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Salt Induced Morpho-biochemical Changes inVegetatively Propagated Back Mangrove *Cerbera manghas* during Hardening

Pradeep Kumar Maharana and Uday Chand Basak*

Department of Seed bank and Seed Biology Division, Regional Plant Resource Centre (R&D institute of Forest and Environment Department, Govt. of Odisha) Bhubaneswar-751015, Odisha, India.

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ABSTRACT

This work evaluated the effect of salt (NaCl) on morphological changes followed by both enzymatic and non-enzymatic antioxidant activity in vegetatively propagated plantlets of *Cerbera manghas* during hardening. The vegetatively propagated *Cerbera manghas* plants were exposed to 0, 100, 200, 300, 400 and 500 mM NaCl for 28 days where the enzymatic and non-enzymatic parameters were observed at zero, 7th, 14th, 21st and 28th day of NaCl treatment along with morphological parameters in between zero and 28th day i.e. Height, Collar Perimeter, Number of Branches and Number of Leaves. On the basis of Height, Collar Perimeter and Number of Leaves, the maximum growth took place in 300 mM NaCl treated plantlets. The Protein content was recorded maximum at 400 mM NaCl treated plantlets in 7th day. In enzymatic analysis, both Peroxidase (POX) and Superoxide dismutase (SOD) acted as marker enzymes in 500 mM NaCl treated plantlets at 14th and 28th day respectively. Both total Phenol content and total flavonoids content showed their maximum value at 28th day in 300mM and 400 mM NaCl treated plantlets respectively. This piece of work provides a baseline data regarding salt induced changes on morphological as well as biochemical parameters during hardening of vegetatively propagated *Cerbera manghas* plantlets to facilitate further course of research on successful re-establishment of mangroves in the wild.

KEY WORDS: *Cerbera manghas*, vegetative propagation, NaCl, hardening, morphological parameters, enzymes and non enzymes.

INTRODUCTION

Cerbera manghas is a medium-sized, salt-sensitive landward tree mangrove species found sporadically in the mangrove ecosystems of coastal Odisha having ecological as well as economical importance. Continuous habitat destruction, human interference and changes in soil or water salinity caused great challenge for its survival and natural regeneration and thus categorized as 'vulnerable' species (Ved et al., 2008). Alternatively, successful artificial regeneration and establishment of mangroves demand species-specific salt tolerance (acclimatization) of the planting materials against salt stress of various magnitudes prior to undertake any field restoration programme. Salt stress induces ion toxicities especially production of reactive oxygen species (ROS) (Patel et al., 2010). Changes in the morphological growth parameters and antioxidant (both enzymatic & non-enzymatic) activities may address the optimum salt tolerant behaviour of mangrove planting materials (grown *ex situ*) (Dasgupta et al., 2012). General growth of mangrove plants usually declines at high salinity, but optimal growth obtained at moderate salinity (Clough, 1984). The activities of major antioxidant enzymes i.e. Superoxide dismutase (SOD), Peroxidise (POX) and Catalase (CAT) enhanced under stress condition when more ROS was produced (Prasad et al., 2004). Again the enzyme superoxide dismutase (SOD) catalyzes the conversion of superoxide to hydrogen peroxide and oxygen. Besides, many secondary metabolites like phenols, flavonoids etc. increased at salt stress and involved in resistance against different types of stress conditions (Ayaz et al., 2000). Till date, most of the research works focussed on salinity-induced physiological and bio-molecular changes in certain growth and developmental stages of naturally occurring mangroves. The current research work is based on salt-tolerant behaviour of vegetatively propagated land-ward mangrove species *Cerbera manghas* through observations on morphological changes and evaluation of relevant antioxidant activities at different stages of salt stress (NaCl) during their hardening period under captive conditions to facilitate successful field transfer.

MATERIAL AND METHODS

Plant materials and Salt-treatment

The *Cerbera manghas* were vegetatively propagated from the stock plants available in the nursery of RPRC through stem cuttings following standard methods (Eganathanet al., 2000; Basak et al., 2010, 2000, 1995) during the period from April to June in 2014 and were allowed to grow in polybags (8"×6") kept under

***Corresponding Author:** Uday Chand Basak, Department of Seed bank and Seed Biology Division, Regional Plant Resource Centre (R&D institute of Forest and Environment Department, Govt. of Odisha) Bhubaneswar-751015, Odisha, India. Email. uc_basak07@yahoo.co.in

shade-net house in the institutional premises for two months. The hardening experiment was set up under shade-net house where rooted plantlets (having an average height of 25.37 cm; collar perimeter of 0.45 cm; branches of 1.33 nos and leaves of 9.82 nos) were treated with six different treatments of NaCl-salinity i.e. Control (T0, zero salinity), 100 mM (T1), 200mM (T2), 300mM (T3), 400mM (T4) and 500mM NaCl (T5) treated up to 28 days. The varied salinity levels were maintained regularly by adding either NaCl and/or freshwater following measurement through portable salinometer (Eutech make Salinity Meter PCSTestr ^{TM} 35). Each treatment had 10 replicates.

