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Research Article

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Response of enzymatic and non-enzymatic antioxidant systems in *Excoecaria agallocha* to salt stress

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ABSTRACT

The effect of NaCl stress on antioxidant enzymes activities was investigated in the leaf and root of *Excoecaria agallocha*. Plants were subjected to different levels of NaCl. 100 to 1000 mM. Above 500 mM these mangrove seedlings did not survive. The leaves of 60 day old plants were used for the analysis of enzyme activities. Parameters of enzymatic and non-enzymatic antioxidants such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), polyphenol oxidase (PPO), superoxide dismutase (SOD), ascorbic acid (A_SA) and alpha tocopherol were determined. The highest CAT, POD, APX, PPO and SOD activities in the leaf and root enhanced gradually up to 300 mM of NaCl, the highest A_SA and tocopherol activities in the leaf and root were observed at 500 mM of NaCl. These data suggest that the capacity to limit oxidative damage is important for the salt tolerance of *E. agallocha*.

KEYWORDS: Salinity, *Excoecaria agallocha*, antioxidant activity

INTRODUCTION

Soil salinity is among the most devastating environmental stressors that negatively affect crop production, affecting millions of hectares of land around the world and causing significant economic losses each year (Chung *et al.*, 2019). Approximately 62 million hectares (20%) of irrigated land are currently affected by high salt content (Chung *et al.*, 2019), and it has been estimated that more than 50% of arable land will be salinized by 2050 (Shrivastava & kumar, 2015).

Soil salinity limits crop productivity by impairing root growth, nutrient uptake, and metabolic processes (Chung *et al.*, 2019). In addition, salinity stress affects physiological, morphological, and biochemical processes, which decrease crop biomass and productivity (Chung *et al.*, 2019).

Salinity stress has a drastic effect on plant morphology and physiology due to the physiologically mediated osmotic stress. This can result imperfections in plant water relations and ionic balance that eventually leads to ionic toxicity of plant metabolic processes (Semida *et al.*, 2016; Al-Ashkar *et al.*, 2019; Seleiman *et al.*, 2020). Moreover, salinity stress can induce the overproduction of reactive oxygen species (ROS), which triggers the oxidative stress in different plant tissues, and causes chlorophyll degradation and oxidation of significant molecules including lipids, proteins and DNA (Radi, 2018). Additionally, elevated salt stress can reduce the photosynthetic efficiency, plants growth and productivity and can induce the accumulation of toxic ions (Abd El-Mageed *et al.*, 2017; Taha *et al.*, 2021). Therefore, to mitigate the negative impacts of salt stress, plants have different mechanisms to protect themselves from the effects of OS by inducing the activities of various enzymatic and non-enzymatic antioxidants (Semida *et al.*, 2014).

Various environmental stresses frequently disturb the homeostasis and ion distribution in plant cells and induce osmotic stress, leading to an augmentation in the accumulation of reactive oxygen species (Herbette *et al.*, 2011). The production and accumulation of reactive oxygen species in the plants result in brutal damage of cell organelles and functions cause membrane peroxidation, leading to damage in the cell membrane, restricts the normal metabolism of cells and destroys through oxidation of DNA, proteins, lipids, and other macromolecules of the cell and ultimately cell death (Ahanger *et al.*, 2020).

The ability of plants to scavenge the toxic effects of reactive oxygen species seems to be the most important determinant for their tolerance to various stresses. Antioxidants are the first line of defence against the damages caused by free radicals and are vital for the optimum health of plant cells (Rajput *et al.*, 2016). Plant antioxidants play a significant role in assisting

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plant development through a wide variety of mechanisms and functions. There are numerous antioxidant enzymes associated with ROS scavenging in plants, and the synthesis of these enzymes is known to be improved during the exposure to oxidative stresses (Eltayeb *et al.*, 2007). The reactive oxygen species contain free radicals, such as superoxide radicals (O_2^-), hydroxyl radicals (OH), perhydroxyl radicals (HO_2^-) and alkoxy radicals, and non-radical forms, i.e., hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), present in the intra- and extracellular locations of the plant. Superoxide radicals (O_2^-) can be generated by a single electron transfer (e^-) to dioxygen (O_2).

