International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 06-04-2020; Accepted: 21-04-2020: Published: 10-05-2020
www.botanyjournals.com
Volume 5; Issue 3; 2020; Page No. 41-45

Effect of NaCL on bio-molecular changes during hardening of vegetatively propagated mangrove species *Cerbera manghas*

Pradeep Kumar Maharana[1]*, Uday Chand Basak[2]
[1, 2] Department of Seed Bank and Seed Biology Division, Regional Plant Resource Centre (R&D institute of Forest and Environment Department, Govt. of Odisha) Bhubaneswar, Odisha, India

Abstract
This research work determined the effect of salt (NaCl) on protein, antioxidant potential changes and osmolyte contents in vegetatively propagated plantlets of *Cerbera manghas* during hardening. The vegetatively propagated *C. manghas* plants were exposed to 0, 100, 200, 300, 400 and 500 mM NaCl for 28 days where bio-molecular changes were observed at zero, 7th, 14th, 21st and 28thday of NaCl treatment. Both 20.1 and 43 kDa are identified as the peptide maker for this species both in control and saline treatment plantlets. The highest value of proteins (at 7th, 400mM), reducing power (at 7th, 400mM), Proline (at 28th, 400mM), Glycine betaine (at 28th, 300mM) and lowest IC_{50} (21st, 300mM and 7th, 400mMNaCl) could be the bio-molecular marker.

Keywords: *C. manghas*, NaCl, vegetative propagation, antioxidant potential, osmolytes, proteins

1. Introduction

Cerbera manghas is an economically and medicinally important landward mangrove species found in coastal region of Odisha. This mangrove species are belongs to vulnerable category due to anthropogenic pressure and changes in soil or water salinity [1]. Continuous habitat loss, anthropogenic disturbances and changes in soil/water salinity are the great obstacles for the natural regeneration of this species. Now, there is great demand of artificial regeneration of mangroves followed by its establishment in the wild. The equilibrium between ROS (Reactive oxygen species) production and their scavenging may be perturbed by salinity which may lead to significant oxidative damage [2]. Plants initiate some defensive machinery in order to cope with stress.

In general, the protein content increased with increasing concentration up to an optimal level. Beyond the optimum level, the protein content decreased in *Phalaria arundinacea* [3] and *Sesuvium portulacastrum* [4]. Osmolytes are compatible metabolites and do not interfere with normal metabolism of plant even when present at high concentrations. Osmolytes are typically rises during exposure to stresses such as salinity, water deficit etc. The accumulation of osmolytes could be the part of a plants adaptation against adverse environmental conditions. Proline influence cell proliferation or cell death and trigger specific gene expression, which helps the plant to recover from stress [5].Again Proline and Glycine betaine serve as metabolites for the cellular storage of carbon and nitrogen during stress, which would be used by the cell once stress has ceased [6].

Antioxidant potential can determined as the free radical scavenging ability using a stable radical, diphenyl-picryl-hydrazyl (DPPH) and ascertained by measuring reducing power [7].The DPPH is a stable free radical and is widely accepted for estimating free radical scavenging activities of antioxidants because it accepts an electron or hydrogen radical to become a stable diamagnetic molecule [8]. The reduction capability of DPPH radical is induced by antioxidants. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating hydrogen atom [9]. In this study, the salt tolerance behavior of vegetatively propagated *Cerbera manghas,* (a back-mangrove species) was determined through protein, antioxidant activities (through DPPH and reducing power methods) and osmolytic analysis (through proline and glycine betaine estimation).

2. Materials and Methods

2.1 Plant material and growth condition

The *Cerbera manghas* were vegetatively propagated from the stock plants available in the nursery of RPRC through stem cuttings with the application of suitable synthetic hormone combination i.e. IBA (Indole Butyric Acid) and NAA (Naphalene Acetic Acid) in a ratio of 5:5 Parts Per Thousand (PPT) following standard methods [10,11]. The rooted plantlets were transferred to the polybags (8”×6”) containing the mixture of soil, sand and cow dung in 1:1:1 ratio. Then they were kept under shade-net house and allowed to grow for a period of two months.