Biochemical analysis

Quantitative analysis of proteins

The extract was prepared by grinding 1.0 g of leaf sample in chilled pestle and mortar by adding 5ml of protein extraction buffer (pH 7.9).The extraction buffer (50 ml) was consisting of Tris (4.0 gm), Glycine (5.0 gm), Polyvinylpyrrolidone (5.0 gm) and 5N HCL. The crushed material was centrifuged for 30 min at 7500 rpm at 4°C (Eppendorf cold centrifuge, Model No. 5437).Supernatant was collected and treated with 10%TCA for isolation of total protein content. The total protein content was quantified following the method Lowry et al., (1951) and unit was expressed in mg/gram leaf fresh tissue.

Peroxidase (POX) assay

Enzyme extract was prepared by grinding 0.5 g of leaf sample in chilled pestle and mortar by adding 5 ml of phosphate buffer (pH 6.5). The crushed material was then centrifuged for 30 min at 7500 rpm at 4°C (Eppendorf cold centrifuge, Model No. 5437). Supernatant was poured into a new Eppendorf. To measure Peroxidase activity, a reaction mixture consisting of 3.5 ml 0.1 M phosphate buffer (pH 6.5), 0.2 ml of 0.1% Methanolic solutions of O-dianisidine and 0.5 ml sample extract was incubated in a water bath at a constant temperature of 28 °C for 10 min. Then 0.2 ml of 0.2 M hydrogen peroxide was added to the reaction mixture and the optical density (OD) was recorded at 530 nm in 1 min interval up to 10 min in UV-VIS Spectrophotometer (UV-VIS Spekol 2000, Analytik Jena, Germany) following the method of (Quesada *et al*., 1992).The specific enzyme activity was expressed in terms of U/mg protein; where U is the multiplication of rate of reaction and reaction volume. The rate of reaction is an average increment in OD per minute.

Catalase (CAT) assay

Catalase enzyme extract was prepared from 0.2 g of leaf sample with the application 4.0 ml of phosphate buffer (pH 7.0) mixed with polyvinylpyrrolidone (PVP 1%). The crushed material was centrifuged for 15 min at 4000 rpm in 4°C (Eppendorf cold centrifuge, Model No. 5437). Supernatant was poured into a new Eppendorf for further use. Antioxidant activity was monitored using protocol set by Chandlee and Schanalios (1984). For Catalase assay, 0.2 ml of enzyme extract was taken in a test tube and mixed with 0.5 ml of phosphate buffer. Then, 0.1 ml of 3% H_2O_2 was added to the previous mixture and immediately after adding H_2O_2, optical density was measured at 240 nm as initial reading. Another reading of the same sample was taken after 3 min as final reading. These two readings were recorded. The specific enzyme activity was expressed in terms of U/mg protein; where U is the multiplication of rate of reaction and reaction volume. The rate of reaction is the decrease in absorbance per minute.

Superoxide dismutase (SOD) assay

The extract for SOD enzyme assay was prepared by grinding 0.2 g of leaf sample in 5.0 ml of phosphate buffer (pH 7.8) with the help of chilled pestle and mortar. The crushed material was centrifuged for 30 min at 7500 rpm in 4°C (Eppendorf cold centrifuge, Model No. 5437). Supernatant was poured into a new Eppendorf.SOD activity was estimated by monitoring the inhibition of photo-chemical reduction of nitroblue tetrazolium (NBT) in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.5), 13 mM methionine, 75 µM riboflavin, 0.1 mM EDTA and 0.1 ml of enzyme extract as described by Giannopolitis and Ries (1977). The reaction mixture was irradiated for 15 min and OD was read at 560 nm against the non-irradiated blank. The specific enzyme activity was expressed in terms of U/mg protein; where U is the multiplication of rate of reaction and reaction volume. The rate of reaction is an average decline in OD per five minutes.

