



## Changes in proteins and antioxidative enzymes in tree mangroves *Bruguiera parviflora* and *Bruguiera gymnorrhiza* under high NaCl stress

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### Abstract

Mangroves possess special cellular mechanisms to cope with the salty and uncompromising environment. In order to access the role of protein and antioxidative enzymes defense system during high salt shock, two *Bruguiera* species *B. parviflora* and *B. gymnorrhiza* of the family Rhizophoraceae were exposed to high salt shock (500 mM NaCl) for a short period of 6days. Total protein content in both the species decreased upon salt shock but the rate of degradation was more rapid in *B. parviflora* than in *B. gymnorrhiza*. SDS-PAGE protein profiling revealed that the protein having apparent molecular mass 90kDa, 49kDa, 33kDa, 23kDa, 10kDa reduced very first after 4days of salt treatment of *B. parviflora* than in *B. gymnorrhiza*. The specific activities of catalase increased 2.4 times and 2.1 times in *B. parviflora* and *B. gymnorrhiza* upon exposure to high salt. Out of the four isoforms of catalase, CAT-2 activities enhanced 1.5 times and 1.2 times upon initial salt treatment for 4 days and 6 days. APX activity increased 1.3 times and 2.3 times in *B. parviflora* and *B. gymnorrhiza* under salt shock. A single isoform of APX enhanced 1.3 times and 1.7 times in *B. parviflora* and *B. gymnorrhiza*. The GPX activity increased 1.5 times and 1.3 times in leaves of salt treated seedlings of *B. parviflora* and *B. gymnorrhiza*. The ability of salt adaptability in both the species were discussed in context to their afforestation programme.

**Key words:** Mangroves, *Bruguiera parviflora*, *Bruguiera gymnorrhiza*, Antioxidative enzyme, Sodium chloride

### 1. Introduction

Mangrove forests are widely distributed in the inter-tidal zones of the tropical and subtropical areas of the globe (Hogarth, 1999). The mangrove plants in the forest are adapted to harsh environments like marshy anoxic anaerobic soil and fluctuating salinity of the water bodies with of several morphological and anatomical features (Nandy Datta et al., 2007). The mangroves are divided in to secretor (possess salt gland or salt hairs) and non-secretor group (do not possess such salt secreting features) on the basis of their salt management strategies. They manage salt stress in variety of ways like ultrafiltration by roots, Na<sup>+</sup>/ H<sup>+</sup> exchange and sodium accumulation in the xylem sap (Takamura et al., 2000). *Bruguiera parviflora* (BP) and *Bruguiera gymnorrhiza* (BG) are representative of non-secretor true tree mangroves with widespread large-leaf which are known to own differential limits for salt tolerance (Sevam et al., 2002). Some of the earlier reports describe the effect of salt stress on plants, which are complex, but largely it imposes a water deficit because of osmotic effects on a wide variety of metabolic activities (Green way and Munns, 1980; Cheesman, 1988). Mangroves synthesize osmotically active metabolites, specific salt inducible proteins and regulate water fluxes and support scavenging oxygen radicals and chaperons to avoid this adverse condition (Jithesh et al., 2006). Soluble protein contents of leaves usually get reduced in response to salinity (Gadallah, 1999; Wang and Nil, 2000; Parida et al., 2002). Agastiana et al. (2000) have reported that soluble leaf protein were increased at low salinity level and decreased at high salinity in mulberry, a glycophyte. It has also been reported that high salinity could decrease of several proteins of molecular mass 17, 23, 32, 33 and 34 kDa in *B. parviflora* (Parida et al., 2004b). In this

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investigation, we have analyzed the salt tolerance mechanism in two related species of *Bruguiera*, *B. parviflora* and *B. gymnorrhiza* by taking protein and antioxidative enzymes as potential indicators of salt shock.

