646}$; Carotenoids (µg/ml) = [1000 × A$_{470}$ (2.86 × Chl a) - 129.2 × Chl b]).

Salt secretion rate — Salt secretion rate was measured under culture room conditions (see plant materials and growth conditions). The leaves were washed with distilled water followed by blotting with a paper towel just prior to onset of photoperiod. Secreted salt (NaCl) was weighed at an interval of 24 hr. After 24 hr, the leaves were rinsed with distilled water and again blotted with paper towel\textsuperscript{4}.

Results and Discussion

Salt secretion — Aegiceras corniculatum secretes salt on the upper surface of the leaves. The secreted salt constitutes of number of ions\textsuperscript{22}, but the secretion consists mainly of NaCl\textsuperscript{3}. It has been reported that salts are accumulated during day and removed during the night and therefore, there is an increase in salt secretion at night. In this way, mineral content of the plant is regulated and compartmented in the leaves that contained the salts during the day are emptied during the night to be available for a new supply in the following day\textsuperscript{29}. Salt secretion is very effective in maintaining salt balance at low to moderate salinity and ineffective at high salinity\textsuperscript{28}. At moderate salinity, salt secretion increases with increasing salinity\textsuperscript{16}.

Plants of A. corniculatum were raised in different concentrations of NaCl (100, 150, 200, 250 and 300 mM) supplemented with Hogland’s solution (Fig. 1). The plant could survive in 300 mM of NaCl for 10 days. With increase in salt treatment period of A. corniculatum, the rate of salt secretion increased linearly. After 24 hr of NaCl (300 mM) treatment, 2.95 µmole cm$^{-2}$ of salt secreted and after 96 hr salt secretion increased to 13.732 µmole cm$^{-2}$ with a secretion rate of 0.135 µmole cm$^{-2}$ hr$^{-1}$ (Fig. 2). No salt was found to be deposited on lower surface of the leaves. These results are different from that of Ball\textsuperscript{1}, may be due to light intensity and humidity.

Spectral characteristics of thylakoids — There was not much change in spectral characteristics of thylakoids upon salt treatment (Fig. 3). The spectrum showed slight decrease in intensity and broadening of peak at 680 nm in salt treated thylakoids. Some of the cross over points were observed at 695.5 and 665 nm after normalization of spectrum at 680 nm.
Measurement of photosynthetic pigments, proteins and biomass — Effect of NaCl (300 mM) on the fresh weight, dry weight and leaf area of control and treated plants has been shown in Table 1. Biomass did not change significantly after 96 hr of salt treatment. Fresh weight to dry weight ratio remained same (~4.0) and leaf area to dry weight ratio remain unaltered. Photosynthetic pigments (Chl a, Chl b and carotenoids) did not change significantly upon high salt treatment. Total soluble root proteins were measured at an interval of 24 hr and it was found that there was not appreciable change in the protein concentration (Fig. 4). SDS-PAGE analysis of proteins of salt-treated and control roots also showed no change in protein profile (Fig. 6) that ranges from ~14 to 94kDa. This is expected as there was no significant change in fresh wt and dry wt during the experimental period.

**Effect of NaCl on activity of antioxidant enzymes** — Root is the primary sensor of salt stress. In response to salinity, number of antioxidant enzymes increase or decrease invariably including catalase, peroxidase and ascorbate peroxidase. Therefore, we examined effect of high NaCl on three antioxidant enzymes, catalase (CAT); guaiacol peroxidase (GPX); and ascorbate peroxidase (APX), in roots. There was an inhibition of activity of these antioxidant enzymes. Activity of CAT and GPX was inhibited more than APX. Activity of these enzymes decreased by about...

![Graph showing salt secretion on the leaf of A. corniculatum in control and treated plants. Secreted salt was measured at intervals of 24 hr and amount of salt expressed in leaf area basis. Data are mean of two independent experiments.](image)

**Table 1** — Effect of NaCl treatment on plant growth parameters. Samples were taken after 96 hours. Data are mean of two independent experiments. Each value represents mean of 5 replicates.

<table>
<thead>
<tr>
<th>Fresh wt/Dry wt</th>
<th>Leaf area/Dry wt</th>
<th>Photosynthetic pigments mg g⁻¹ dry wt</th>
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<tbody>
<tr>
<td></td>
<td>Chl a</td>
<td>Chlb</td>
</tr>
<tr>
<td>Fresh wt/Dry wt</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>Root</td>
<td>4.5</td>
<td>4.17</td>
</tr>
<tr>
<td>Leaf</td>
<td>4.37</td>
<td>4.05</td>
</tr>
<tr>
<td>Leaf area/Dry wt</td>
<td>0.093</td>
<td>0.12</td>
</tr>
</tbody>
</table>

![Image showing hydroponic culture of Aegiceras corniculatum exposed to different concentrations of NaCl exposure for long term; (b) Three months old nursery grown plants were taken for hydroponic in Hoagland’s medium in a culture room.](image)
Fig. 3 — Room temperature absorption spectra of thylakoid membranes isolated from control and NaCl treated *A. corniculatum*. Thylakoids equivalent to 6µg Chl ml⁻¹ were suspended in a buffer containing 100 mM sorbitol; 10 mM NaCl; 5 mM MgCl₂; and 20 mM Hepes (pH 7). The dotted line and the solid line graph represent the spectra of control and salt-treated samples respectively.

90% of environmental control (Fig. 5). Activity of APX, CAT, and GPX decreased by 58, 72 and 80% respectively as compared to that of hydroponic control (Fig. 5 inset). Staining of peroxidase (POX) and catalase (CAT) showed presence of 4 isoforms of peroxidase (POX 1, 2, 3 and 4) and two of catalase (CAT 1 and 2) in roots and there was also decrease in activity of these enzymes in salt treated roots (Fig. 7a, b). Guaiacol peroxidase activity is a general measure of peroxidase activity and its decrease reflects the loss of ability of plants to sustain oxidative damage by salt shock.

Decrease in activity of enzymes upon salt treatment was also reported in roots of glycophytes²² and
Fig. 6 — SDS-PAGE showing the protein patterns of control and salt-treated roots of A. corniculatum. Root proteins were extracted after 96 hr of salt treatment [Lane M: Molecular mass markers in kDa; Lane C — Control; Lane T—salt-treated].

Fig. 7 — Effect of NaCl on antioxidant enzymes extracted from roots of Aegiceras corniculatum. Enzymes were activity stained following standard procedure: (a) Activity staining of peroxidase; and (b) Catalase. [Lane C — control; and Lane T — salt treated]

halophytes. Ashiara et al. examined seven enzymes from the leaves of secretor mangrove A. marina and found that six of the enzymes have decrease in their activity as NaCl concentration increases. All seven enzymes have inhibition at 500 mM of NaCl. Therefore, present results are in agreement with earlier results on mangroves. Although the antioxidative enzyme, superoxide dismutase (SOD), plays important role against oxidative stress, the levels of SOD in the present study was not measured.

It was concluded from the present study that the high salt concentration (≥ 300 mM) suppressed activity of antioxidative enzymes without losing the ability to secrete salt. This immediate loss of activity of antioxidative enzymes might induce leaf senescence after short term salt exposure.

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