Total phenol content

One gram of each fresh leaf sample was weighed and grounded in a chilled mortar and pestle with 10 ml buffer solution containing Tris HCl 0.05 M (pH 7.0), 3mM $MgCl_2$ and 1mM EDTA. The extract centrifuged at 4°C for 10 min at 5000 rpm (Eppendorf cold centrifuge, Model No. 5437) and the supernatant was used for the determination of non enzymatic antioxidant i.e. total phenol. The amount of total phenol in extracts was determined according to the Folin- ciocalteu procedure (Singleton and Rossi 1965). To a 200 µl sample extract, 1.0 ml of Folin Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added. The mixture was then

allowed to stand for 30 min and OD was measured at 765 nm. The total phenol content was expressed as Gallic acid equivalents (GAE) in milligrams per gram leaf sample calculated against Gallic acid as standard.

Total flavonoid content

The extract was prepared from 1 gram of fresh leaf of with the application of 10 ml buffer solution containing Tris HCl 0.05 M, (pH 7.0) of 3 mM $MgCl_2$ and 1 mM EDTA. The extract centrifuged at 4°C for 10 min at 5000 rpm (Eppendorf cold centrifuge, Model No. 5437)and the supernatant obtained was used for the determination of non enzymatic antioxidant i.e. total flavonoid. Total flavonoid content of the buffer extracts was determined according to a modified method of Bao *et al*. (2005). Leaf extract (1.0 ml) was mixed with 1ml of distilled water, and 75 µl of 5% $NaNO_2$ solution. After 5 min, 75 µl of 10% $AlCl_3$ H_2O solution was added. After 5 min, 0.5 ml of 1M Sodium hydroxide was added. The solution was mixed well and kept for 15 min. The increase in absorbance was measured at 510 nm using a UV-Visible spectrophotometer. The total flavonoid content was calculated using quercetin as standard and expressed as milligram of quercetin equivalent (QE) per gram leaf sample.

Statistical analysis

All the data, obtained in this experiment, were presented as mean values of 10 replicates for morphological analysis and triplicate for both enzymatic and non enzymatic observations and the difference between control and treatments were analysed using two way ANOVA and Holm-Sidak's multiple comparisons test with alpha value 0.05(Graph Pad Prism, Version 6).

RESULTS

The morphological parameters of *Cerbera manghas* plantlets were found changed at different concentration of salt stress after 28 days of exposure. The maximum growth in height took place in 300mMNaCl treated plantlets (T3) i.e. about 12.1% increase (Table 1 &2).Both Day factor and interaction (day x treatment)were significant with P value 0.0223 and 0.0076 respectively. According to multiple comparisons test, the comparisons like T0 vs. T3 was highly significant ($P < 0.0001$)followed by T1 vs. T3 ($P < 0.0010$) and T2 vs. T3 ($P < 0.0007$). The maximum growth in Collar Perimeter obtained in 300mM treated plantlets (T3) which was 18.6% increase over period of 28 days (Table 1 &2).However, the day factor, treatment factor and their interactions were not found significant.

The maximum branches was obtained in 200mM NaCl treated plantlets with30%increase and no further development was noticed in 400mM and 500mM NaCl treated plantlets (Table 1&2).Number of Leaves was found higher in 300mM treated plantlets (T3)with an increase of 31.52%(Table-1 &2). However, the interaction of day and treatment factors was not significant.

Again there is variation in total protein contents at different stages of salt stress. The protein content was recorded maximum (2.88±0.5374 mg/g) at 7th day of 400 mM NaCl treated plantlets (T4) i.e. (Figure 1). Both day and treatment factor were significant with P value 0.0025 and 0.0008 respectively; while Interaction (day × treatment) were highly significant with P value <0.0001. According to multiple comparisons test, the comparison likeT0 vs. T4 and T4 vs. T5 were significant with P value 0.0004 and 0.0030 respectively.

Antioxidant activities

Peroxidase enzyme activity showed major variation at 14th day followed by 28th day. The enzyme activity was recorded highest (0.379±0.003 U/mg protein) at 14thday of 500mM NaCl treated plantlets (T5)(Table 3). Day factor ($p<0.0032$) and treatment factor ($p<0.0006$) were significant. According to multiple comparisons test, the comparisons likeT0 vs. T5, T1 vs.T5, T2 vs. T5, T3 vs. T5 and T4 vs. T5 were highly significant ($P < 0.0001$).