## 2. Materials and methods

Propagules of *B. parviflora* and *B. gymnorrhiza* were collected from the mangrove forest of Bhitarkanika deltas of Orissa, India (latitude 20°4'N to 20°8'N; longitude 86°45'E to 87°50'E). Seedlings were raised in a greenhouse under PAR of 677 to 1040  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , and were grown in non saline and non-brackish water using standard nursery practices. Three months old, healthy seedlings of six leaved propagules were used for hydroponics culture in full strength Hoagland's nutrient medium (Hoagland and Arnon, 1940). These cultures were aerated continuously and were maintained in a growth chamber at  $22\pm 2^\circ\text{C}$ , 80% RH, 14h photoperiod, and a light intensity of 300  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . A pilot experiment was carried out with BP and BG using 400, 500, 600 and 800 mM NaCl for 0d, 7d, 14, 21 days in order to determine the lethal dose of salinity. Since both the species survived for 7 days in 500 mM NaCl concentration in the above experiment this experiment was conducted in a single 500mM NaCl dose for 0, 2, 4, 6 days. Second pairs of laves from the apical leaf of plant from different days of treatment were collected to measure the experimental parameters and the experiments were repeated twice with three replicas in each treatment.

### 2.1. Extraction and estimation of total leaf protein

For estimation of total soluble protein, 0.5 g chilled sample tissue was homogenize in ice cold 10% TCA and was incubated over night at 4°C. The precipitates were centrifuged at 10000  $\times g$  for 10 min, pellets were washed with 100% acetone and pigment free pellets were successively washed with 80% ethanol, 3:1 (v:v) ethanol:chlorophorm and ether to remove phenolic compounds. Pellets were suspended in known volume of 0.1N NaOH and estimation of protein was made according to Lowry et al., (1951). Proteins in the unknown sample were estimated at 660nm wavelength, using de-fatted bovine serum albumin (fraction V, Sigma, USA) as a standard; the results were expressed on the basis of grams per unit fresh wt (gm/fresh wt) basis.

### 2.2. Analysis of protein profile of leaf by SDS-PAGE

Leaf samples (0.5 g) each of control and treated plants were homogenized with 2 ml of 50 mM Tris-Glycine buffer (pH 8.3) in chilled mortar and pestle at 4°C. The homogenate was centrifuged at 14,000  $\times g$  for 10 min at 4°C and the protein concentration of the supernatant samples were estimated according to the method of Bradford (1976). The supernatant sample containing 40 $\mu\text{g}$  of protein were mixed with equal volume of solubilizing buffer containing 62.5 mM Tris-HCl (pH 6.8), 20% glycerol, 25(w/v) SDS, 0.5% 2-mercaptoethanol and 0.01% bromophenol blue and heated for 4 min at 95°C. Gel was made according to Laemmli (1970) with some modification. A 12.5% SDS-PAGE separating gel containing 375 mM Tris-HCl (pH 8.8), 0.1 % (w/v) ammonium persulfate and 0.4 $\mu\text{l ml}^{-1}$  TEMED where as 4% stacking gel containing 125 mM (pH 6.8), 0.1 % (w/v) SDS, 0.05 (w/v) ammonium persulfate and 0.5  $\mu\text{l ml}^{-1}$  TEMED was used to concentrate (stack) the polypeptides. The electrophoresis running buffer consisted of 25 mM Tris, 192 mM glycine (pH 8.3), 0.1% SDS. Gel was run at a constant 35 mA for 2 h using Consort N.V. (Belgium) mini vertical electrophoresis system with running buffer used by Laemmli (1970). Gel staining was made in 0.1% Coomassie Brilliant Blue R-250 for overnight and de-stained with 5% methanol and 10% acetic acid until background was clear. The gel was photographed and scanned using densitometer (GS-710, Bio-Rad, USA). The protein profile was analyzed using Quantity One software (Bio-Rad, USA) for quantification of each bands and determination of relative molecular weight of each protein band.

