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RESEARCH ARTICLE

SALT-INDUCED MODULATION IN GROWTH AND BIOCHEMICAL CONSTITUENTS IN THE MANGROVE PLANT OF *CERIOPS ROXBURGHIANA* ARN

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ABSTRACT

In the present investigation, the effect of different concentrations of NaCl on growth and development, organic constituent of *Ceriops roxburghiana,* a dicotyledonous halophyte has been studied. *Ceriops roxburghiana* survived a wide range of NaCl salinity ranging from 100 to 700 mM. The upper limit for the survival of this species to NaCl salinity was 700 mM. Results of the present study indicated that the optimal salt concentration for the overall better performance of the seedlings of *Ceriops roxburghiana* was 300 mM NaCl. Since the growth and development of this species were improved in the presence of NaCl and it could tolerate high concentration of Cl^- in the cell sap, it could be designated as "facultative halophyte". The growth parameters such as shoot and root length, fresh and dry weight increased with increasing salinity upto 300 mM NaCl.

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INTRODUCTION

Salinity is one of the major environmental stresses that can limit the growth and development of salt-sensitive plants. Salinity generally, affects the plant growth adversely and these adverse effects may be attributed to non-availability of water and ion toxicity to the plants. Extra expenditure of energy of osmotic adjustment under salinity causes growth reduction (Pasternak, 1987). Halophytic environments though dominated by NaCl contain a variety of other salts Na_2SO_4, $MgSO_4$, $CaSO_4$, $MgCl_2$, KCl and Na_2CO_3. About 71 per cent of the world's surface is occupied by oceans and 9.0 million km^2 of coastal land in the world is salt-affected. In India, about 30 million hectares of coastal land remain barren and uncultivable because of saline soil. Concentration of the salts in the seawater is measured as the chloride concentration and it is estimated to be 35g/l. The most important ions in the seawater are the sodium and chloride with the concentrations of 480 mM and 560 mM respectively. Plant growth and productivity is significantly compromised by various biotic and abiotic factors. Salinity is one of the major abiotic stresses affecting crop yield and quality. More than 800 million hectares (approximately 6%) of world's total land area is salt affected (Munns and Tester, 2008). This is mostly contributed by natural causes such as accumulation of salts over time in arid and semiarid regions, release of salts from weathering rocks and deposition of oceanic salts brought in by wind and rain. Besides natural causes, agricultural methods of land clearing, usage of poor quality of irrigated water and poor drainage also lead to salt accumulation in cultivated agricultural lands. Saline soils are characterized by electrical conductivity (ECe) of ≥ 4 dSm^{-1} which is equivalent to 40 mM NaCl and an osmotic pressure of approximately -0.2 MPa (Munns, 2005). Plant type, soil type and climatic conditions determine threshold ECe values beyond which plant growth is severely reduced. Salinity negatively affects plant growth by causing water-deficit and ion-specific damage. Munns and Tester (2008) distinguished two distinct growth phases in response to salt stress. An osmotic phase starts as soon as the plant is exposed to salinity beyond threshold limits. Osmotic stress affects the growth rate due to loss of cell turgor. The second phase is dominated by ion specific toxicity, resulting in rapid leaf senescence and nutrient imbalances and deficiencies caused by reduced uptake of essential ions like K^+, Ca^{2+}, NO^3- and other growth-limiting nutrients (P, Fe, and Zn) from soil (Hu and Schmidhalter, 2005). Osmotic shock, ion cytotoxicity and nutrient imbalances lead to disruption of metabolic processes like photosynthesis and respiration. Metabolic disturbances cause accumulation of harmful reactive oxygen species (ROS), which bring about oxidative damage to cells and adversely affect plant growth and survival (Jithesh *et al.,* 2006).

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Halophytes have evolved characteristics to adjust to the stress conditions in their native habitats by means of a number of different adaptive responses at the germination stages of development. The level of expression of salt tolerance by plant at germination stages cannot always be correlated with tolerance at later stages of development. Halophytes survive salt concentration equal to or greater than that of seawater and possess physiological mechanism that maintains a lower Water potential than that in the soil (Ungar, 1991). A variety of mechanisms contributes to the salt tolerance of halophytes. Adaptation of halophytes to the saline environment includes high tolerance for the negative effect of salinity as well as positive reaction towards it. It is suggested that compartmentation of ions in vacuoles and accumulation of compatible solute in the cytoplasm, as well as presence of genes for salt tolerance, confer salt resistance to halophytes (Gorham, 1995). Salt tolerance is brought about by the development of succulence, transportation of salts to bladders or hair, secretion through salt glands and accumulation of a variety of organic compounds such as proline, glutamic and aspartic acid in their tissues, all of which mitigate the salt stress in saline habitats.