The tolerance mechanisms in stressed plant include a number of physio-biochemical strategies, which includes many enzymatic components, such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione peroxidase (GPX), monodehydroascorbate reductase and dehydroascorbate reductase, and non-enzymatic components, such as ascorbic acid (AsA), phenolic compounds, carotenoids and Alpha-tocopherols (Zhou *et al.*, 2018). The antioxidant defence system protects the unsaturated membrane lipids, nucleic acids, enzymes and other cellular structures from the negative impacts of free radicals (Dumont & Rivoal, 2019). Therefore, the antioxidant defence system of plants has been attracting considerable interest of the scientific community (Dumont & Rivoal, 2019). A correlation between the antioxidant capacity and NaCl tolerance has been demonstrated in numerous plant species such as *Aegiceras corniculatum* (Mohanty *et al.*, 2013), *Avicennia marina* (Maqtari & Nagi, 2014) and *Bruguiera cylindrica* (Sruthi & Puthur, 2018).

In the present study the effect of different concentrations of NaCl salinity on the activities of enzymatic (Catalase, Peroxidase, Ascorbate peroxidase, Polyphenol oxidase and Superoxide dismutase), and non enzymatic (Ascorbic acid and Alpha-tocopherol) antioxidant enzymes in *E. agallocha* have been monitored.

MATERIALS AND METHODS

Plant Material and Salt Stress Application

E. agallocha L., 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, 900,1000 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 60th day after salt treatment.

EXTRACTION OF ENZYMES AND ASSAYS

Determination of Enzymatic Antioxidants

Two grams of young leaves were macerated to powder with liquid nitrogen with a mortar-pestle; then 0.1 g PVP and 5 ml of extraction buffer (consisting of 1 M Sucrose, 0.2 M Tris-HCl and 0.056 M β-Marcaptoethanol; pH adjusted at 8.5) was added and homogenized. The extracts were centrifuged at 10,000 rpm for 20 min at 48°C; supernatants were used as samples for enzyme assay.

ESTIMATION OF CATALASE: (CAT: E.C.1.11. 1.6)

Catalase (CAT: EC.1.11. 1.6) was measured according to Chandlee & Scandalios, (1984) by change in absorbance at 240nm. An assay mixture contained 2.6ml of 50mM potassium phosphate buffer (pH7.0), 0.4ml of 15mM H_2O_2 and 0.04ml of enzyme extract. The decomposition of H_2O_2 was followed by the decline absorbance at 240nm. The enzyme activity is expressed in units per min per mg protein.

ESTIMATION OF PEROXIDASE: (POX: E.C.1.11.1.7)

Peroxidase (POX: E.C.1.11.1.7) activity was measured by Kumar & khan, (1982) following the change in absorbance at 470nm due to 2ml of 0.1 M phosphate buffer (pH 6.8), 1ml of 0.001 M pyrogallol, and 1ml of 0.0054 M hydrogen peroxide and 0.5ml of enzyme extract. The reaction mixture was incubated for 5 minutes at 25°C, after which the reaction was terminated by adding 1ml of 2.5N sulphuric acid. The activity is expressed in unit per minute per mg protein.

ESTIMATION OF ASCORBATE PEROXIDASE (APX E.C. 1.11.1.11)

APX (EC: 1.11.1.11) activity was assayed following the method of Asada & Takahashi, (1987). The reaction buffer solution contained 50 mM K-phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA and enzyme extract in a final volume of 0.7 ml. The reaction was initiated by the addition of H_2O_2 and activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

ESTIMATION OF POLYPHENOL OXIDASE (PPO E.C.1.10.3.1)

Polyphenol oxidase activity was assayed by the method of Kumar & khan, (1982). Assay mixture for polyphenol oxidase contained 2ml of 0.1 M phosphate buffer (pH 6.0), 1ml of 0.1M catechol and 0.5ml of enzyme extract. This was incubated for 5 minutes at 25°C, after which the reaction was stopped by adding 1ml of 2.5N sulphuric acid. The absorbance of the purpurogallin formed was recorded at 495nm. The enzyme activity is expressed in units. One unit is defined as the amount of purpurogallin formed, which raised the absorbance by 0.1 per minute under the assay condition.