### 2.3. Extraction of antioxidative enzymes and their assays

#### 2.3.1. Preparation of enzyme extract

Leaf samples (1 gm) was homogenized with pre-chilled motor and pestle with 2ml of 50mM potassium phosphate buffer (pH 7.0), 1mM EDTA, 1mM D-isoascorbic acid, 2%(w/v) PVP (polyvinyl polypyrrolidone) and 0.05 % (w/v) Triton X-100 following the procedure of Gossett et al. (1994). The homogenate was centrifuged at 10,000  $\times g$  for 10 min at 4°C. The supernatant were collected and used for the assay of catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX).

#### 2.3.2. Assay and activity staining of catalase (CAT) (EC 1.11.1.5)

CAT activity was determined spectrophotometrically by following the rate of  $\text{H}_2\text{O}_2$  disappearance at 240nm taking change in  $\Delta\epsilon$  240 nm at 36  $\text{M}^{-1}\text{cm}^{-1}$  (Patterson et al., 1948). The reaction mixture contains 50 mM potassium phosphate (pH 7.0); 10.5 mM  $\text{H}_2\text{O}_2$  and the enzyme extract containing 20 mg of protein (Miyagawa et al., 2000). The reaction was run at 27°C for 2 min and initial liner rate of decrease in O.D. was used to calculate the activity. A polyacrylamide gel (7.5%) containing soluble starch was prepared and stained for catalase activity following the

staining procedure of Thorup et al., (1961). The gel was incubated in 18 mmol/L sodium thiosulphate and 679 mmol/L  $H_2O_2$  for 30 sec at room temperature (25°C). The gel was then rinsed with distilled water and flooded with 90 mmol/L potassium iodide solution acidified with 0.5% acetic acid. Deep blue bands were visualized on light blue background of gel.

### 2.3.3. Assay and activity staining of ascorbate peroxidase (APX) (EC 1.11.1.11)

APX was assayed at 25°C as described by the method of Nakano and Asada (1994). The reaction mixture contains 50 mM potassium phosphate (pH 7), 0.2 mM EDTA 0.5 mM ascorbic acid and 0.25 mM  $H_2O_2$ . The reaction was started by addition of  $H_2O_2$  after adding the enzyme extract containing 50µg of protein. The decrease in absorbance at 290 nm for 1min was recorded and the amount of ascorbate oxidized was calculated from the extinction coefficient  $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ . APX in-gel assay (Mittler and Zilinskas, 1993) was run using a 10% separating and 4% stacking gel. For the aforesaid assay, 2 mM ascorbic acid was added to the electrode buffer and the gel was pre-run for 30 min before the samples were loaded. After electrophoresis the gel was immersed in a solution of 50 mM sodium phosphate solution containing 2 mM ascorbic acid for 30 min, changing in the solution three times in every 10 min. The gel was then soaked in 50 mM sodium phosphate buffer pH 7.0 containing 4 mM ascorbic acid and 20 mM  $H_2O_2$  for an additional 20 min before washing briefly 50 mM sodium phosphate buffer (pH 7.0). Finally, the gel was incubated in a solution of 50 mM sodium phosphate buffer (pH 7.8), 28mM TEMED except at position and 2.45 mM NBT until the gel turned uniformly blue except at position exhibiting APX activity and the reaction was stop by washing it with distilled water.

### 2.3.4. Assay and activity staining of guaiacol peroxidase (GPX) (EC. 1.11.17)

GPX activity was measured spectrophotometrically at 25°C by following the method of Tatiana et al. (1999). The reaction mixture (2ml) consisted of 50 mM potassium phosphate (pH 7.0), 2 mM  $H_2O_2$ , and 2.7 mM guaiacol. The reaction was started by the addition of an enzyme extract equivalent to 5 µg protein. The formation of tetra guaiacol was measured at 470nm ( $\epsilon=26.6 \text{ mM}^{-1} \text{ .cm}^{-1}$ ). Activity of GPX was visualized on 7.5% PAGE according to staining procedure of Birecka and Faraway (1975). The gels were washed in distilled water and incubated at room temperature (in dark) in staining solution containg 50 ml of 50 mmol/L sodium acetate buffer (pH 5.0), 330 µl of 30%  $H_2O_2$  until reddish brown bands appeared on gel. The reaction was arrested by immersing the gel into 7% acetic acid for 10min after bands stained sufficiently.