Plants are either termed as glycophytes or halophytes on the basis of their salt tolerance capabilities. Glycophytes are salt-sensitive plants whereas halophytes can survive and complete their life cycle in salt concentrations of 200 mM NaCl or more (Flowers and Colmer, 2008). Although halophytes constitute approximately 1-2 % of world's flora, they are present in almost half the higher plant families (Glenn *et al*., 1999; Flowers and Colmer, 2008). Halophytes can be further categorized as euhalophytes (true halophytes), pseudohalophytes (salt avoiders) and crinohalophytes (salt excreters) depending on the strategy used to adapt to the saline environment (Glenn *et al.,* 1999; Parida and Das, 2005). Mangroves are unique halophytes, mostly trees and shrubs, thriving in the intertidal zones of tropical and subtropical coastal regions of the world (Tomlinson, 1994). Mangrove habitats show varying degree of salinity gradients, from fresh water to seawater (~ 500 mM NaCl) or sometimes greater than levels of seawater. Such broad ranges of salinity give rise to conspicuous zones of mangrove species, which define the area's most suitable for their growth. True mangroves grow only in mangrove environments and show features like presence of aerial roots for gas exchange, vivipary and hydrochory of propagules, which differentiate them from 'non-exclusive mangrove species" or "mangrove associates." The latter are mostly halophytes which grow on the landward margin of mangrove habitats. These include halophytes such as *Suaeda spp*., *Salicornia spp*. and *Sesuvium spp*. One of the striking features of most of these halophytes is the correlation between uptake of cations and whole plant succulence. Succulence balances out ion toxicity arising due to salinity and also increases the total plant water content (Waisel, 1972). Mangroves are classified as 'secretors' or 'non-secretors' on the basis of their salt exclusion mechanisms. The secretors possess special morphological structures like salt glands and salt hairs on the upper leaf surface, which eliminate excess salt actively by accumulating salt crystals. These include mangrove genera like *Avicennia*, *Acanthus*, *Aegialitis*, and *Aegiceras* where salt concentration of the leaf sap is one magnitude lower than that of surrounding saline medium. On the other hand, non-secretors show no morphological features like salt glands for salt exclusion. They can maintain a 1:100 salt concentration gradient against seawater via ultrafiltration. The non-secretors are considered better salt excluders than secretors and include the genera *Bruguiera*, *Rhizophora*, *Kandelia*, *Lumnitzera*, *Sonneratia* (Hanagata *et al.,* 1999). In the present investigation, effect of different concentrations of NaCl on the commonly occurring halophyte in the Pichavaram mangrove area *Ceriops roxburghiana* with regard to its growth and organic compounds was studied. The upper limit of the salt for the survival of the seedlings of *Ceriops roxburghiana* and the optimal level of salinity for its favourable growth and development were also assessed for their possible utility in cultivation of the species in salt-prone barren coastal environment.

MATERIALS AND METHODS

Identification of plant

The plant material used for the presence study was the seedlings of salt secretor mangrove, *Ceriops roxburghiana*, Arn. These species are typical woody mangrove shrubs with opposite leaves belonging to the family *Rhizophoraceae*. They were found growing in the tidal forests of mangrove belt of Pichavaram on the north east coast of Tamil Nadu, India, about 10 Km away from Annamalai University Campus (11° 24' N and 79° 44' E) Fig. 1.

Plant culture

One month old healthy seedlings of *Ceriops roxburghiana* of uniform size were uprooted from the mangrove soil without damaging the root system. This seedling were washed thoroughly with tap water and brought to the botanical garden of the Annamalai University. Polythene sleeves (7" X 5") were filled with homogenous mixture of garden soil, comprising of red earth, sand and farm-yard manure in the ratio of 1: 2: 1. Healthy seedlings were selected and planted in the polythene sleeves. These were irrigated with tap water and allowed to establish well. The seedlings established well within a month and then they were transferred to the experimental site roofed with transparent polythene sheet for Protection from rain water. The plants had an approximate 12 h photoperiod, a mean day temperature of 36^{o} C and night temperature of 27^{o} C.