ESTIMATION OF SUPEROXIDE DISMUTASE: (SOD: E. C. 1. 15.1.1)

Superoxide dismutase (SOD: E. C. 1. 15.1.1) was assayed as described by Beauchamp & Fridovich, (1971). The reaction mixture contained 1.17 μM × 10^{-6}M riboflavin, 0.1 M methionine, 2μM × 10^{-5}M potassium cyanide and 5.6 μM × 10^{-6}M Nitroblue tetra-zolium salt (NBT) dissolved in 3ml of 0.05 M sodium phosphate buffer (pH7.8). Three ml of the reaction medium was added to 1 ml of enzyme extract. The mixtures were illuminated in glass test tubes of selected uniform thickness. The illumination was performed by two sets of Philips 40W fluorescent tubes. The test tubes were arranged in a single row, with a set of tube lights fixed on either side. Illumination was started to initiate the reaction at 30°C for an hour. Identical solutions were kept under dark served as blanks. The absorbance was read at 560nm in the spectrophotometer against the blank. Superoxide dismutase activity is expressed in units. One unit is defined as the amount of change in the absorbance by 0.1 per hour per mg protein under assay condition.

DETERMINATION OF NON-ENZYMATIC ANTIOXIDANT

Ascorbic Acid (A_SA)

Ascorbic acid was analyzed by Omaye *et al.* (1979). Ascorbate was converted into dehydroascorbate on treatment with activated charcoal, which in turn reacted with 2,4-dinitrophenylhydrazine to form osazones. These osazones produce an orange coloured solution when dissolved in H_2SO_4, whose absorbance can be measured spectrophotometrically at 540nm.

Ascorbate was extracted from 1g of the plant sample using 4 per cent TCA and the volume was made up to 10 mL with the same. The supernatant obtained after centrifugation at 2000rpm for 10 minutes was treated with a pinch of activated charcoal shaken vigorously using a cyclomixer and kept for 5 minutes. The charcoal particles were removed by centrifugation and aliquots were used for the estimation. Standard ascorbate ranging between 0.2 and 1.0 mL of the supernatant were taken. The volume was made up to 2.0 mL with 4 per cent TCA. DNPH reagent (0.5 mL) was added to all the tubes, followed by 2 drops of 10% thiourea solution. The contents were mixed and incubated at 37°C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5 mL of 85% H_2SO_4 in cold. To the blank alone, DNPH reagent and thiourea were added after cooled in ice and the absorbance was read at 540nm in a spectrophotometer. The concentrations of ascorbate in the samples were calculated using a standard curve and expressed in terms of mg/g FW of sample.

Alpha-tocopherol

Alpha -tocopherol was analyzed by Backer *et al.* (1980). Five hundred milligrams of fresh tissue was homogenized with 10 mL of a mixture of petroleum ether and ethanol (2:1. 6 v/v) and the extract was centrifuged at 10,000rpm for 20 minutes and the supernatant was used for the estimation of Alpha-tocopherol. To one mL of extract, 0.2 mL of 2% 2,2-dipyridyl in ethanol was added and mixed thoroughly and kept in dark for 5 minutes. The resulting red colour was diluted with 4 mL of distilled water and mixed well. The resulting colour in the aqueous layer was measured at 520nm. The Alpha -tocopherol content was calculated using a standard graph made with known amount of Alpha -tocopherol.

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

Effect of Salinity on Enzymatic Antioxidant

The effect of NaCl on the catalase, peroxidase, ascorbate peroxidase, polyphenol oxidase and superoxide dismutase activity in the leaf and root at various NaCl concentrations is presented in Figure 1-5. There was a steady increase in all the enzyme activities up to 300 mM NaCl. At higher concentrations, these enzymes activities reduced gradually. These enzyme leaf activities were higher than that of root.

Effect of Salinity on Non-enzymatic Antioxidant

The effect of NaCl on the Ascorbic acid and Alpha-tocopherol content in the leaf and root at various NaCl concentrations is presented in Figure 6 and 7. There was a steady increase in both enzymatic antioxidants up to 500 mM NaCl.

DISCUSSION AND CONCLUSION

In this study, enzymatic and non enzymatic antioxidants were estimated, *i.e.* CAT, POD, APX, PPO, SOD, A_SA and tocopherol.