## 3. Results

### 3.1. Changes in total leaf soluble protein content

Total soluble protein content in both species of *Bruguiera* decreased upon exposure to 500mM NaCl for a short period of 6 days (Figure 1). Total soluble leaf protein content in *B. parviflora* and *B. gymnorrhiza* decreased 4.5fold and 3.4 fold respectively after 6 days of salt treatment as compared to control.

### 3.2. Changes in leaf protein SDS-PAGE profiling

The leaf protein were extracted from control and salt treated plant leaves samples and analyzed by SDS-PAGE (Figure 2). SDS-PAGE analysis of total protein profiling indicated that no differences was found in number of protein band upon exposure to salt treatment. But the intensities of several protein bands having apparent molecular mass like 90kDa, 50kDa, 33kDa, 23kDa, 16kDa reduced severely in salt treated samples compared to control in both the species.

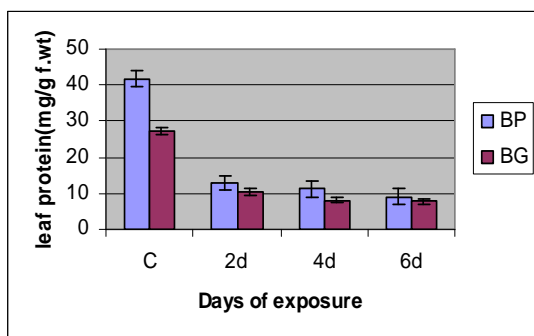


Figure 1. Effect of NaCl on total soluble proteins in leaves of *B. parviflora* and *B. gymnorrhiza* in different days for 500mM NaCl treatment.

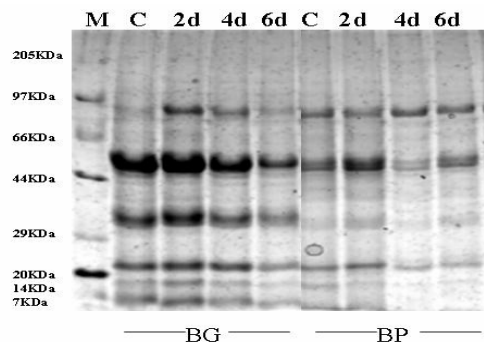


Figure 2. Effect of 500mM NaCl on SDS PAGE profile of leaves of *B. parviflora* and *B. gymnorrhiza* in different days of treatment.

From densitometry scan of individual band showed that in *B. parviflora* relative densities of these protein bands reduced nearly two folds compared to control (Table 1). In 4d salt treated sample where as the changes in these protein bands was more in 6d treated plant compared to control.

Table 1. Relative changes in average density of leaf protein bands in response to 500mM NaCl treatment in *B. parviflora* and *B. gymnorrhiza*

Days/Species	Protein bands with relative density				
	90 kDa	50 kDa	33 kDa	23 kDa	16 kDa
<b><i>B. parviflora</i></b>					
Control	1	1	1	1	1
2 days	1.1	1.2	1.2	1.13	0.93
4 days	0.99	0.73	0.88	1.08	0.88
6 days	1.12	0.89	0.93	0.99	0.73
<b><i>B. gymnorrhiza</i></b>					
Control	1	1	1	1	1
2 days	1.35	1.06	1.32	1.35	0.88
4 days	1.18	0.87	0.82	0.84	0.61
6 days	0.97	0.71	0.85	0.73	0.35

### 3.3. Changes in catalase activity

Catalase activity increased 2.4 times and 2.1 times in *B. parviflora* and *B. gymnorrhiza* up on exposure to NaCl compared to control (Figure 3a). Native PAGE and activity staining data revealed that four isoforms of catalase (CAT-1, CAT-2, CAT-3, CAT-4) enhanced in salt treated sample in both the species. But in *B. gymnorrhiza*, only one isoform of catalase (CAT-2) was found in control sample. In *B. gymnorrhiza*, the intensity of CAT-2 enhanced 1.5 times and 1.2 times upon initial salt treatment for 4 days and 6 days compared to control. Where the other isoforms, the changes were not so significant. In *B. parviflora*, all the four isoforms of catalase increased continuously upon exposure to high salt for 6 days (Figure 3b).