NaCl treatment

Seedlings (two to three centimeters in height) were selected and kept in 11 plots, each consisting of 100 plants for salt treatments. Different millimolar concentrations of sodium chloride solution were prepared in distilled water. The treatment constituted 0 (Control) and 100 to 1000 mM NaCl. The salt treatment continued until each plant received the required millimolar NaCl. The control plants were maintained without the addition of NaCl. After the completion of salt treatment, the seedlings were irrigated with tap water. Since the seedlings of *Ceriops roxburghiana* treated with NaCl above 700 mM could not survive a week after the treatment, seedlings treated upto 700 mM NaCl were alone maintained in the experimental yard. Samples were collected periodically at bimonthly intervals for different analyses.

Growth

The total length of the seedlings, leaf and root length were measured before and after the treatment of sodium chloride. Five plants were collected from each concentration and used

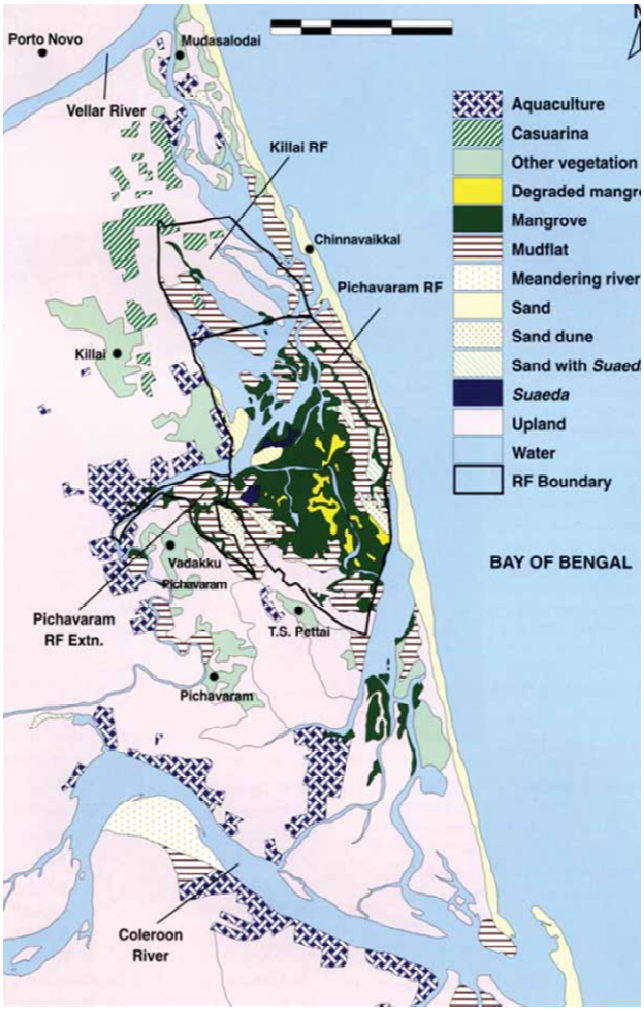

Fig. 1. Distribution of mangrove area in Pichavaram. Inset map showing the location of Pichavaram in India

for studying the morphological parameters. For the estimation of fresh weight, shoot and root portions were separated and weighed. To estimate the dry weight, the different plant organs were dried at 80°C for 48 h in an oven and weighed.

Amino acids

Shoot and root tissues were treated with 80 per cent boiling ethanol for taking extraction (5 ml extract representing 1 g tissue). Three readings for each sample were taken. One ml of ethanol extract was taken in 25 ml test tube and neutralized with 0.1 N sodium hydroxide using methyl red indicator. One ml of ninhydrin reagent was added (800 mg stannous chloride in 500 ml citrate buffer, pH 5.0, 20 g ninhydrin in 500 ml methyl cellosolve; both solutions were mixed). The contents were boiled in a water bath for 20 min 5 ml of diluting solution (distilled water and n-propanol mixed in equal volume) was added, cooled and diluted to 25 ml distilled water. The absorbance was measured at 570 nm in a spectrophotometer. The standard graph was prepared using Lucience (Moore and Stein, 1948).

Protein

Five hundred mg of plant sample was macerated with a mortar and pestle with 10 ml of 20 per cent trichloroacetic acid (Lowery *et al.,* 1951). The homogenate was centrifuged for 15 min at 600 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 upto 100 ml) was added and centrifuged. The supernatant was taken and made upto 5 ml with 0.1 N NaOH. This extract was used for the estimation of total protein. To 0.5 ml protein extract, 5 ml of the reagent C were 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 upto 100 ml). To this solution, 2 g of Na_2CO_3 was added. Reagent B: 2 g of $CuSO_4$ was dissolved in distilled water and made upto 100 ml and 2 g of sodium potassium tartarate was dissolved in distilled water and made upto 100 ml both solution were mixed with 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 absorbence was read at 660 nm in a spectrophotometer. The protein contents of unknown samples were calculated from Bovine serum albumin standards.