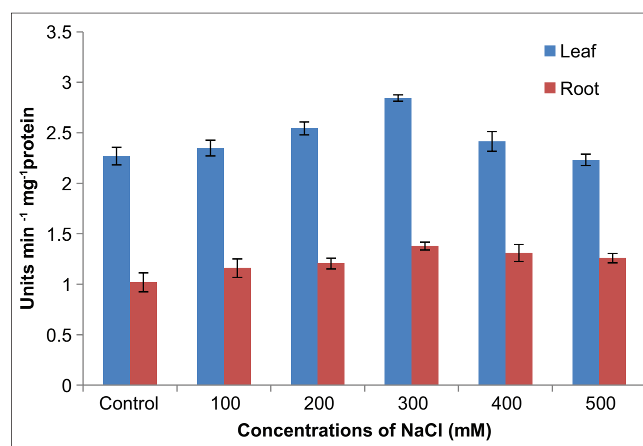

Figure 1: Effect of different concentrations of NaCl on catalase activity (Units min^{-1} mg^{-1} protein) in leaf and root of *Excoecaria agallocha* 60th day after salt treatment

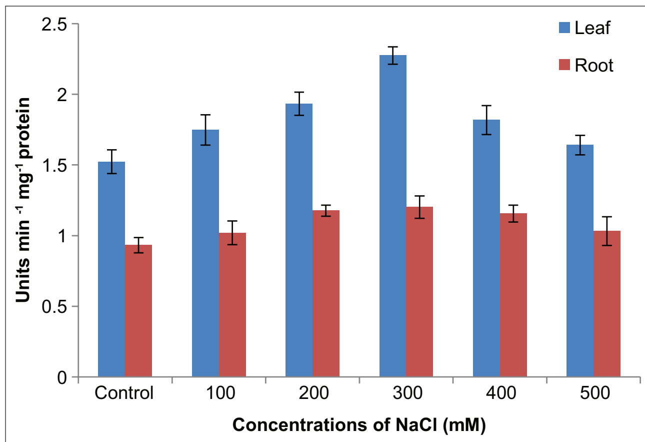

Figure 2: Effect of different concentrations of NaCl on peroxidase activity (Units min^{-1} mg^{-1} protein) in leaf and root of *Excoecaria agallocha* 60th day after salt treatment

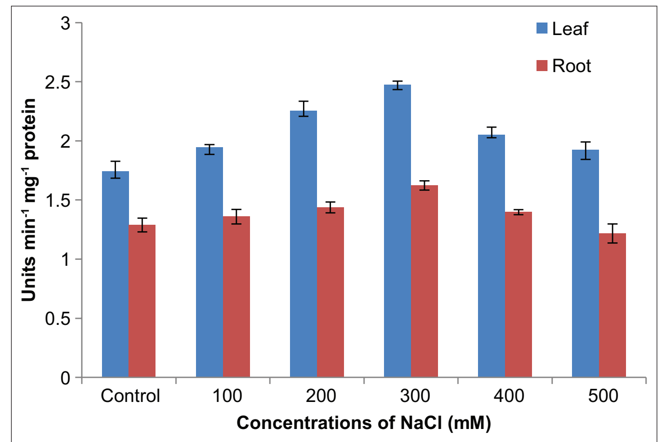

Figure 5: Effect of different concentrations of NaCl on superoxide dismutase activity (Units min^{-1} mg^{-1} protein) in leaf and root of *Excoecaria agallocha* 60th day after salt treatment

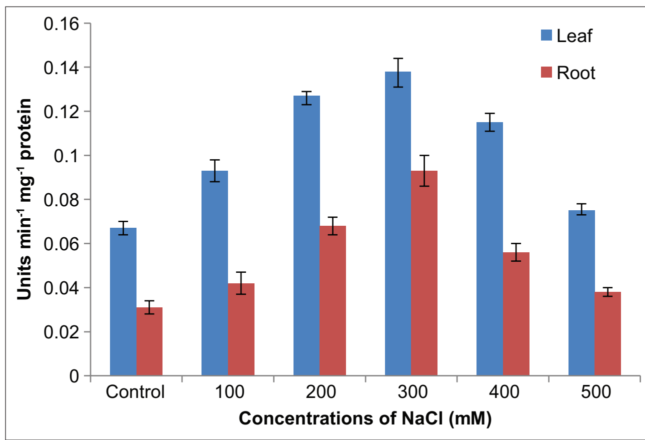

Figure 3: Effect of different concentrations of NaCl on ascorbate peroxidase activity (Units min^{-1} mg^{-1} protein) in leaf and root of *Excoecaria agallocha* 60th day after salt treatment

