

# Biochemical constituents of *Excoecaria agallocha* L. under different levels of NaCl stress

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## ABSTRACT

The present investigation was made to study the effect of different concentrations of NaCl on biochemical constituents of the seedlings of *Excoecaria agallocha*. The plant could survive a wide range of 100-1000 mM NaCl concentrations. The upper limit for the survival of the seedlings was 500 mM NaCl. Above 500 mM, the seedlings did not survive. The plant parts (leaf, stem, and root) of after salt treatment 60 days, old plants were used for the analysis of biochemical activities. The NaCl promoted the compatible solutes such as starch, proline, and protein. The Sugar content decreased up to the optimum level of 300 mM NaCl and increased at the higher concentrations. The starch and chlorophyll content increased up to 300 mM NaCl, beyond 300 mM, the contents decreased marginally. The proline content increased with the increasing salinity. The protein increased with increasing salt concentration up to the optimal level of 300 mM NaCl, and decreased at the higher concentrations. The plant can able to survive up to 500 mM NaCl concentration and remarkable accumulations of compatible solute make the plant survive salinity stress.

**KEY WORDS:** Compatible solutes, *Excoecaria agallocha*, salinity

## INTRODUCTION

Mangrove ecosystem is a distinctive transitional, coastal ecosystem, amid marine, and terrestrial environments, generally confined to the tropical and subtropical regions. The largest percentage of mangroves is found between 5° N and 5° S latitude, but occurs all the way to 32° N and 38° S Latitude (Friess *et al.*, 2012a). Mangroves are intertidal dicots adapted to high temperatures, fluctuating salinities and changing aerobic and anaerobic substrates. Mangroves, fringe, riverine, and basin are represented by 70 tropical and subtropical species, 28 genera and 19 families (Duke *et al.*, 1998). The worldwide coverage of these halophytic spermatophytes has been estimated as 152,308 km<sup>2</sup> (Spalding *et al.*, 2010) and 137,760 km<sup>2</sup> (Giri *et al.*, 2011). Salinity is regarded as one of the majority vital environmental extremes that affect plant growth and metabolism harmfully predominantly in the arid and semi-arid regions in the globe (Munns and Tester, 2008). This environmental crisis is becoming more ubiquitous throughout the planet due to the intensification of agriculture and universal climate change (Ashraf and Arkam, 2009).

The effects of salinity on mangroves have been studied in relation to anti-oxidative enzymes (Parida *et al.*, 2004b) leaf structure, rates of transpiration, stomatal conductance and rates of photosynthesis (Santiago *et al.*, 2000) and changes in chloroplast structure and function (Parida *et al.*, 2003). Tanaka *et al.* (2000) reported that Na<sup>+</sup>/H<sup>+</sup> anti-port catalyzed exchange of Na<sup>+</sup> for H<sup>+</sup> across the vacuolar membrane of the cells of *Bruguiera sexangula* offered tolerance to ionic stress imposed by NaCl and this mechanism was important for cellular salinity adjustments. Also, the mechanism of acclimation to salt in mangroves was suggested to be linked to the changes in the vacuolar size in *B. sexangula* (Hotta *et al.*, 2000). Further, one of the biochemical mechanisms by which mangroves counter the high osmolarity of salt was an accumulation of compatible solutes (Takemura *et al.*, 2000). Although, pinitol and mannitol were the most common compatible solutes of a number of mangrove species, proline (in *Xylocarpus* species), methylated quaternary ammonium compounds (in *Avicennia* species), and carbohydrates (in *Acanthus ilicifolius*, *Heritier littoralis*, and *Hibiscus liliaceous*) were found to be dominant osmoregulating compounds (Popp *et al.*, 1985).

Biochemical studies have shown that plants under salt stress accumulate a number of metabolites, which are termed compatible solutes because they do not interfere with the plant metabolism and the accumulation of these solutes contribute to turgor maintenance and osmoprotectance in plants. In different plant species, it was found that salinity stress caused accumulation of soluble sugars, proline, and proteins in leaf growth is most inhibited under salinity (Zennek and Lips, 2001). Salinity causes a range of venomous effects such as inhibition of photosynthetic rate, chlorophyll content, and damage to plasma membrane permeability and other metabolic disturbances (Karimi *et al.*, 2005). In this paper, we present the effect of NaCl on chlorophyll, proline, sugars, starch, and protein content in *Excoecaria agallocha* under salinity stress with an aim to obtain insights into the changes in osmotic composition associated with salt accumulation.