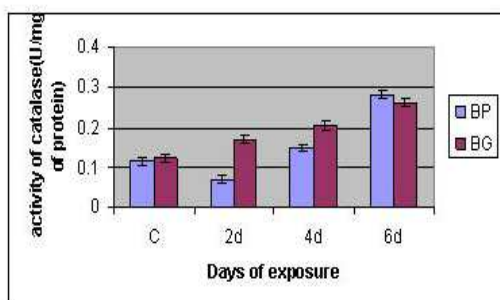


Figure 3a. Effect of 500mM NaCl on catalase activity of *B. parviflora* and *B. gymnorrhiza* in different days of treatment

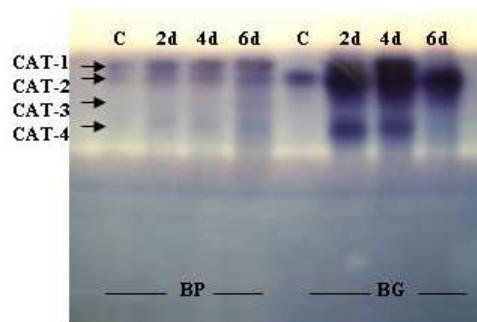


Figure 3b. Effect of 500mM NaCl on CAT profile of *B. parviflora* and *B. gymnorrhiza* in different days of treatment

### 3.4. Changes in Ascorbate peroxidase activity (APX)

*B. parviflora* and *B. gymnorrhiza* upon exposure to high salt for 6days showed an enhancement in APX activity 1.3 times and 2.3 times compared to control (Figure 4a).The activity staining of gel exhibited only one prominent band of APX and the densitometry analysis of this band revealed a marginal increase 1.2 times and 1.7 times in salt treated *B. parviflora* and *B. gymnorrhiza* (Figure 4b).

### 3.5 Changes in guaiacol peroxidase activity (GPX)

GPX activity increased 1.5 times and 1.3 times in leaves of salt treated seedlings of *B. parviflora* and *B. gymnorrhiza* (Figure 5a).Activity staining data showed an enhancement in 6 isoforms of GPX in both the species.But the enhancement in these isoforms were more in *B. parviflora* than in *B. gymnorrhiza* (Figure 5b).

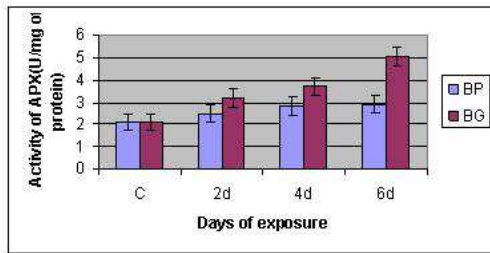


Figure 4a. Effect of 500mM NaCl on ascorbate peroxidase activity of *B. parviflora* and *B. gymnorrhiza* in different days of treatment

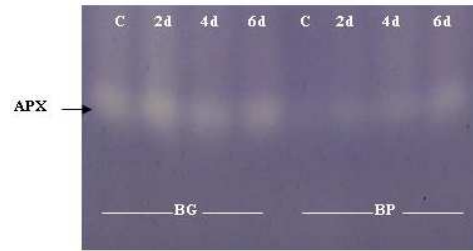


Figure 4b. Effect of 500mM NaCl on ascorbate peroxidase profile of *B. parviflora* and *B. gymnorrhiza* in different days of treatment