Total Sugars

One ml of ethanol extract taken in the test tube was evaporated in a water bath (Nelson, 1944). To the residue, 1 ml of distilled water and 1 ml of 1 N sulphuric 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's reagent was added to each test tube prepared by mixing reagent A and B 25:1 ratio (Reagent A: 25 g sodium carbonate, 25 g sodium potassium tartarate, 20 g sodium bicarbonate and 200 g anhydrous sodium sulphate in 1000 ml: Reagent B: 15 g cupric sulphate in 100 ml of distilled water with 2 drops of concentrated sulphuric acid). The test tubes were heated for 20 min in a boiling water bath, cooled and 1 ml of arsenomolybate reagent (25 g ammonium molybdate, 21 ml concentrated sulphuric acid, 5 g sodium arsenate dissolved in 475 ml of distilled water and incubated at 37°C in a water bath for 48 h) was added. The solution was thoroughly mixed and diluted to 25 ml and read at 495 nm in a spectrophotometer. The reducing sugar contents of unknown samples were calculated from glucose standards.

RESULTS

Ceriops roxburghiana, the *Rhizophoraceae* is a family of shrubs or small trees, often with stilt roots clustered around base of trunk; knee roots present or absent. Leaves crowded near tips of shoots, entire; veins obscure on both surfaces. Inflorescence usually 4- to many-flowered; bracteoles connate at base. Sepals usually 5, ovate, acuminate. Petals as many as sepals, free or cohering at base by marginal hairs, involute. Stamens usually 10, in unequal pairs opposite petals and enclosed by inrolling of petal margins; anthers 4-locular. Ovary half-inferior, 3-locular; style simple; stigma simple or obscurely lobed. Fruit elongating above rim of hypanthium. Seeds viviparous. Hypocotyl of propagule narrowly club-shaped, terete or ridged. The seedlings survived upto 300 mM NaCl and at higher concentrations of the salt, seedlings mortality occurred within a week after the salt treatment. The experimental plants were therefore, maintained upto 300 mM NaCl. Observation on the growth morphology and biochemical constituents were made at a month intervals on the 60th, 90th and 120th day after salt treatments. The data gathered from periodical observations were processed and presented in the form of figures. The data were also statistically analysed.

Fresh weight

The results on the effect of NaCl on the fresh weight of leaf and root of *Ceriops roxburghiana* are given in Fig. 1. Increase in the fresh weight of leaf and root upto optimum concentration of 300 mM NaCl. The percent increase in the leaf was found to be 35.14 %, 53.42 % and 31.15 % and root fresh weight was 25.45 %, 50.50 % and 43.48 % respectively when compared to that of control. At higher concentrations, there was reduction in the fresh weight. The fresh weight of shoot was always higher than that of root. All values were expressed as mean ± standard error and per cent over control. The results on the effect of NaCl on the dry weight of leaf and root of *Ceriops roxburghiana* are given in Fig. 2. The dry weight of leaf and root increased with increasing NaCl salinity upto 300 mM on all the sampling days.

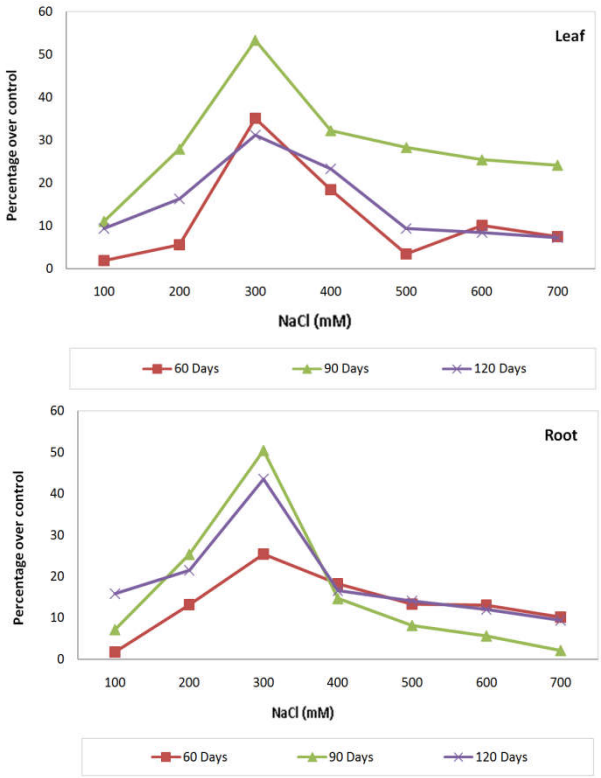

Fig. 1. Per cent increase or decrease in fresh weight of *Ceriops roxburghiana* at various concentration of NaCl at 60, 90 and 120 days of treatment

