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Modulation of various physiological changes in *Excoecaria agallocha* L. under NaCl stress

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Abstract
Excoecaria agallocha is a most important mangrove species in the tropical mangrove ecosystems and it grows in an extensive range of salinities. The adaptation of this milky mangrove to salinity could be at the physiological and biochemical level. The one month old plants of *E. agallocha*, were treated with different levels of NaCl (Control (0mM), 100mM-1000mM). Above 500 mM this mangrove seedlings did not survive. The modulation of different physiochemical changes in *E. agallocha*, such as photosynthetic pigments, fresh weight, dry weight and water content, compatible solutes were studied. Total chlorophyll and carotenoid content increased at 300 mM NaCl after salt treatment and the water content percentage was increased in leaf, stem and root tissues with increasing concentration. A significant increase of compatible solutes content such as amino acid, proline and glycine betaine showed an increasing pattern with increasing NaCl concentration as compared to the control in leaf, stem and root tissues. The sugar content increased with increasing concentrations of NaCl. The result establishes that *E. agallocha* tolerates 500 mM of NaCl concentration, without any foremost reticence on photosynthesis and metabolite accumulation. Understanding the modulation of various physiological and biochemical changes of *E. agallocha* at high levels of NaCl and remarkable accumulations of compatible solute make the plant survive salinity stress.

Keywords: mangroves, *E. agallocha*, photosynthetic pigments, compatible solutes, NaCl stress

Introduction
Mangrove forest consists of tropical trees and woody shrub like plants growing in the intertidal zone and highly productive ecosystem. Sternberg *et al.* (2007) [48] describes mangrove is a diverse group of tropical plants that are well adapted for the life in a tidal habitat. The biggest percentage of mangroves is establish between 5° N and 5° S latitude, but occurs all the way to 32° N and 38°S Latitude (Friess *et al.* 2012) [13]. Mangroves have 70 tropical and subtropical species, 28 genera and 19 families (Duke *et al.* 1998) [11]. The worldwide coverage of these halophytic spermatophytes has been estimated as 152,308 km^2 (Spalding *et al.* 2010) [47] and 137,760 km^2 (Giri *et al.* 2011) [15].

Plants are affected often due to abiotic stress like high or low temperature, drought, and salinity, flooding, chilling and freezing which pose hazardous risk to the crop plant productivity and quality, and negative trends in sustainable agriculture (Shrivastava & Kumar 2015) [45]. Among the abiotic stresses salinity is one of the serious factors of abiotic stress that substantially diminishes crop growth, development and production (Seleiman *et al.* 2020) [43]. Globally, 20 per cent of total cultivated and 33 per cent irrigated agricultural land affected by salinity (Abobatta, 2020) [1].

Salt stress has three fold effects; viz. i) the plant growth is impaired by osmotic stress ii) cell functions and structure damaged by ionic stress iii) finally suppress the yield (Mann *et al.* 2019a) [26]. Moreover, salinity stress can induce the overproduction of reactive oxygen species (ROS), which triggers the oxidative stress in various plant tissues, and causes chlorophyll degradation and oxidation of significant molecules including lipids, proteins and DNA (Radi, 2018) [38]. Furthermore, salt stress affects all the major processes such as growth, membrane damage, nutrient imbalance enzymatic inhibition and metabolic dysfunction, and photosynthesis which ultimately leads to plant death (Seleiman *et al.* 2020) [43].

Mangroves in the inter-tidal regions are exposed to high salinity due to evaporation of water and withdrawal of the sea due to tide. The water is swept off and salinity concentration increases (Perri *et al.* 2017) [36]. The salinity tolerance potential of mangroves is mainly the sum effects of its varied capacities such as effective ion compartmentation, osmoregulation, selective uptake of ions and its transport for compartmentation, regulating the movement of toxic ions to the shoot regions and capacity to regulate salt influx (Parida & Jha 2010) [32].