## MATERIALS AND METHODS

### Plant Material and Salt Stress Application

*E. agallocha* L., an evergreen mangrove species belonging to the family Euphorbiaceae was used for the present investigation. This species is naturally growing in abundance in the salt marshes of Pichavaram on the east coast of Tamil Nadu, India about 10 km east of the Annamalai University campus. The mature seedlings were collected from Pichavaram. Healthy seedlings with uniform size were planted individually in polyethene bags (7"×5") filled with homogenous mixture of garden soil containing red earth, sand, and farmyard manure mixed in the ratio of 1:2:1, and polyethene bags were irrigated regularly. One-month-old seedlings were subjected to salt stress with different NaCl concentrations. The treatment constituted (control), 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 mM NaCl. Fifty plants were treated with each of the NaCl concentrations. A control was maintained without any exogenous addition of salts. First, sampling for these studies was collected on the 60<sup>th</sup> day after salt treatment.

### Estimation of Sugar

Sugar content was estimated as described by Nelson (1944). One ml of ethanol extract taken in the test tubes was evaporated in a water bath. To the residue, 1 ml of distilled water and 1 ml of 1 N sulfuric acid were added and incubated at 49°C for 30 min. The solution was neutralized with 1 N sodium hydroxide using methyl red indicator. One ml of Nelson reagent was added to each test tube. The test tubes were heated for 20 min in a boiling water bath, cooled, and 1 ml of arsenomolybdate reagent was added.

The solution was thoroughly mixed and diluted to 25 ml and read at 495 nm in a Spectrophotometer (U-2001, HITACHI). The reducing sugar content of the unknown samples was calculated from glucose standards.

### Estimation of Starch

Starch was determined according to the method described by Summner and Somers (1949). The ethanol insoluble residues taken from ethanol extraction were dried at 60°C for 48 h in an oven. To 200 mg of the powdered residue, 3 ml of 6 N HCl was added and autoclaved at 100°C for 1 h. The flask was cooled, and the volume was raised to 25 ml with distilled water. One ml of aliquot was withdrawn, neutralized with 1 N NaOH and sugar was estimated by Nelson's (1944) method. The amount of starch was arrived at by multiplying the sugar by the factor 0.9.

### Estimation of Proline

Proline accumulation was determined as described by Bates *et al.* (1973). Five hundred mg of plant tissue was homogenized in 10 ml of 3% sulfosalicylic acid. The homogenate was filtered through Whatmann No.42 filter paper. Two ml of acid ninhydrin (1.25 g ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid) and 2 ml of glacial acetic acid in a test tube was heated for an hour at 100°C. The reaction mixture was extracted with 4 ml of toluene and mixed vigorously by using a vortex mixture for 15-20 s. The chromophore containing toluene was aspirated from the aqueous phase. The absorbance of the toluene layer was measured in a Spectrophotometer (U-2001, HITACHI) at 520 nm using toluene as blank.

### Estimation of Protein

Protein was determined according to the method described by Lowry *et al.* (1951). Five hundred mg of plant sample was macerated with a mortar and pestle with 10 ml of 20 percent trichloroacetic acid. The homogenate was centrifuged for 15 min at 6000 rpm. The supernatant was discarded. To the pellet, 5 ml of 0.1 N NaOH (400 mg of NaOH was dissolved in distilled water and made up to 100 ml) was added and centrifuged. The supernatant was taken and made up to 5 ml with 0.1 N NaOH. This extract was used for the estimation of total protein.

To 0.5 ml of protein extract, 5 ml of the reagent C was added (prepared by mixing reagent A and reagent B in 25:1 ratio; Reagent-A: 400 mg of NaOH was dissolved in distilled water and made up to 100 ml. To this solution, 2 g of Na<sub>2</sub>CO<sub>3</sub> was added. Reagent-B: 2 g of CuSO<sub>4</sub> was

dissolved in distilled water and made up to 100 ml and 2 g sodium potassium tartrate was dissolved in distilled water and made up to 100 ml, both the solutions were mixed equal volume) and it was allowed to stand for 10 min at 28°C. 0.5 ml of folin-phenol reagent (Folin-Ciocalteu and distilled water were mixed in the ratio 1:2 [v/v]) was added to this solution and kept at room temperature (30°C) for 10 min and the absorbance was read at 660 nm in a spectrophotometer. The protein contents of unknown samples were calculated from bovine serum albumin standards.

### Estimation of Chlorophyll

Chlorophyll content was estimated as described by Arnon (1949). Five hundred mg of leaf tissue was taken in a pestle and mortar with 10 ml of 80% acetone, and it was ground well. Then, the homogenate was centrifuged at 800 g for 10 min and the supernatant was saved. The pellet was re-extracted with 5 ml of 80% acetone each time till the pellet become colorless. All the extracts were pooled, and the chlorophyll content was determined by using the formula.

