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ORIGINAL ARTICLE

Morphological and biochemical responses of *Aegiceras corniculatum* L. to salinity stress

Pritinanda Mohanty, Jyoti Ranjan Rout, Chinmay Pradhan and Santi Lata Shaoo*

Biochemistry and Molecular Biology Laboratory, P. G. Department of Botany, Utkal University, Vani Vihar, Bhubaneswar-751004, Odisha, India

*Tel.: +91-9937062620; Fax: +91674-2567798
*E-Mail: santi_bot_uu@yahoo.com

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Key words: Aegiceras corniculatum L., Biochemical, Mangrove, Morphological, Salt stress

Mangroves are marine halophytes, salt tolerant woody plants found growing at the seashore in tropical and subtropical areas. More than 100 species of several families (Tomlinson, 1986), including the genus *Aegiceras* belong to this group of plants. The mechanisms of salt tolerance of mangrove plants at the organ level (Ball and Farquhr, 1984; Wermer and Stelzer, 1990) have been reported. There are few reports dealing with the mechanism of salt tolerance at the cellular and biochemical level (Clough *et al.,* 1982). *A. corniculatum* is a small evergreen true mangrove species belonging to the family Myrsinaceae and is one of the three pioneer mangroves which can thrive in 3 % salinity (Duck *et al.,* 1998) by secreting salt though its leaf glands (Ball, 1988). The growth

of mangrove like *A.corniculatum* is regulated by tidal inundation and other factors like salinity of surface and soil water, availability of nutrients and the degree of soil saturation. Spatial and temporal changes in salinity could affect the growth and physiology of plants (Naidoo, 1985). General growth of mangrove plants usually declines at high salinity, but optimal growth obtained at moderate salinity (Clough, 1984). As a function of tolerance, salinity stress decreases the leaf water potential of the plant (Clough, 1984). Retarded growth of plants and lowered water potential resulting variation in sap osmotic pressure, salt exclusion at the root level and active salt excretion through leaves were routine observation with the plants exposed to saline stress (Hutchings and Saengar, 1987). *A. corniculatum* secretes excess salt through salt glands present in the leaves. The plant adopts the changes in salinity gradient habitats. The effect of salt stress had been studied in relation to leaf structure, rates of transpiration, stomatal conductance and rates of photosynthesis (Parida *et al.,* 2002; Santiago *et al.,* 2000) and changes in chloroplast structure and function (Parida *et al*., 2003). One of the biochemical mechanisms by which mangroves counter the high osmolarity of salt was accumulation of compatible solutes (Takemura *et al.,* 2000).The present study was designed to elucidate the morphological and biochemical responses of *A. corniculatum* when exposed to various concentrations of NaCl solution in *ex-situ* condition.

MATERIALS AND METHODS

Six months old plants (generated from propagules) were collected from Goverment temporary nursery of forest, Dangamal, Bhitarkanika sanctuary area, Kendrapada, Odisha. The plants were maintained in green house under natural conditions and watered for two months through regular watering. After two months, plants of same height approximately 60 cm were selected for further study.

Healthy and morphologically young plantlets were planted in the earthen pots of uniform size (one ft. height and 1.5 ft. diameter), filled with garden soil. In these pots, plants were periodically treated with different increasing concentrations (0 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM) of NaCl. The salinity level of the culture solution was maintained in the pots through periodic watering with saline water in 7 days interval.

The different growth parameters like plant height, dry weight, stem diameter, number of leaves and number of branches per plant were measured at the end of 30 and 60 days of NaCl treatment. Dry mass was determined after drying the plant sample in a fan-forced oven at 80 °C. The leaves were plucked at 0, 30, 60 days intervals to measure the biochemical parameters.

Leaves (0.5 g) were homogenized in chilled 80 % acetone in a mortar and pestle in dark at 4 °C and the homogenates were centrifuged at 8800 rpm for 10 min. The supernatants were collected and absorption spectra at 663, 645 and 470 nm were recorded using Perkin Elmer UV–Vis spectrophotometer Lambda 25 for estimation of Chl *a*, Chl *b*, and carotenoids. The total Chl and Chl *a* and *b* ratio was also calculated following the procedure of Porra *et al*. (1989). The total carotenoids were calculated, according to the method of Arnon (1949).