At higher concentrations, there was a gradual decease in the dry weight. The percent increase in the dry weight of leaf was 110.71 %, 64.45 % and 29.19 % and root dry weight was 45.03 %, 27.38 % and 54.55 % on 60th, 90th and 120th day respectively when compared to that of control. Even at extreme salinity of 700 mM, the maximum reduction of leaf and root dry weight was found to be 3.69 % and 25.78 % on 120th day when compared to control. The leaf dry weight was always higher than the root. All values were expressed as mean ± standard error and per cent over control. Changes in the total free amino acids at different levels of NaCl salinity were observed and the results are presented in Fig. 3. The amino acids content in leaf and root decreased with increasing salinity upto 300 mM on all the sampling days and the maximum decrease was observed (3.55 and 2.60 (mg g-^{1} dr. wt.) on 90th day respectively and this was 15.67% and 19.25% decreased when compared to their control. All values were expressed as mean ± standard error and per cent over control.

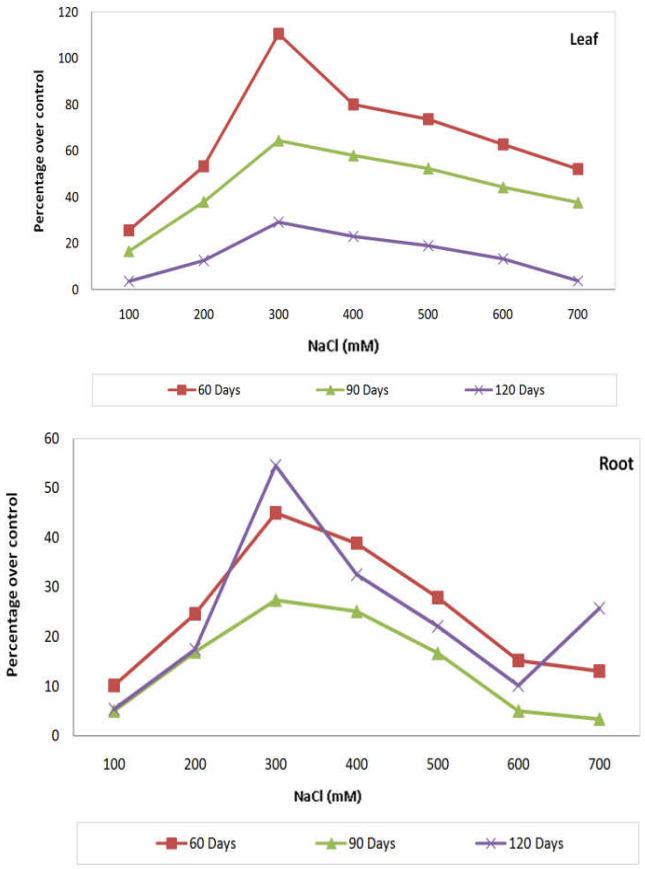

Fig. 2. Per cent increase or decrease in dry weight of *Ceriops roxburghiana* at various concentration of NaCl at 60, 90 and 120 days of treatment