The plants exposed to high NaCl concentration have developed various biological responses (Parida & Das 2005) [31]. The mangroves are classified into three chief groups based on salt eliminating mechanisms i) salt excluders ii) salt secretors iii) salt accumulators (Parida & Jha 2010) [31]. Wang *et al.* (2002) [52] describes some mangroves have adapted the mechanisms for eliminating overload salt by ultrafiltration in root cell membranes. Salt secreting mangroves regulate the internal level of salt by expelling the excess salt through leaf glands (Selvam, 2003) [44]. Salt accumulating type mangroves accumulates high level of salts in the cells and tissue and at the same time avoid toxicity due to these salts by sequestering toxic ions into the vacuoles of leaves, translocate the ions out of leaves by cuticular transpiration (Aziz & Khan 2001b) [5].

The success of the mangroves to establish and survive under harsh conditions is by their specialized adaptations such as salt-excreting leaves and viviparous nature of the propagules (Parida & Jha 2010) [32]. Plants have evolved a variety of protective mechanisms viz. accumulation of different ions and osmolytes like proline, protein, sugar etc. which allowed them for survival and growth at the unfavorable environmental condition. Therefore, accumulation of these compounds prevents water loss and ionic toxicity of the plant cells (Rajaravindran & Natarajan 2012) [40]. Therefore, biochemical mechanisms of mangroves counter the high osmolarity of salts by accumulation of compatible solutes (Parida *et al.* 2004) [33] which do not interfere with the plant metabolism but contribute to turger maintenance and osmoprotectance in plant (Sozharajan & Natarajan 2015) [46].

The plant material used in the present study was *E. agallocha* L. It belongs to Euphorbiaceae family, a tree or shurb mangrove species. It is an important medicinal plant and among the few non-viviparous mangrove species, it also known as milky mangrove and blinding tree. In the present investigation the effect of NaCl on growth, photosynthetic pigments, biochemical constituents and accumulation of compatible solutes of *E. 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 an evergreen mangrove species 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 10km east of Annamalai University campus. The mature seedlings were collected from Pichavaram. Healthy seedlings with uniform size were planted individually in polythene 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 polythene 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, 900,1000mM 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 60th day after salt treatment.

Morphological studies

Shoot length (cm/plant)

Plant height was recorded by measuring the height of the plant from the surface of the soil to the tip of the top most leaf. This was recorded on 60th day after treatment and expressed in cm plant^{-1}.

Root length (cm/plant)

The root length was measured from the point of first cotyledonary node to the tip of longest root and expressed in cm plant^{-1}

Physiological studies

Dry weight and water content

To record the fresh weight, leaf, stem and root portions were separated and weighed. They were dried in a hot air oven at 80ºC for 24 hours. Then, the dry weight was taken by using an electronic balance.

Water content (g) = fresh weight − dry weight

Total chlorophyll and carotenoid content

Total chlorophyll and carotenoid content were calculated according to the method of Arnon (1949) [2].

Biochemical estimations

Amino acid content

Free amino acids were extracted from plant samples using 70 per cent ethanol and estimation was carried out following the method of Moore and Stein (1948) [28] using ninhydrin reagent. Total free amino acids were calculated from a standard curve prepared with glycine.

Total sugar content

Total soluble sugar content was extracted from plant samples by 80 per cent ethanol and estimated following the method of Dubois *et al.* (1956) [10]. Standard curve was plotted with d-glucose as standard.

Protein content

Protein content was extracted from plant using 20 per cent trichloro acetic acid (TCA) and estimated following the method of Lowry *et al.* (1951) [22].

Proline content

Free proline content was extracted from plant using 3% sulphosalicylic acid and estimated following the method of Bates *et al.* (1973) [7] using l-proline as standard.

Glycine betaine (GB) content

GB activity was assayed by the method of (Grieve & Grattan 1983) [16].

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 Dunkan ($P < 0.05$) with the help of SPSS 16.0 software package. Means and standard deviation were calculated from three replications.