Variation in the Catalase enzyme activity was major at 28th day. The activity was highest (0.862±1.025 U/ mg protein)in 400mM (T4) treated plantlets at 28th day (Table 3). Day factor was highly significant ($P <0.0001$); while the interaction is significant with P value 0.0006. According to multiple comparisons test, the comparisons like T0 vs. T4, T1 vs. T4, T2 vs. T4 and T4 vs. T5 are highly significant with P value < 0.0001.

Superoxide dismutase (SOD) enzyme activity exhibited maximum variation at 28th day followed by 7th day. The SOD enzyme activity was recorded highest (31.94±8.755 U/mg protein) at 28th day in 500mM treated plantlets (T5)(Table 3). The day factor, treatment factor and interaction were significant with P value 0.0402, 0.0252 and 0.0276 respectively. According to multiple comparisons test, the comparisons likeT1 vs. T5 and T2 vs. T5 are highly significant with P value < 0.0001; while T0 vs. T5 (0.0003), T3 vs. T5 (0.0009) and T4 vs. T5 (0.0016) were also significant.

Remarkable variation in total Phenol content was noticed during the studied periods (14th-28th day) of salt treatment. The highest total Phenol content (1.5±0.035 mg/g)was recorded at 28th day in 300mM treated (T3) plantlets(Table 4). Both the day and treatment factors and their interaction (day x treatment) were significant

(p value =0.0218, 0.0147 and 0.0009 respectively).According to multiple comparisons test, the comparisons like T0 vs. T3 (0.0252) and T2 vs. T3 (0.0485) were significant.

The total flavonoid contents showed major variation at 14th and 28th days of salt treatment. The flavonoid content was recorded highest (0.925±0.106 mg/g)in 400mM NaCl(T4) treated plantlets at 28th day(Table 4). The variation due to interaction (day × treatment) is significant with P value 0.0014. According to multiple comparisons test, the comparisons like T1 vs. T4 (0.0039) and T2 vs. T4 (0.0018) were also found significant.

Table 1. Morphological parameters of *Cerbera manghas* at zero and 28th day of different concentration of salt stress.

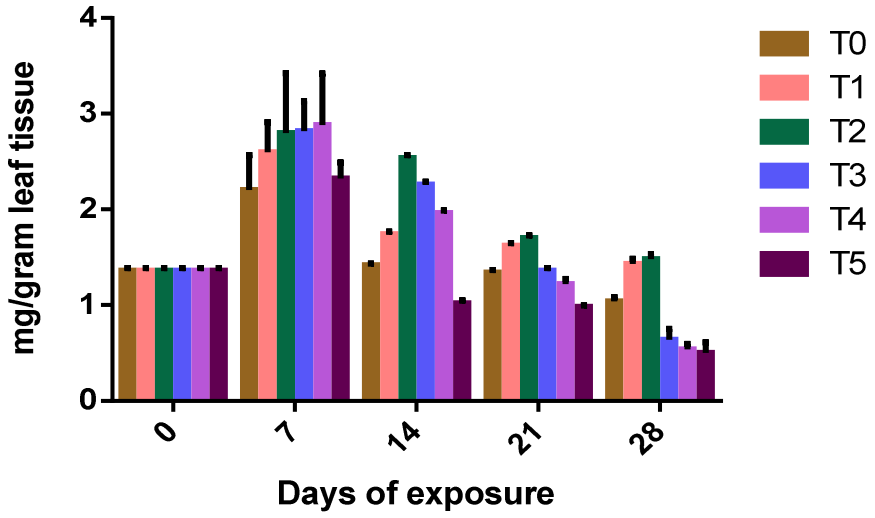
	Days of exposure	T0	T1	T2	T3	T4	T5
Height (cm)	**zero day**	30.5±0.98994	25.2±3.39411	25.8±4.24264	22.3±2.40416	24.2±3.95979	24.2±5.09116
	28th day	32.4±1.13137	28±2.54558	28.5±4.38406	25±1.97989	25.3±4.10121	24.4±5.09116
Collar Perimeter (cm)	**zero day**	0.51±0.04242	0.46±0.028284	0.43±0.01414	0.43±0.01414	0.4±0.02828	0.45±0.01414
	28th day	0.55±0.04242	0.52±0.02828	0.5±0.05656	0.51±0.04242	0.44±0.05656	0.47±0.01414
No. of Branches	**zero day**	1.5±0.14142	1.1±0.14142	1±0.2357	1.2±0.28284	1.2±0.28284	1.8±0.28284
	28th day	1.6±0.28284	1.3±0.14142	1.3±0.14142	1.5±0.14142	1.2±0.28284	1.8±0.28284
No. of Leaves	**zero day**	10.1±0.42426	9.2±0.56568	9±0.56568	9.2±1.13137	8.5±0.7071	12.9±1.83847
	28th day	10.4±1.13137	11.4±0.84852	11.5±1.27279	12.1±2.687	8.9±0.42426	12.2±1.13137

The data represent mean ±SD of replicates (n = 10).