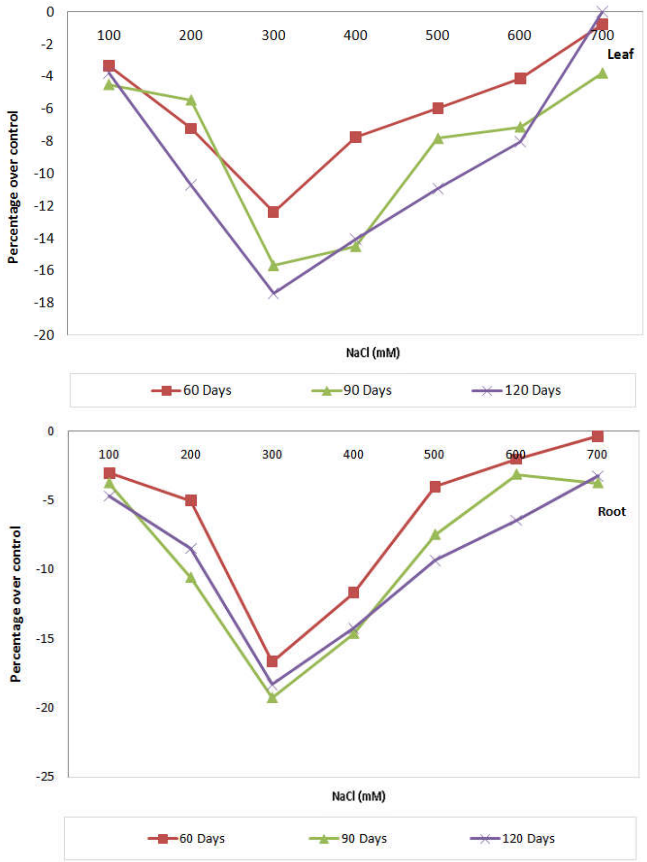

Fig. 3. Per cent increase or decrease in amino acids content of *Ceriops roxburghiana* at various concentration of NaCl at 60, 120 and 180 days of treatment

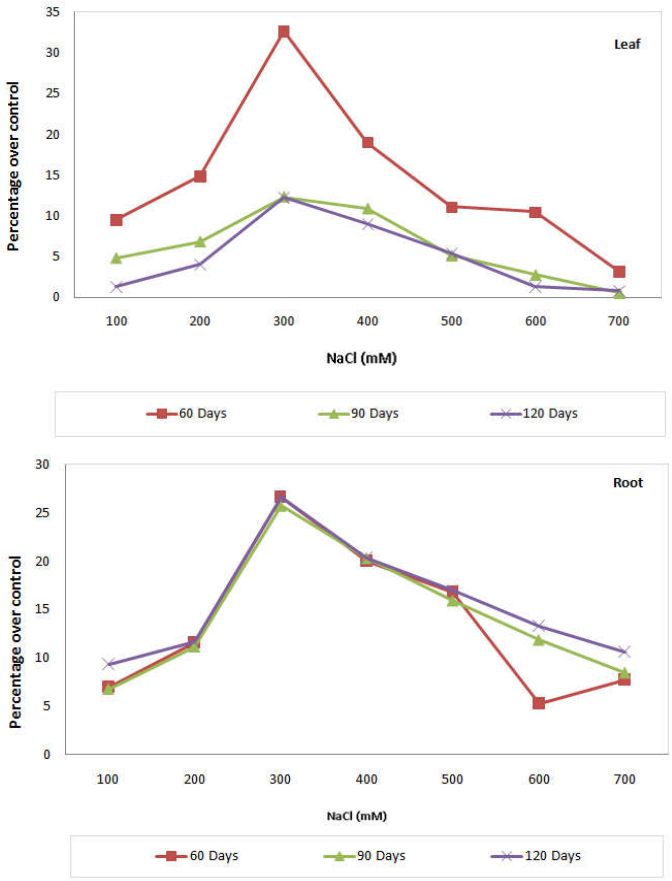

Fig. 4. Per cent increase or decrease in protein content of *Ceriops roxburghiana* at various concentration of NaCl at 60, 120 and 180 days of treatment