Total chlorophyll (mg/ml)

$$(0.0202) \times (\text{O.D. } 645) + (0.00802) \times (\text{O.D. } 663)$$

Chlorophyll 'a' (mg/ml)

$$(0.0127) \times (\text{O.D. } 663) - (0.00269) \times (\text{O.D. } 645)$$

Chlorophyll 'b' (mg/ml)

$$(0.0229) \times (\text{O.D. } 645) - (0.00468) \times (\text{O.D. } 663)$$

### Statistical Analysis

The experiment was placed in a completely randomized block design with three replicates of the each treatment. The results were analyzed by one-way ANOVA with a significance level of  $P \leq 0.05$  and means were separated by Duncan ( $P < 0.05$ ) with the help of SPSS 16.0 software package. Means and standard deviation were calculated from three replications.

## RESULTS

### Total Sugar

Total sugar content declined in the leaf, stem, and root with augmenting NaCl up to 300 mM and at higher concentrations the total sugar content increased gradually. The leaves showed more total sugar content than the stem and root.

### Starch

The results on the starch content of the leaf stem and root at various salinity levels of NaCl are given in Table 2. The starch content in all the three tissues increased with increasing NaCl up to 300 mM. At still higher concentrations, there was a gradual decrease in starch content.

### Proline

The effect of NaCl on the proline content in the leaf, stem, and root are given in Table 3. There was a gradual raise in the level of proline in all the three tissues with increasing NaCl concentrations up to 500 mM. The leaf had more proline than that of stem and root.

### Protein

The result on the effect of NaCl on the protein content of leaf, stem, and root of *E. agallocha* is given in Table 4. The protein content increased with increasing concentrations up to 300 mM and at higher concentrations it steadily declined.

### Chlorophyll

Sodium chloride treatment stimulated the chlorophyll synthesis in the leaves with increasing NaCl concentrations up to 300 mM and thereafter it declined steadily. The highest accumulation of total chlorophyll synthesis was recorded at 300 mM.

## DISCUSSION

Sugars are the source of energy and carbons needed for adaptive and/or defensive responses of stresses. In addition, sugars such as raffinose and sucrose are indicated to have important roles in protecting cells from water stress; they are solutes available for osmoregulation or function as protectants of molecules and membranes (Bray, 1997). Under severe salinity stress, the decrease in sugar content can be either due to high respiration or a decrease in photosynthetic activity accompanied by a reduction in growth rate. An increasing sugar content and a corresponding decrease in the starch at higher salinities have been reported in several halophytes (Prado *et al.*, 2000; Joshi *et al.*, 2002). A salt induced reduction in the amount of sugar has also been reported by Singh and Singh (1995).

The increase in starch may be due to increase in the nitrogen content, which plays an important role in photosynthesis (Cook and Evens, 1983). Like other cellular constituents, starch, and sugar levels are also

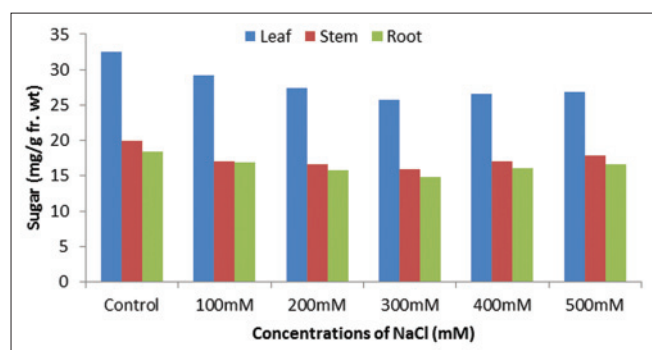
affected by stress (Prado *et al.*, 2000). In *E. agallocha* total sugar content decreased by salinity whereas starch content increased. Our results suggest that NaCl induces sugar starch interconversion. Our results are supported by (Gadallah, 1999) who reported that salinity decreases soluble and hydrolysable sugars in *Vicia faba*.

The changes in soluble protein showed a reverse trend to that of free amino acids implying that the increase in protein content may be at the expense of amino acids and that the salinity changes influenced the interconversion of these compounds. In halophytes, the protein content increased with increasing concentrations such as *Atriplex*

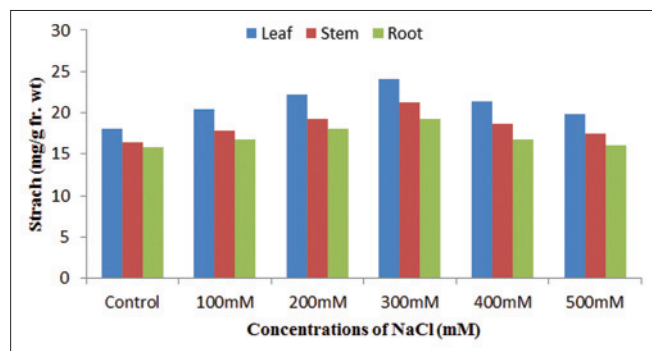
*satidea* (Flowers and Dalmond, 1992), *Helochola setulosa* (Joshi *et al.*, 2002), and *Thellangiella halophila* (M'rah *et al.*, 2006). In general, the protein content increased with increasing concentration up to an optimal level. Beyond the optimum level, the protein content decreased in *Phalaris arundinacea* (Maeda *et al.*, 1995) *Sesuvium portulacastrum* (Venkatesalu *et al.*, 1994).