Table 2. Percentage increase (in growth parameters) of salt acclimatized *Cerbera manghas* plantlets after 28 days of hardening with salt.

Percentage of increase after 28th day	T0	T1	T2	T3	T4	T5
Height	6.229508	11.11111	10.46512	**12.10762**	4.545455	0.826446
Collar Perimeter	7.843137	13.04348	16.27907	**18.60465**	10	4.444445
Number of Branches	6.666667	18.18182	**30**	25	0	0
Number of leaves	2.970297	23.91304	27.77778	**31.52174**	4.705883	-5.42636

Figure 1. Total protein content (mg/ gram leaf tissue) of *Cerbera manghas* at different stages of salt stress.



The data represent mean ±SDof replicates (n = 3).

Table 3. Enzymatic activity (U/mg protein) of *Cerbera manghas* at different stages of salt stress.

Enzymes	Days of exposure	T0	T1	T2	T3	T4	T5
POX	0	0.0963± 0.029	0.0963±0.029	0.0963±0.029	0.0963±0.029	0.0963±0.029	0.0963±0.029
	7	0.0557±0.001	0.0638±0.005	0.0484±0.004	**0.037±0.001**	0.0788±0.051	0.0512±0.002
	14	0.1183±0.041	0.1559±0.017	0.1497±0.021	0.1953±0.005	0.2015±0.033	**0.3795±0.003**
	21	0.1027±0.006	0.0894±0.003	0.0996±0.003	0.1599±0.015	0.1499±0.005	0.193±0.005
	28	0.0865±0.00936	0.0651±0.0124	0.0761±0.01306	0.2209±0.01114	0.2405±0.01961	0.2417±0.049
CAT	0	0.0105±0.009	0.0105±0.009	0.0105±0.009	0.0105±0.009	0.0105±0.009	0.0105±0.009
	7	0.0189±0.01	0.0757±0.065	0.1051±0.011	0.01349±0.008	0.0425±0.053	**0.0096±0.006**
	14	0.0112±0.008	0.0143±0.013	0.0107±0.009	0.0402±0.035	0.0408±0.025	0.0412±0.035
	21	0.1032±0.129	0.0564±0.013	0.0241±0.02	0.0822±0.03	0.0223±0.038	0.0502±0.105
	28	0.0805±0.277	0.21±0.456	0.292±0.348	0.4912±0.45	**0.862±1.025**	0.1394±0.24
SOD	0	6.244±3.189	6.244±3.189	6.244±3.189	6.244±3.189	6.244±3.189	6.244±3.189
	7	2.71±2.377	2.48±1.841	**1.784±1.022**	5.877±4.696	6.0377±5.556	15.91±10.699
	14	3.776±2.750	2.616±1.813	3.959±3.629	2.946±2.087	11.93±5.862	7.718±2.152
	21	11.67±10.261	9.228±8.852	5.101±3.503	10.2384 ± 2.806	7.527±6.445	10.09±6.805
	28	11.19±3.007	7.412±6.114	2.867±1.609	13.02±9.923	13.89±13.324	**31.94±8.755**

The data represent mean ± SD of replicates (n = 3).

Table 4. Non Enzymatic activity of *Cerbera manghas* plantlets at different stages of salt stress.

	Days of exposure	T0	T1	T2	T3	T4	T5
Total phenol contents (mg GAE/g leaf)	0	0.85±0.07	0.85±0.07	0.85±0.07	0.85±0.07	0.85±0.07	0.85±0.07
	7	0.5±0.194	0.6625±0.053	1.25±0.141	0.6±0.141	0.525±0.247	0.7±0.265
	14	0.5625±0.247	0.75±0.035	1.175±0.07	1.4625±0.583	0.9875±0.017	0.8875±0.017
	21	**0.3125±0.035**	1.525±0.141	1.475±0.318	0.7±0.247	0.725±0.229	0.5±0.07
	28	0.7875±0.229	1.2±0.124	0.85±0.212	**1.5±0.035**	1.25±0.247	1.125±0.035
Total flavonoids contents (mg QE/g leaf)	0	0.6375±0.159	0.6375±0.159	0.6375±0.159	0.6375±0.159	0.6375±0.159	0.6375±0.159
	7	0.4±0.017	0.625±0.017	0.575±0.141	0.5375±0.0176	0.4375±0.035	0.5375±0.053
	14	0.65±0.141	0.5±0.07	0.625±0.035	0.725±0.106	0.775±0.106	0.79375±0.185
	21	0.6125±0.035	0.6375±0.0176	0.5875±0.088	0.525±0.01	0.45±0.017	0.5±0.017
	28	0.75±0.035	0.625±0.07	0.6±0.053	0.775±0.07	**0.925±0.106**	0.875±0.035