Results

Shoot length and root length of NaCl treated plants were increased with increasing concentration up to 300 mM compared to control plants (Figure 1). Fresh weight, dry weight and water content of leaf, stem and root were increased when treatment was done with 300 mM NaCl as compared to control plants (Figures 2, 3, 4). The total chlorophyll and carotenoid content in leaves was showing an increasing pattern upon increasing concentration of NaCl, i.e., 14%, 10% increase in the treatments of 300 mM NaCl, respectively on 60 days after salt treatment (Figures 5, 6). The highest amino acids content was recorded on 60 days in leaf, stem and root tissues, subjected to treatment with 500 mM NaCl concentration and the increase was 67%, 43% and 21% higher in leaf, stem and root respectively on 60 days over the control plants (Figure 7). The result on the effect of NaCl on the protein content of leaf, stem and root of *E. ahallocha* is given in Figure 8. The protein content increased with increasing concentrations up

to 300 mM and at higher concentrations it steadily declined. Total sugar content increased in leaf, stem and root tissues of *E. agallocha* when exposed to increasing concentration of 500 mM NaCl. The leaves showed more total sugar content than the stem and root (Figure 9). The effect of NaCl on the proline content in the leaf, stem and root are given in Figure 10. 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. The results on the glycine betaine content of the leaf stem and root at various salinity levels of NaCl are given in Figure 11. The glycine betaine content in all the three tissues increased with increasing NaCl up to 500 mM.

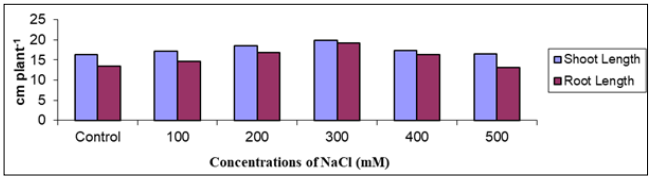

Fig 1: Effect of NaCl on shoot and root length of (cm plant^{-1}) of *Excoecaria agallocha* on 60th day after salt treatment

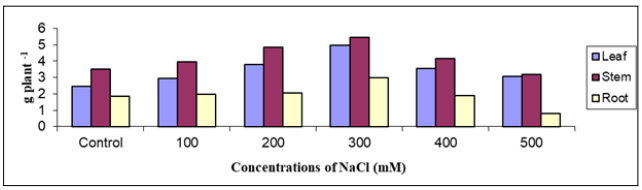

Fig 2: Effect of NaCl on whole plant fresh weight (g plant^{-1}) of *Excoecaria agallocha* on 60th day after salt treatment

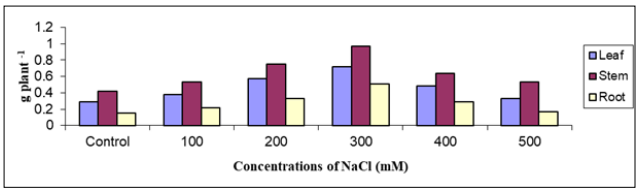

Fig 3: Effect of NaCl on whole plant dry weight (g plant^{-1}) of *Excoecaria agallocha* on 60th day after salt treatment

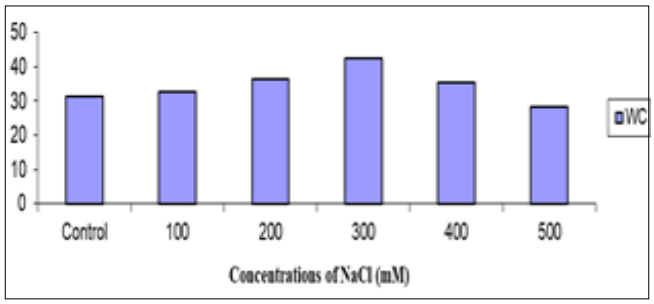

Fig 4: Effect of NaCl on water content (g plant^{-1}) of *Excoecaria agallocha* on 60th day after salt treatment

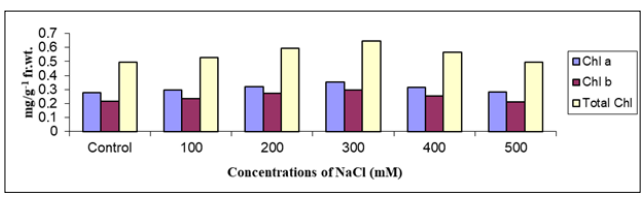

Fig 5: Effect of NaCl on chlorophyll ʿaʾ chlorophyll ʿbʾ and total chlorophyll content of *Excoecaria agallocha* in 60th day after salt treatment