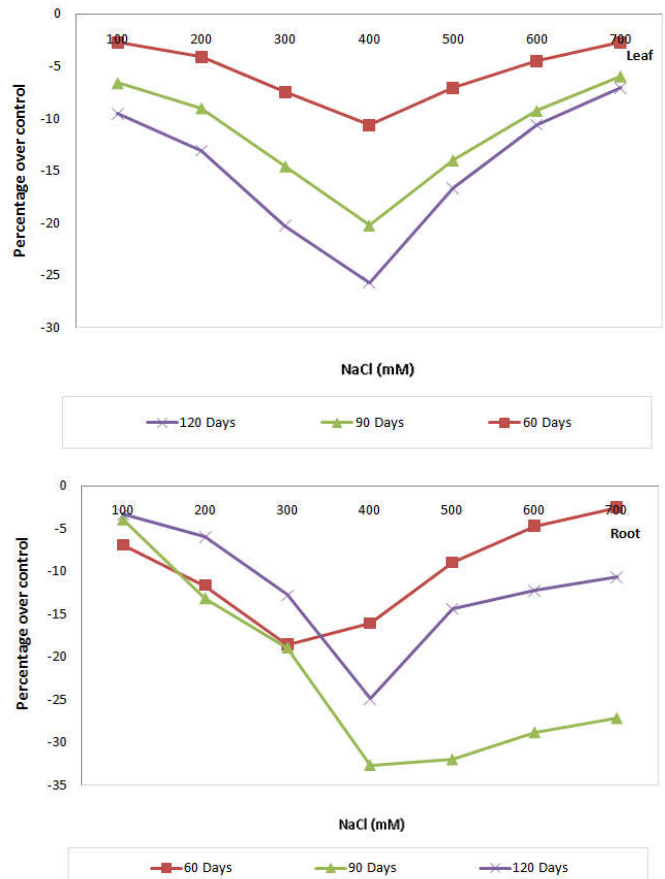

Fig. 5. Per cent increase or decrease in sugar content of *Ceriops roxburghiana* at various concentration of NaCl at 60, 120 and 180 days of treatment

The results on the effect of NaCl on the protein content in leaf and root are presented in Fig. 4. and the percent increase or decrease in protein content of leaf and root is shown in Fig. 4. The protein content increased with increasing salinity upto 300 mM and all the sampling days, with the maximum increase on the 90th day (3.91 and 3.71 mg g-^{1} dr. wt). The leaf had more protein than the root. The increase in protein content in the leaf and root was maximum at 300 mM on 90th day and this was 12.35 % and 25.76 % higher than that of control. At concentration above 300 mM there was a steadily decrease in protein in leaf and root. All values were expressed as mean ± standard error and per cent over control. Changes in the total sugar content in leaf and root in response to different concentrations of NaCl are given in Fig. 5. There was a gradual decrease in the total sugar content with increasing salinity upto the optimal level in leaf and root with 8.20 and 5.80 (mg g-^{1} fr. wt) on 90th day respectively and this was 7.13 % and 18.89 % higher than control. The leaf had more sugar content then the root. Beyond this optimum level the sugar content gradually increased at all the sampling days. At the extreme level of 700 mM, the sugar content was 3.21 % and 27.17 % higher than control. All values were expressed as mean ± standard error and per cent over control.

DISCUSSION

Salinity is one inimical factor of the environment to plant life. Crop plants are very sensitive to the presence of high concentrations of salt. The work on the salt induced damage to plant is quite extensive and has been reviewed from time to time (Flowers *et al.,* 1977). Most naturally occurring salinity problems are caused by excessive levels of sodium salt especially NaCl. During the past few decades, considerable attention has been given to the effect of NaCl on plant growth (Greenway and Munns, 1980). Excess NaCl in the soil medium causes injury to the crop plants by inhibiting growth followed by isolated brown areas of leaves, decreased cellular hydrostatic pressure, leaf drop and progressive death of the entire plant (Levitt, 1972). The exotic effect has been attributed to a specific influence of chloride ion, although excessive concentration of the sodium ion is also believed to cause detrimental effect (Brawley and Maths, 1990). The knowledge of the salinity effect on plant growth is fundamental to investigation on the physiological basis of salt tolerance. Earlier studies in halophytes have provided information on upper limits of salt tolerance together with crude estimates of optimal salt levels for growth and development of halophytes are either unaffected or stimulated (Pollak, 1967). In the present investigation, *Ceriops roxburghiana* was found to survive NaCl concentrations upto 700 mM. However, the favorable effect for maximum growth and development was noticed at 300 mM NaCl. At this optimal NaCl concentration, the percentage increase in the plant height was greater during the first 60 days after salt treatment when compared to those of later 60 days. This could be due to greater accumulation of salt in the tissues over prolonged period of salt treatment. The data also showed that there was depressed growth at high salinities. A stimulation of growth in response to moderate levels of NaCl salinity has been reported for several halophytes such as *Atriplex griffithii* which produce high yield in the presence of 360 mM NaCl and *Atriplex halimus* tolerates 480 mM NaCl (Gale *et al.,* 1970) and *Allendrolfea occidentalis* tolerates upto 600 mM NaCl (Gul *et al.,* 2000). Most halophytic species are inhibited by high salt concentration, with none of them show optimal growth at