Total leaf protein was extracted by the polyvinyl polypyrrolidone (PVP) precipitation method (Ferreira *et al.,* 2002). Fresh leaf tissues (0.5 g) were

homogenized in 50 mM sodium phosphate buffer containing 10% (w/v) insoluble PVP using a prechilled mortar and pestle and incubated overnight at 4^0C. The homogenates were centrifuged at 14000 rpm for 20 min at 4^0C (Remi Instruments, India). The supernatant was kept under -20^0C for protein estimation and enzyme assay. The protein estimation was done by the method of Lowry *et al.* (1951). Protein in the unknown sample was estimated by measuring the absorbance at 750 nm using bovine serum albumin as standard and expressed as mg per gram fresh weight basis (mg/ g f. w.).

For extraction of total soluble sugar, 1 g of leaf tissue was homogenized in 80 % ethanol, re-fluxed for 15 min in water bath at 60 °C and centrifuged at 4400 rpm for 10 min. The pellet was re-extracted twice with 80 % ethanol and the supernatants were pooled. Pigments were removed from the supernatant by adding 1–2 ml of saturated neutral lead acetate and precipitated out with slight excess of Na_2HPO_4. The supernatant was filtered through Whatman no.1 filter paper. To the filtrate 0.2 ml of 0.3 N $Ba(OH)_2$ was added per ml of the filtrate and mixed well. Then, 0.2 ml of $ZnSO_4$ was added and shaken thoroughly and filtered through Whatman no. 1 filter paper after 10 min to precipitate out the proteins by $Zn(OH)_2$ to obtain a protein-free sugar extract (Kumar and Sharma, 1995). The OD was measured at 630 nm in a spectrophotometer after setting for 100 % transmission against the blank. A standard curve was prepared by using known concentration of glucose. The quantity of sugar was expressed as mg/ g f.w. of leaf tissue.

All results are the mean of three independent experimental replicates (n = 5). The data were analyzed by analysis of variance (ANOVA) and tested for least significance differences (LSD) by Duncan's multiple range test at $P \leq 0.05$.

RESULTS

A. corniculatum could tolerate maximum NaCl up to 300 mM and could be maintained for more than 60 days. All the plants were grown up to 60 days under increasing concentrations (0 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM) of NaCl. Morphologically, it was observed that the plant height was enhanced with increasing concentration of salt both in 30 and 60 days of treatment (Tables 1 and 2). However the maximum growth was obtained at 250 mM NaCl and then declined. The similar variations were also observed in case of other parameters like dry weight, stem diameter, number of leaves and number of branches per plant with supplementation of 250 mM NaCl (Tables 1 and 2). A remarkable observation was also noticed that the 250 mM concentration of treatment able to produced flowering buds after 30 days of treatment (Fig. 1a) and remained up to 60 days (Fig. 1b).

The decreasing trend of total contents of Chlorophyll and carotenoids were found under long time exposure of NaCl. The total Chl expressed on mg/ g fresh wt. basis decreased by 23.52 and 32.20 % upon 30 and 60 days treatment, respectively in 300 mM NaCl treatment as compared to control. The Chl *a* and *b* ratio in the control and treated plant remained between 2.88 to 3.33 (Table 3).

The total soluble protein content of leaves was measured on initial days of plantation as well as 30 and 60 days of experiment. The protein content (20.48 and 20.70 mg g^{-1}) was increased in control plants grown for a duration of 30 and 60 days. However, a decreasing activity was observed in both the 30 and 60 days treated plants. About 10.64 and 21.49 % less protein was found in 30 and

60 days of treatment, respectively at 300 mM concentration of NaCl than that of control (Table 4).

Similar results were observed in leaf samples of *A. Corniculatum* that the sugar content was decreased with supplementation of increasing concentration of NaCl. The maximum decrement was noticed in 300 mM of NaCl treatment in which total soluble sugar decreased to 29.90 and 52.95 % (30 and 60 days, respectively) to that of the control.

Table 1: Effects of NaCl on plant height, dry weight, stem diameter, leaf number and leaf sizes after 30 days of exposure.

Growth parameters	Concentration of NaCl (mM)					
	0	100	150	200	250	300
Plant height (cm)	76.2 ± 4.2^{b}	77.0 ± 4.8^{b}	80.6 ± 3.1^{a}	76.8 ± 2.7^{b}	84.9 ± 4.5^{a}	82.6 ± 3.6^{a}
Dry weight (g)	11.9 ± 0.6^{d}	13.3 ± 1.2^{c}	13.4 ± 1.2^{c}	13.0 ± 1.1^{c}	18.6 ± 0.9^{a}	15.8 ± 0.7^{b}
Stem diameter (cm)	3.9 ± 0.6^{c}	4.1 ± 0.9^{c}	4.4 ± 0.5^{b}	5.0 ± 0.6^{ab}	5.8 ± 0.7^{a}	4.8 ± 0.9^{ab}
No. of leaves/ plant	26.0 ± 2.0^{d}	23.0 ± 0.7^{d}	32.0 ± 2.5^{cd}	44.0 ± 1.5^{c}	85.0 ± 3.0^{a}	60.0 ± 2.5^{b}
No. of branches/ plant	5.0 ± 1.3^{c}	5.0 ± 1.1^{c}	6.0 ± 1.1^{b}	6.0 ± 0.8^{b}	9.0 ± 0.6^{a}	5.0 ± 0.9^{c}

The data represent mean ± SE of replicates (n = 5). Values in the same rows carrying different letters are significantly different between treatments and control by Duncan's multiple range test at $P \leq 0.05$.