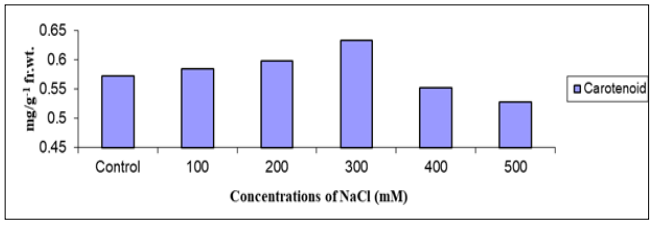

Fig 6: Effect of NaCl on carotenoid content of *Excoecaria agallocha* in 60^{h} day after salt treatment

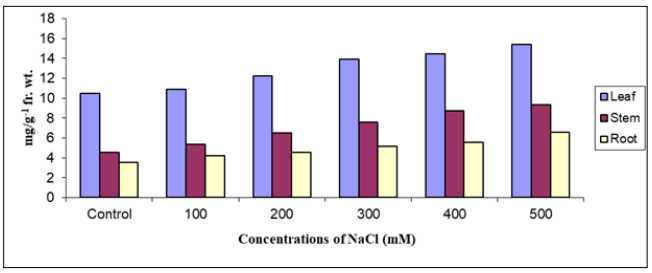

Fig 7: Effect of NaCl on amino acid content (mg/g fr. wt.) of the leaf, stem and root of *Excoecaria agallocha* in 60th day after salt treatment

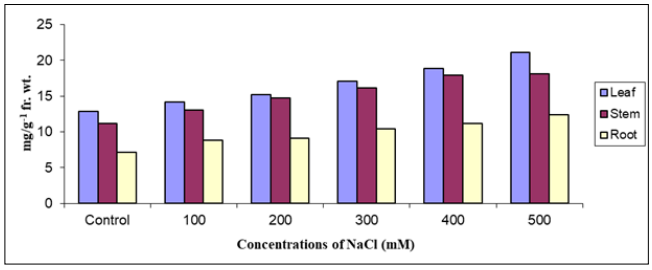

Fig 8: Effect of NaCl on total sugar content (mg/g fr. wt.) of the leaf, stem and root of *Excoecaria agallocha* in 60th day after salt treatment

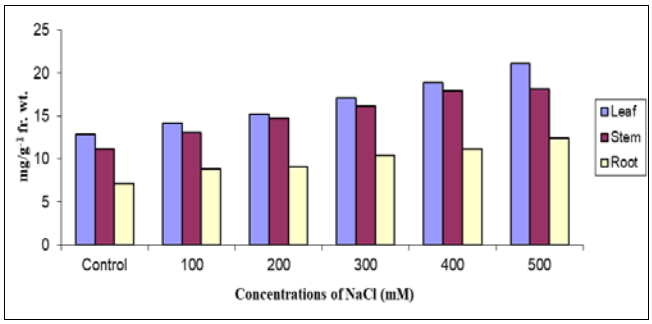

Fig 9: Effect of NaCl on protein content (mg/g fr. wt.) of the leaf, stem and root of *Excoecaria agallocha* in 60th day after salt treatment

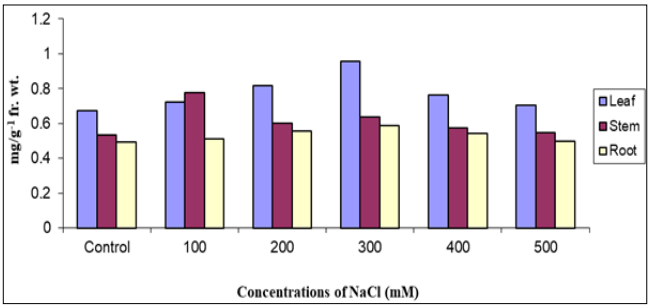

Fig 10: Effect of NaCl on proline content (mg/g fr. wt.) of the leaf, stem and root of *Excoecaria agallocha* in 60th day after salt treatment