Proline showed the highest absolute accumulation in response to salinity (Misra and Gupta, 2005). Proline has several functions, such as osmotic pressure regulation, protection of membrane integrity, and scavenger of free radicals (Hare and Cress, 1997). Accumulation of proline under stress conditions such as high salinity, in plants, has been correlated with stress tolerance (Misra and Gupta, 2005). Many plants accumulate proline as a nontoxic and protective osmolyte under saline conditions (Lee and Liu, 1999; Singh *et al.*, 2000). It has been reported that proline levels increase significantly in leaves of non-secretor mangrove, *Bruguiera parviflora* (Parida *et al.*, 2002).

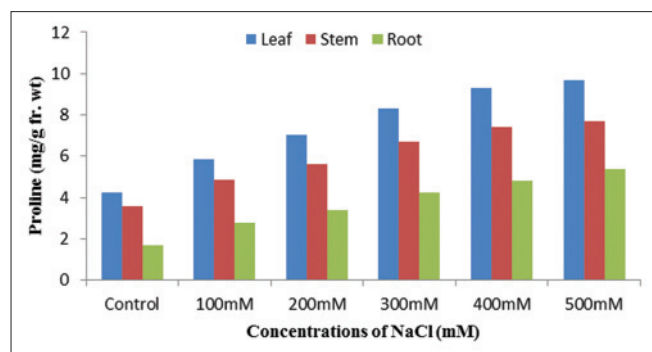
The effect of NaCl salinities favored chlorophyll synthesis in the leaves of *E. agallocha* up to the optimum concentrations of the respective NaCl salts. However, at higher concentrations, the salts had decreased the



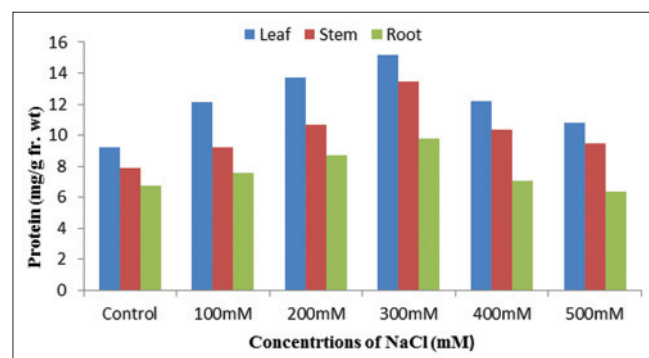
**Figure 1:** Effect of NaCl on sugar content (mg/g fr. Wt) of the leaf, stem, and root of *Excoecaria agallocha* on 60<sup>th</sup> day after salt treatment



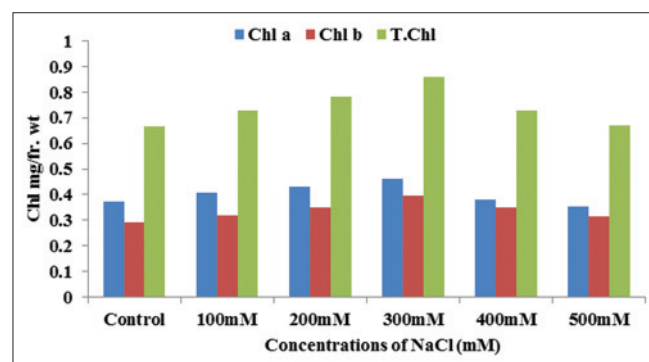
**Figure 2:** Effect of NaCl on starch content (mg/g fr. Wt) of the leaf, stem, and root of *Excoecaria agallocha* on 60<sup>th</sup> day after salt treatment



**Figure 3:** Effect of NaCl on proline content (mg/g fr. Wt) of the leaf, stem, and root of *Excoecaria agallocha* on 60<sup>th</sup> day after salt treatment



**Figure 4:** Effect of NaCl on protein content (mg/g fr. Wt) of the leaf, stem, and root of *Excoecaria agallocha* on 60<sup>th</sup> day after salt treatment



**Figure 5:** Effect of NaCl on chlorophyll a, chlorophyll b and total chlorophyll (mg/g fr. Wt) content of *Excoecaria agallocha* on 60<sup>th</sup> day after salt treatment



chlorophyll content. A positive effect of NaCl salinity on chlorophyll synthesis in the halophyte has been reported (Singh and Dubey, 1995; Khan *et al.*, 2000a). Increasing of leaf chlorophyll content under salinity stress was reported by Pinheiro *et al* 2008 in *Ricinus communis*.

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