Table 2: Effects of NaCl on plant height, dry weight, stem diameter, leaf number and leaf sizes after 60 days of exposure.

Growth parameters	Concentration of NaCl (mM)					
	0	100	150	200	250	300
Plant height (cm)	88.3 ± 2.5^{b}	88.7 ± 3.5^{b}	91.2 ± 5.7^{a}	89.6 ± 5.8^{b}	96.4 ± 6.2^{a}	94.5 ± 2.9^{a}
Dry weight (g)	14.5 ± 1.1^{bc}	11.2 ± 1.3^{c}	12.7 ± 1.2^{c}	12.1 ± 1.3^{c}	22.0 ± 1.5^{a}	18.9 ± 1.1^{b}
Stem diameter (cm)	4.2 ± 0.6^{c}	4.4 ± 0.9^{c}	4.8 ± 0.7^{b}	5.2 ± 1.0^{b}	6.5 ± 0.5^{a}	5.0 ± 0.9^{b}
No. of leaves/ plant	49.0 ± 2.5^{c}	42.0 ± 2.5^{c}	52.0 ± 3.0^{c}	65.0 ± 5.0^{b}	111.0 ± 5.0^{a}	67.0 ± 2.5^{b}
No. of branches/ plant	6.0 ± 1.3^{c}	6.0 ± 1.1^{c}	6.0 ± 1.1^{c}	7.0 ± 1.0^{b}	11.0 ± 1.0^{a}	5.0 ± 0.9^{d}

The data represent mean ± SE of replicates (n = 5). Values in the same rows carrying different letters are significantly different between treatments and control by Duncan's multiple range test at $P \leq 0.05$.

Table 3: Changes of Chl a, Chl b, total Chl, Chl a/b ratio and carotenoid content in leaf samples of *A. corniculatum* under different concentrations of NaCl treatment for 0, 30 and 60 days.

Duration of treatment (d)	NaCl (mM)	Chl *a* (mg g^{-1} f.w.)	Chl *b* (mg g^{-1} f.w.)	Total Chl (mg g^{-1} f.w.)	Chl *a/b*	Carotenoid (mg g^{-1} f.w.)
Initial day	0	0.49±0.12^{b}	0.17±0.007^{a}	0.66±0.06^{a}	2.88	0.18±0.007^{a}
	100	0.49±0.03^{b}	0.17±0.003^{a}	0.66±0.03^{a}	2.88	0.17±0.008^{a}
	150	0.48±0.06^{b}	0.16±0.005^{a}	0.64±0.02^{a}	3.0	0.17±0.005^{a}
	200	0.49±0.05^{b}	0.16±0.006^{a}	0.65±0.04^{a}	3.06	0.18±0.007^{a}
	250	0.50±0.02^{a}	0.17±0.007^{a}	0.67±0.01^{a}	2.94	0.18±0.007^{a}
	300	0.49±0.01^{b}	0.17±0.006^{a}	0.66±0.02^{a}	2.88	0.17±0.003^{a}
30 days	0	0.52±0.03^{a}	0.16±0.003^{a}	0.68±0.07^{a}	3.25	0.19±0.008^{a}
	100	0.40±0.03^{c}	0.13±0.002^{c}	0.53±0.05^{b}	3.33	0.15±0.007^{b}
	150	0.41±0.01^{c}	0.13±0.002^{c}	0.54±0.02^{b}	3.15	0.14±0.006^{b}
	200	0.41±0.02^{c}	0.14±0.001^{b}	0.55±0.02^{b}	2.92	0.13±0.005^{b}
	250	0.42±0.02^{c}	0.14±0.003^{b}	0.56±0.05^{b}	3.0	0.15±0.007^{b}
	300	0.40±0.03^{c}	0.12±0.006^{c}	0.52±0.03^{b}	3.33	0.13±0.003^{c}
60 days	0	0.44±0.02^{bc}	0.15±0.005^{b}	0.59±0.05^{ab}	2.93	0.16±0.007^{b}
	100	0.36±0.03^{d}	0.11±0.003^{d}	0.47±0.02^{bc}	3.27	0.12±0.006^{c}
	150	0.37±0.02^{d}	0.11±0.005^{d}	0.48±0.02^{bc}	3.36	0.11±0.005^{c}
	200	0.37±0.03^{d}	0.12±0.006^{c}	0.48±0.03^{bc}	3.08	0.10±0.003^{d}
	250	0.38±0.01^{d}	0.13±0.005^{c}	0.51±0.03^{b}	2.92	0.12±0.007^{c}
	300	0.30±0.02^{e}	0.10±0.009^{d}	0.40±0.03^{c}	3.0	0.09±0.008^{d}