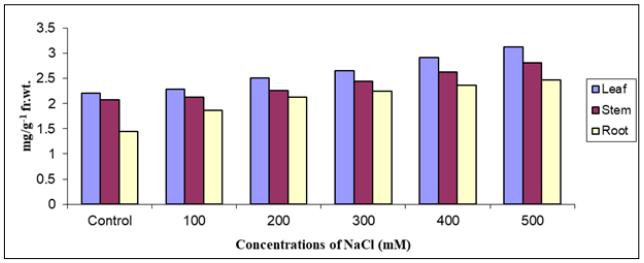

Fig 11: Effect of NaCl on glycine betaine content (mg/g fr. wt.) of the leaf, stem and root of *Excoecaria agallocha* in 60th day after salt treatment

Discussion and Conclusion

In the present investigation, *E. agallocha* was found to survive NaCl concentration up to 500 mM. However, the favorable effect for maximum growth and development was noticed at 300 mM NaCl. Shoot and root length of *E. agallocha* plants was stimulated by salinity at 300 mM.

The elongation of the shoot and root length when treated with low concentration of salt may induce osmotic adjustment activity in the plants which may develop growth. On the other hand, the noticed decrease in the length of the shoot and root, also due to treatment with NaCl solution, could be due to the harmful effect of salt on the rate of photosynthesis, the changes in enzyme activity and also decrease the level of protein synthesis, which can lead to inhibition of the growth (Mazher *et al.* 2007) [27].

NaCl salinity augmented the fresh weight of the plant organs up to the optimal concentration of 300 mM NaCl. At the elevated NaCl concentrations, the fresh weight of leaf, stem and root was reduced. The increase in fresh weight of the leaf tissue can be attributed to the increase in leaf thickness (Clipson, 1987) [8] and the accumulation of ions and water in the tissues (Lee *et al*. 2008) [21]. Also, the fresh weight increase could be largely attributed to cell extension by water absorption, cell vacuolation and turgor driven wall expansion (Ayala & O'Leary 1995) [4].

The dry weight of the leaf, stem and root also increased with increasing concentration up to 300 mM. At higher concentrations, the dry weight of leaf, stem and root was reduced. The dry weight increase could be attributed to the accumulation of inorganic salts and organic matter in the plant tissue.

In the dicotyledonous halophytes, it was observed that Na^+ and Cl^- ions were 30-50 per cent of the dry weight (Flowers *et al*. 1986) [12]. The results of the present study also indicated the obligate requirement of optimum concentration of NaCl for cell growth and increase in dry weight. The accumulation of salt has a positive function. Similar observations have been observed in certain halophytes such as *Helechola setulosa* (Joshi *et al*. 2002) [19] and *Aegiceras corniculatum* (Manikandan & Venkatesan 2004) [24].

Increased dry weight and water content of leaf, stem and root tissues of *E. agallocha* was observed and the maximum increase was observed in leaf tissue than in stem and root during optimum level of NaCl treatment (300 mM), the carbon allocation from the stem to the root and the consequent increase of the dry matter at the expense of the water content, and this help the plant to tolerate the high salinity stress (Barr, 2013) [6]. At higher salinity *Aeluropus lagopoides* showed distinguishing variation in fresh and dry plant biomass indicating its higher tolerance. Thus mangrove species with maximum dry weight percentage at higher salinity are more tolerant (Rao *et al.* 2005) [41].

Sodium chloride salinity stimulated photosynthetic pigments such as chlorophyll and carotenoid synthesis up to the optimum concentration of 300 mM and at higher concentrations, the photosynthetic pigments decreased gradually. The increase in chlorophyll pigments up to 300 mM NaCl in the leaves of *E. agallocha* in the present investigation indicates that optimal salinity neither affected protein-pigment-lipid complexes nor the chlorophllase activity. Further increase in chlorophyll content could be correlated with the increase in photosynthesis. Though chlorophyll content increased upto optimal NaCl salinity of 300 mM, there were differences in the amount of chlorophyll content at different duration of salt treatment.