seawater concentration (Ungar, 1991). Sodium chloride increased the fresh weight of the plant upto the optimal concentration of 300 mM NaCl. At higher NaCl concentration, the fresh weight o f shoot and root was reduced. In relation to seedlings growth, the cotyledons and the embryonic axis were suppressed by NaCl. They were smaller than in distilled water because of reduced fresh weight resulting from reduced water absorption (Prado *et al*., 1995). The fresh weight increase could be largely attributed to cell alignment by water absorption, cell vacuolation and turgor-driven wall expansion (Ayala and O'Leary, 1995). The dry weight of the shoot and root also increased with increasing concentrations upto 300 mM. Even at extreme salinity of 700 mM, though the dry weight was less than that of optimal concentration, it was higher than that of control plants. The dry weight increase may be attributed to the accumulation of organic and inorganic constituents in their tissues. In the dicotyledonous halophytes, Na^+ amd Cl^- ions were 30 to 50 % of the dry weight (Flowers *et al.,* 1986). The results of the present study reveal the obligate requirement of 300 mM NaCl for the optimal growth and maximum increase in dry weight. The accumulation of salt may have positive functions. Similar observations have been observed in certain other halophyte such as *Halosarcia pergranulata* (Short and Colmer, 1999), *Heliocola Setulosa* (Joshi *et al.,* 2002), *Chenopodium quinoa* (Prado *et al.,* 2000), *Kandelia candel* (Hwang and Chen, 2001; and *Aegiceras corniculatum* (Manikandan and Venkatesan, 2004).

Free amino acids in the shoots and roots of *Ceriops roxburghiana* significantly decrease with increasing concentrations of NaCl upto 300 mM. Beyond this concentration an increase in amino acids was observed (Table 18). Similar observations were made in other halophytes such as *Salicornia europaea* (Shamsutdinov *et al*., 1995), *Ipomoea pes-caprae* (Venkatesan and Chellappan, 1998) and *Helleochloa setulosa* (Joshi *et al*., 2002). Rao and Rao (1981) have reported that certain halophytes under moderate salinity accumulated free-amino acids and it was believed to be in response to change in the osmotic potential in their external environment by osmotic adjustment of cellular content and the increase in the amino acids at higher salinity levels may be due to degradation of protein. The increase in amino acids could be correlated to the decrease in the protein content in the present study. Accumulation of amino acid in the plant tissues occur not only under salinity stress, but also under water stress in higher plants (Treichel, 1975; Sivasankaramoorthy, 2013; Rasmia and Darwesh, 2013).

The pattern of 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 the amino acids and that the salinity changes influenced the inter conversion of these compounds. Similar findings were observed in various halophytic species such as *Sprobolus madraspatanus* (Joshi *et al*., 1996) and *Heleochloa setulosa* (Joshi *et al*., 2002). Increase in protein was association with the decrease in the amino acids content under moderate salinity and a reverse trend was noticed at higher salinity ranges. Protein synthesis responded dramatically to environmental stress such as heat shock (Key *et al*., 1982) and anaerobiosis (Sachs, 1980), water stress (Singh *et al*., 1985), osmotic shock (Fleck *et al*., 1982) and salt stress (Ericson and Alfinito, 1984) have also been shown to increase the net synthesis of protein. Salinity was shown to reduce the protein content accompanied by a considerable increase in the pigment and amino acid content (ABD EI-Samad, 1993; Saxena *et al.,* 2013; Silambarasan and Natarajan, 2014). There was a decrease in the sugar content upto optimum salinity level of 300 mM NaCl. Beyond this level, it gradually increased. Under severe salinity stress, the decrease in sugar content could be either due to high respiration or a decrease in photosynthetic activity accompanied by reduction in growth rate. An increasing sugar content and corresponding decrease in the starch at higher salinities have been reported in several halophytes (Prado *et al*., 2000; Joshi *et al.,* 2002; Ashraf and Hanis, 2004; Cabello *et al.,* 2014). An increase in soluble carbohydrate content, principally sucrose in both monocotyledons and dicotyledons has been associated with an adaptation to saline conditions among higher halophytes (Wang *et al*., 1996). It might also be mentioned that the adaptive value of sucrose in salt stress is questionable in species exhibiting a weak potential to store carbohydrates (Jefferies *et al*., 1979). Although some researchers agree that the salinity and water stress induce soluble sugar accumulation (Wang and Stutte, 1992; Kameli and Losel, 1995), there are objections to the suggestion that metabolically labile primary metabolites, such as reducing sugars, are "compatible" cytosolutes, since many of them have effects on cytoplasmic enzymes and could be incompatible in high concentration (Rozema *et al*., 1978). So it is concluded that this species could be recommended for cultivation in salt affected soils to reduce the soil salinity level and the redamated soil can be utilized for cultivation of crop.

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