The data represent mean ± SD of replicates (n = 3).

DISCUSSION

The maximum growth in Height, Collar Perimeter and Number of Leaves were obtained in 300 mM NaCl treated plantlets (T3). In a recent report it was found that maximum growth in plant height, stem diameter, number of branches and number of leaves took place in *Aegiceras corniculatum* L., treated with 250 mM NaCl (Mohanty et al., 2013).

The protein content was recorded highest at 7th day in 400 mM NaCl treated plantlets (T4). The protein content increased in *Sesuvium portulacastrum* with increasing concentration up to an optimal level of 600 mM NaCl and decreased beyond the optimum level (Venkatesalu et al., 1994). However, in this study, the total protein content decreased further at higher concentrations (beyond 400 mM) of NaCl. This was in agreement with the findings of Parida et al. (2004) where increased activities of both acid and alkaline proteases under high salinity caused decrease in protein content and increase in free amino acids content in *Bruguiera parvifora,* a Rhizophoraceae mangrove seedling.

The Peroxidase (POX) enzyme activity was recorded highest at 14thday of 500 mM NaCl treated plantlets (T5). This was in agreement with the findings in *Aegiceras corniculatum,* where Peroxidase activity was found increased along with increasing salt concentration (Manikandan et al., 2004). Increased peroxidase enzyme activity might be useful for adaptation under conditions requiring prevention of peroxidation of membrane lipids (Kalir et al, 1984).The Catalase (CAT) enzyme activity was highest at 28th day of 400mM NaCl treated plantlets (T4). The Catalase enzyme activity increased up to optimum level in *Ipomoea pes-caprae* (Venkatesan et al., 1999). The Catalase activity decreased with increasing concentration in *Phaseolus radiatus* (Saha et al., 1999). The SOD enzyme activity was recorded highest at 28th day of 500 mM NaCl treated plantlets (T5).Effect of salinity stress in halophytes are analysed by studying the total SOD protein activity. SOD enzyme showed its full activity at least up to seawater salt levels (Takemura et al., 2000).

The total Phenol content was recorded highest at 28th day of 300 mM NaCl treated plantlets (T3). Salinity increases the Phenolic compounds at different tissue of the plants but decreases at higher concentration of salt (Agastian et al., 2000). Again phenolics were found to be accumulated in the leaves of *Bruguiera parviflora* with increasing levels of salinity (Parida et al., 2002). In short, phenolics are secondary metabolites and arising from the shikimate-phenylpropanoids-flavonoids pathways as by-product of several plant metabolism (Lattanzio et al., 2006). Phenolics are produced by plants mainly for protection against stress and play important roles in plant development, particularly in lignin and pigment biosynthesis. They also provide structural integrity and support to plants (Bhattacharya et al., 2010). The flavonoid content was recorded highest at 400 mM NaCl (T4) treated plantlets of 28th day. The flavonoids were also shown to protect mangroves from UV radiation (Agati et al., 2007). Flavonoids are bioactive plant secondary metabolites and serve as ROS scavengers by locating and neutralizing radicals before they damage the cell thus important for plants under adverse conditions.

CONCLUSION

In the present investigation, the effect of different concentration of sodium chloride on growth, organic components and activities of certain key enzymes of *Cerbera manghas,* a back mangrove species, has been studied during the hardening process of vegetatively propagated plantlets. The plantlets of the *C. Manghas* could survive a wide range of salinity i.e. from 100-400 mM NaCl concentrations. However, the maximum growth response of *C. manghas* was identified in 300mM NaCl treated plantlets. Besides, both the Peroxidase and Superoxide dismutase acted as marker enzymes in response to the salt stress expressed during 14th and 28th day of 500 mM NaCl treatment respectively.

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