Carotenoids are pigments with numerous functions in plants, besides their direct role in photosynthesis; it has an active part in oxidative stress tolerance (Gill & Tuteja 2010) [14]. Carotenoids play essential role in PS-I assembly and the stability of light harvesting complex proteins as well as thylakoid membrane stabilization (Niyogi *et al.* 2001) [30]. Salinity stress produces an increase in carotenoids levels as a tolerance mechanism.

E. agallocha accumulated amino acids in the leaf, stem and root with increasing NaCl concentration up to 500 mM. The leaf had more amino acids than the stem and root. Vicente *et al.* (2004) [50] reported that the leaves of a halophyte *Plantago crassifolia* accumulated greater content of free amino acids during an extended period of 300 mM NaCl exposure. The increased content of amino acids recorded in *E. agallocha* upon NaCl treatment indicates that this species possessed an effective NaCl stress tolerance mechanism. Although this species proves tolerance to high NaCl concentration, other mangrove species, like *A. corniculatum* are not adaptable to 500 mM NaCl in saline condition (Parida *et al.* 2002) [34].

The total sugar content was found to increase gradually with increasing concentrations of NaCl treatment up to 500 Mm. This was a clear indication that *E. agallocha* can tolerate high NaCl concentration in the initial period of stress and also sugar accretion contributed extensively towards maintaining the osmoticum of the cell sap. But in some mangroves like *A. corniculatum*, total sugar content decreased at 250 mM of NaCl during 30 days of treatment. Sozharajan & Natarajan (2015) [46] reported that total sugar content decreased on exposure to 300 mM of NaCl in *E. agallocha.* Compatible solutes are synthesized in living organisms in response to osmotic stress. Total sugar can contribute significantly towards osmotic stress tolerance by ensuring membrane integrity (Manchanda & Garg 2008) [23], acting as osmoprotectants, not only maintaining cell turgidity but also protecting membranes against increased salinity (Cooper & Farrant 2002) [9].

The protein content increased with increasing concentration up to an optimal level, beyond the optimal level the protein content decreased due to proteolysis and decreased protein synthesis (Sozharajan & Natarajan 2015) [46]. Leaf protein content is one of the crucial indicators of the effects of salt stress (Isayenkov & Maathuis 2019) [17].

Under stress condition, plant accumulates protein that protects cells from environmental stress effects (Wang *et al.* 2003) [53]. Specific expression of stress proteins is an important adaptive manifestation in maintaining the integrity, native configuration and topology of cellular

membrane components to ensure their normal functioning under salinity stress (Wahid *et al.* 2007) [51]. Our results are in conformity with the results of other reports which include the work carried out on *E. agallocha* (Mariam *et al.* 2020) [26].

Proline accretion is one of the familiar physiological responses in higher plants exposed to salinity stress (Per *et al.* 2017) [35]. It functions as a possible molecular chaperone in osmotic adjustment and defense of cellular structures, and membranes during osmotic stress in plants (Zhang & Shi 2018) [54]. Proline protects the protein-lipid components of the cell membranes (Szabados & Savoure 2010) [49], and can act as antioxidant, energy metabolism (Kartashov, 2013) [20]. Our study shows that under high salinity, *E. agallocha* significantly increased the accumulation of proline compared to lower salt conditions. Our results are in conformity with the results of other reports which include the work carried out on *P. triandra*, (Podar *et al.* 2019) [37].

In salt tolerant species, accumulation of glycine betaine under salt stress was high (Jagendorf & Takabe 2001) [18]. GB protects photosynthetic machinery in case of some mangroves such *as A. marina* (Ashihara *et al.* 1997) [3]. The levels of osmoprotectants increased during exposure to stresses such as salinity. Glycine betaine acts as defensive molecules in higher plants at extreme conditions of salt, drought, temperature or light stress (Sakamoto & Murata 2000) [42]. GB is the most common compatible solute; which protects plants photosynthetic machinery and also found in some mangroves such *as A. marina* (Ashihara *et al.* 1997) [3].

In the present study, various levels of salt treatment alone decreased the photosynthetic pigments, while the compatible solutes may have contributed to the reduced oxidative damage and osmotic adjustment in the cytoplasm under salinity stress.

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