The data represent mean ± SE of replicates (n = 5). Values in the same column carrying different letters are significantly different between treatments and control by Duncan's multiple range test at $P \leq 0.05$.

Table 4: Effects of different concentrations of NaCl treatment for varying periods on total sugar content and protein content of the leaves of *A. corniculatum.*

Treatment time (days)	NaCl (mM)	Protein (mg g^{-1} f.w.)	Total sugar (mg g^{-1} f.w.)
Initial day	0	18.32 ± 0.98^{c}	28.76 ± 2.08^{b}
	100	18.52 ± 1.02^{c}	28.43 ± 1.84^{b}
	150	18.09 ± 1.20^{c}	28.51 ± 1.28^{b}
	200	18.22 ± 0.78^{c}	28.73 ± 1.36^{b}
	250	18.48 ± 0.88^{c}	28.22 ± 2.04^{b}
	300	18.10 ± 1.32^{c}	28.69 ± 1.30^{b}
30	0	20.48 ± 1.50^{a}	30.76 ± 2.22^{a}
	100	20.14 ± 0.80^{a}	26.28 ± 1.66^{c}
	150	19.52 ± 2.08^{b}	26.20 ± 2.08^{c}
	200	19.60 ± 1.50^{b}	24.08 ± 1.82^{d}
	250	19.06 ± 1.20^{b}	24.69 ± 0.89^{d}
	300	18.30 ± 2.20^{c}	21.56 ± 1.08^{e}
60	0	20.70 ± 1.52^{a}	29.82 ± 1.70^{a}
	100	19.12 ± 1.03^{b}	23.51 ± 1.64^{d}
	150	18.22 ± 1.44^{c}	23.77 ± 2.08^{d}
	200	18.62 ± 1.50^{c}	20.76 ± 1.55^{e}
	250	18.18 ± 1.21^{c}	20.25 ± 2.90^{e}
	300	16.25 ± 1.30^{d}	14.03 ± 1.75^{f}

The data represent mean ± SE of replicates (n = 5). Values in the same column carrying different letters are significantly different between treatments and control by Duncan's multiple range test at $P \leq 0.05$.