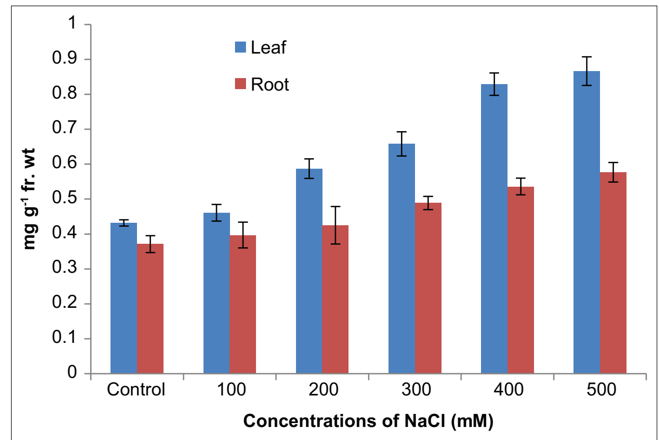

Figure 6: Effect of different concentrations of NaCl on ascorbic acid content (mg g^{-1} fr. wt) in leaf and root of *Excoecaria agallocha* 60th day after salt treatment

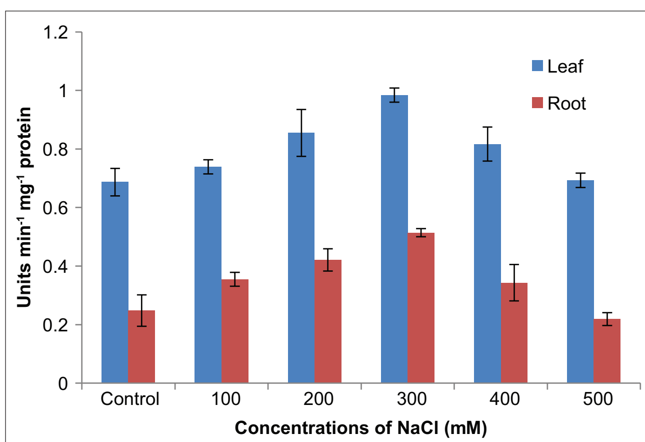

Figure 4: Effect of different concentrations of NaCl on polyphenol oxidase activity (Units min^{-1} mg^{-1} protein) in leaf and root of *Excoecaria agallocha* 60th day after salt treatment

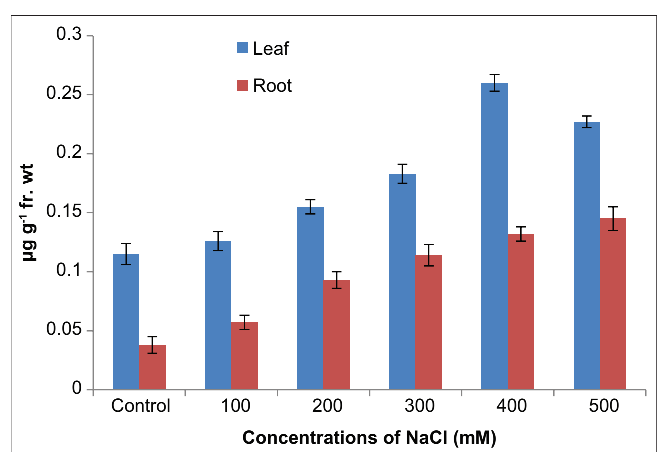

Figure 7: Effect of different concentrations of NaCl on alpha tocopherol content (μg g^{-1} fr. wt) in leaf and root of *Excoecaria agallocha* 60th day after salt treatment

A quantitative study of these enzymes from saline and fresh water grown plants revealed that enzymes activity were higher in salt-stressed plant.

Increase in CAT activity is supposed to be an adaptive trait possibly helping to overcome the damage to the tissue metabolism by sinking venomous levels of hydrogen peroxide

produced during cell metabolism and protection against oxidative stress (Bor *et al.*, 2003). Under stress condition an inductive response in CAT activity in the mangrove *B. gymnorrhiza* (Takemura *et al.*, 2000). Similarly in the present investigation, the NaCl induced enrichment of catalase activity in *E. agallocha* may suggest its effective scavenging mechanism to remove hydrogen peroxide and imparting tolerance against salinity induced oxidative stress.

POD activity of *E. agallocha* was increased with increasing NaCl concentrations. This result agrees with *Avicennia marina* (Jithesh *et al.*, 2006), *Bruguiera conjugate* (Sivasankaramoorthy & Chellappan, 2006). Increase in POD activity indicated the formation of big amount of hydrogen peroxide and which could release enzyme from membrane structure (Zhang & Krikham, 1994). Jiang and Huang (2001) reported that increase in peroxidase activity demonstrates the accumulation of H_2O_2 in salt stress condition, and this enzyme used for adaptation and prevention of peroxidation of membrane lipids (Kalir *et al.*, 1984).