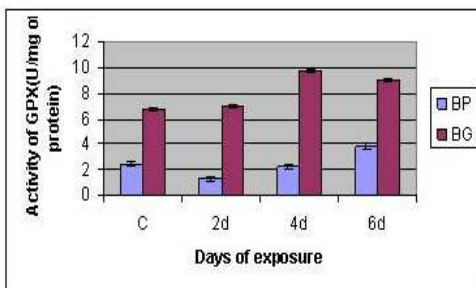


Figure 5a. Effect of 500mM NaCl on guaiacol peroxidase activity of *B. parviflora* and *B. gymnorrhiza* in different days of treatment

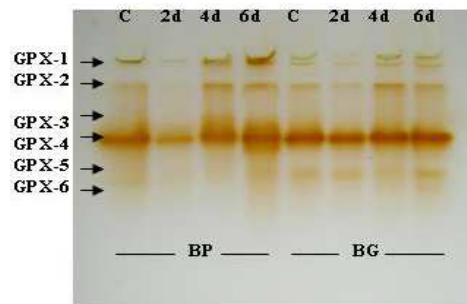


Figure 5b. Effect of 500mM NaCl on guaiacol peroxidase profile of *B. parviflora* and *B. gymnorrhiza* in different days of treatment

#### 4. Discussion

Exposure to high NaCl imposes oxidative stress due to changes in the osmotic and ionic environment in plant (Alakhverdiev et al.2000, Hasehawa et al. 2000). Moreover, mangroves are potential sources of genes that control salt tolerance and also of biochemical strategies linking photosynthesis, plant productivity and abiotic stress conditions (Cheeseman et al.1997). When three month old seedlings of *Bruguiera* were exposed to high salt shock (500mM NaCl), a differential changes were found in protein as well as antioxidative defense system in both the species. The protein content in both the species of *Bruguiera* decreased upon exposure to high salt but the degree of degradation was more rapid in *B. parviflora* than *B. gymnorrhiza*. Salt shock also imposed a differential expression of antioxidative enzymes in both the species of *Bruguiera*. In *B. gymnorrhiza*, the catalase activity increased 2.4 times upon salt shock for 6d compared to control. The activity staining data showed the expression of only one isoform of catalase in control sample of *B. gymnorrhiza* where as upon exposure to high salt four isoforms of catalase was observed. Also, an enhancement in catalase isozymes was found upon salt treatment in this species of *Bruguiera*. Presently, it is difficult to ascertain such types of variation. Where as *B. parviflora* the specific activity of catalase increased 2.1 times compare to control. The four isoforms of catalase as expressed in control samples also enhanced upon exposure to 500 mM NaCl for a short period. *B. gymnorrhiza* showed 1.7 times more APX activity after 6d salt treatment compared to *B. parviflora*. The expression of APX isoform was also more in *B. gymnorrhiza*. But the induction of GPX activity was more in *B. parviflora* than in *B. gymnorrhiza*. Parida et al.(2004) reported an enhancement in APX, GPX but a reduction of catalase activity upon salinization. Takemura et al.(2000) reported an increased in total catalase activity up to sea water concentration. However, mRNA detection study did not showed a change in particular catalase transcript level with salinity stress.

In summury, as *B. parviflora* and *B. gymnorrhiza* are two related species of *Bruguiera* still there was a differential adjustment in protein level expression to salt shock. So, these salt sensitive proteins may be give essential information about salt tolerance mechanism in both species of *Bruguiera*.

#### 4. Conclusions

In this research, 8 applications' effects on seedling characteristics (including planting season, tree species and seedling type) were evaluated in respect of 3 different period. At the end of total evaluation of measured seedling characteristics,

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**Abbreviations:** TCA = trichloroacetic acid, SDS=sodium dodesyl sulfate, kDa=kilo Dalton, PAGE=polyacrylamide gel electrophoresis, Tris=hydroxymethyl amino methane, TEMED=tetra methyl ethylene diamine, AA= ascorbic acid, APX=ascorbate peroxidase (EC 1.11.1.11), CAT=catalase (EC 1.11.1.6), GPX= guaiacol peroxidase (EC. 1.11.1.7), EDTA = ethylenediamine tetra acetic acid (disodium salt), NBT-nitroblue tetrazolium, PVP-polyvinylpyrrolidone.

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