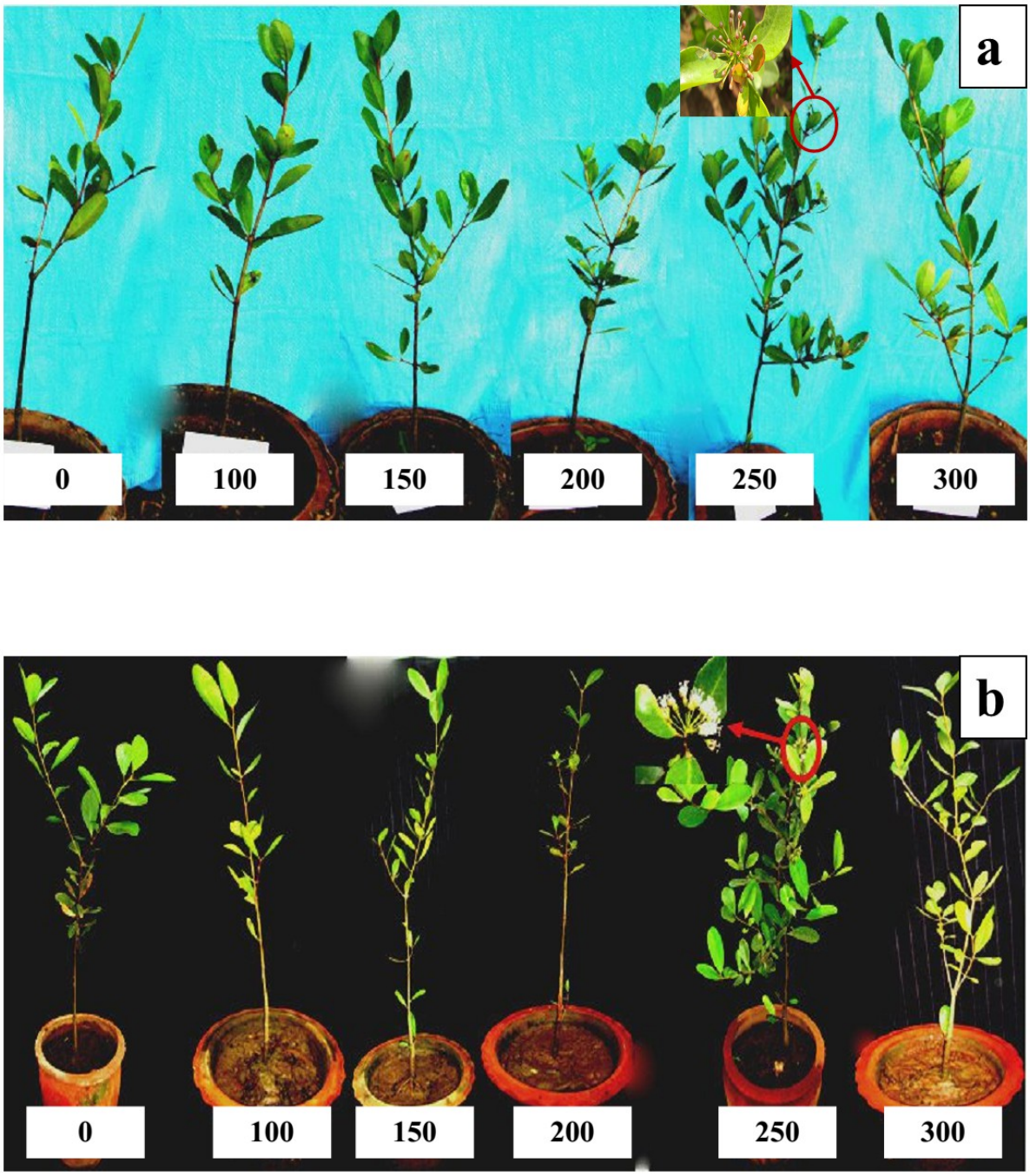

Figure 1. Morphological changes of *Aegiceras corniculatum* L. under 30 days (a) and 60 days (b) increasing concentrations (0 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM) of NaCl stress.

DISCUSSION

Morphological parameters like plant height, stem diameter, dry weight, number of leaves and number of branches per plant of *A. corniculatum* were studied and observed that it was stimulated by low salinity. The optimal growth was obtained at 250 mM NaCl treatment and then declined means the plants were not able to uptake more concentrations beyond this limit. Similar findings have also been reported for other halophytes that have optimal growth in the presence of salt (Khan *et al.,* 2000; Patel and Pandey, 2007). High salinity affects mangrove plant growths due to the low water potential, ion toxicities, nutrient deficiencies

or a combination of all (Khan *et al.,* 2000).

In pigment estimation both Chl and carotenoid content decreased by salinity in *A. corniculatum*. The decrease in Chlorophyll content at 300 mM NaCl is due to changes in the lipid protein ratio of pigment protein complexes or increased chlorophyllase activity (Iyengar and Reddy, 1996). The results agree with several reports of decrease content of Chlorophyll and carotenoids by increasing salinity as reported in a number of glycophytes (Agastian *et al.,* 2000). As the Chl *a*: *b* ratio remained unaffected by NaCl treatment in *A. corniculatum,* it shows that thylakoid membranes are little altered by salt exposure in ex-situ condition.

The total protein content of leaf gradually decreased with increasing concentration of NaCl. This decrease in protein content might be due to the increasing activity of acid and alkaline proteases. A small change in total protein content in *A. corniculatum* suggests that NaCl exposure affect protein synthesis. Agastian *et al.* (2000) reported that soluble protein increases at low salinity and decreases at high salinity in mulberry. In *A. corniculatum* like other cellular constituents, sugar levels are also affected by stress. The total sugar content also decreased with increase in salt concentration. The NaCl concentration of 250 mM is suitable for flowering of the plant. Salinity is an important factor for plant growth, foliage, flowering, leaf size and development. The concentration of NaCl of 250 mM showed the optimal growth in the plant in garden condition.

CONCLUSION

Our results show that the mangrove *A. corniculatum* can easily be propagated under low salinity condition. At 250 mM, the plants become acclimatized to salt after two month of exposure. Therefore by manipulating salt concentration, these plants can be grown under *ex-situ* condition for obtaining various medicinal products without exploiting the plants in its natural habitat.

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