Ascorbate peroxidase activity also increased with increasing salinity up to optimal concentration of 300 mM NaCl. Similar report on increased the ascorbate peroxidase activity was increased with increasing concentration of NaCl up to the optimum level in *Bruguiera cylindrica* (Sruthi & Puthur, 2018). Hydrogen peroxide is reduced to water by APX and plays a role in cell defense mechanism (Ashraf, 2009). APX together with monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase remove H_2O_2 through the Foyer – Halliwell -Asada pathway (Halliwell, 2006). APX activity may have an important role in the mechanism of salt tolerance in plants (Lin & Pu, 2010).

A considerable increase in polyphenol oxidase activity up to 300mM and a gradual decrease at higher concentrations under NaCl salinity was registered in the present study (Figure-4). Similar results were obtained by several authors in a number of halophytes such as A*egiceras corniculatum* (Manikandan & Venkatesan, 2004), A*phis craccinora* (Soffan *et al.*, 2014) and *Medicago sativa* (Babakhani *et al.*, 2017). Under stress high PPO activity indicates its ability to oxidize and to degrade the toxic substances such as phenolic compounds which are generally reported to be accumulated during salt stress (Subhashini & Reddy, 1990). Bendaly *et al.* (2016) reported that halophytes showed upper polyphenol contents with increasing salt stress as a possible defense mechanism against salt toxicity.

NaCl salinity enhanced the superoxide dismutase activity up to 300 mM in *E. agallocha* and at higher concentrations the enzyme activity was reduced (Figure-5). Coinciding with our results, an increase in SOD activity has been recorded in two mangroves, *B. gymnorrhiza* and *B. parviflora* during NaCl stress (Takemura *et al.*, 2000; Parida *et al.*, 2004a). SOD is an essential antioxidant enzyme with the capacity to restore oxidation damage caused by Reactive Oxygen Species, and it maintaining normal physiological functions and coping with oxidative stress in the regulation of intracellular levels of ROS (Foyer and Noctor, 2003).

Sruthi and Puthur (2018) reported that in *Bruguiera cylindrica*, the SOD activity was enhanced progressively with increasing salt concentration up to 600 mM NaCl as compared to control. The increase in SOD activity in response to salt stress can be considered as a defense mechanism for scavenging ROS ultimately leading to salt tolerance. The augmented antioxidant enzyme activities of SOD may be related to an increase in the rate of ROS production as well as to the denova synthesis of enzyme protein.

The results of this study show that the content of increased ascorbic acid in *E. agallocha* with increase in salinity (Figure- 6). Ascorbic acid considered as an effective antioxidant in plants which can play vital role in detoxify free radicals and oxidants (Li *et al.*, 2012) increasing cell division and/or cell enlargement (Athar *et al.*, 2008) and serves as a cofactor for enzymes as well as in in signal transduction (Gallie, 2013).

The increase of ascorbic acid content in *E. agallocha* could in part be due to its participation in the reduction of H_2O_2 to H_2O through the increase in APX activity. AsA is a significant antioxidant, which reacts not only with H_2O_2 but also with O_2^-, OH and lipid hydroperoxidases (Reddy *et al.*, 2004). Under salt stress the increased AsA content is associated with the stress protecting mechanism of the plant (Shalata *et al.*, 2001).

There was an increase in tocopherol content in *E. agallocha* with higher concentration of NaCl treatment were observed. Similar observations were made by Bartoli *et al.* (1999) and Shao and Chu (2005). α-tocopherols are known to quench $_1O^2$ in photosynthetic membranes and limit the extent of lipid peroxidation by reducing lipid peroxyl radicals to their corresponding hydroperoxides (Munné-Bosch, 2007). Of various non-enzymatic antioxidants, tocopherols affect photosynthesis; affect plant development, and stress responses by regulating the redox state of chloroplasts (Hunter & Cahoon, 2007).

CONCLUSION

Results of the present study suggest that enhanced antioxidative enzyme activities in *E. agallocha* play an essential detoxifying role against the oxidative stress caused by higher salinity. These enzymes may contribute in avoidance of ROS accumulation and thus in reticence or damage of PS II. These findings suggest that *E. agallocha* possesses an efficient antioxidative system which is activated by elevated salinity and is capable to scavenge the destructive ROS during oxidative stress caused by high salinity stress.

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