2.2 Experimental set up and NaCl treatments

The hardening experiment was set up under shade-net house where plantlets were arranged in six different groups. Out of the six groups, one group was retained as control (T0, zero salinity); while other five groups were allowed to grow with five different concentration of NaCl i.e. 100mM (T1), 200mM (T2), 300mM (T3), 400mM (T4) and 500mM (T5) treated up to 28 days with an interval of one week.

2.3 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 hydrochloric acid [12].

2.4 Qualitative analysis of proteins
The extraction buffer was prepared from 10% (w/v) SDS, 10 mM b- Marcaptoethanol, 20% (v/v) glycerol, 0.2 M Tris/HCl (pH 6.8) and 0.05% Bromophenol blue. Extraction of proteins for gel electrophoresis was done from 1g of fresh leaf [13].

2.5 DPPH radical scavenging assay
Leaf samples were dried in oven at 50°C for one day (24 hours/overnight). The 0.3 gram dried leaf samples were soaked in 6ml of methanol for 5 days with stirring every 18h using a sterilized glass rod separately. The final extract were passed through No.1 Whatman filter paper (twice).The filtrate part was maintained 10ml by adding methanol and stored at 4°C for future use. The absorbance was taken at 517 nm [14].

2.6 Reducing power assay
For this assay, the extract was prepared from 1 gram of fresh leaf tissue of each sample was weighed and ground in a chilled mortar and pestle with 10 ml buffer solution containing Tris HCl 0.05M (pH 7.0) consisting of 3 mM $MgCl_2$ and 1mM EDTA. The extract was centrifuged at 4°C for 10 min at 5000 rpm in cold centrifuge and the supernatant obtained was used for reducing power assay [15]. The absorbance was measured at 700 nm. The reducing power of the extracts was expressed as Ascorbic acid equivalents (AAE) in mg per gram leaf sample.

2.7 Proline analysis
0.5 g plant tissue was taken and homogenized in 5 ml of 3% sulphosalycylic acid using pre washed mortar and pestle and Filtered with Whatman No. 1 filter paper and collected filtrate was used for the estimation of proline content with standard method [16]. The total proline content was calculated by using L-proline as standard and expressed as milligrams per gram leaf tissue.

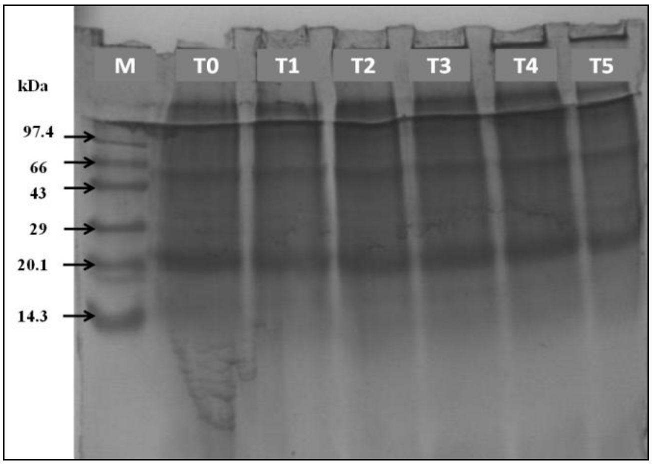
2.8 Glycine betaine analysis
0.5 g dry plant material was mechanically grounded with 20 ml of deionised water by mortar and pestle and shaken for 48 h at 25° C. The samples were then filtered and the filtrate was stored in freezer until analysis. Thawed extracts were diluted 1:1 with 2 N sulphuric acids and with standard method [17]. Reference standards of Glycinebetaine were prepared in 2 N sulphuric acids and the procedure for sample estimation was followed.

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

Table 1: Proteins, DPPH scavenging capacity, Reducing power, proline contents and Glycine betaine (GB) contents of *Cerbera manghas* plantlets at different stages of salt stress.

Days of exposure	NaCl Treatment	Proteins	DPPH (IC_{50}) i.e. mg/ml	Reducing power (AAE mg/g leaf tissue)	Proline contents (mg/g leaf tissue)	GB contents (mg/g leaf tissue)
Zero Day	Control	1.36±0.028	11.7	0.687±0.018	0.1±0.039	0.045±0.007
7th Day	T0	2.2±0.367	11.7	0.836±0.048	0.093±0.024	0.13±0.042
	T1	2.6±0.311	11.85	0.724±0.044	0.11±0.002	0.11±0.042
	T2	2.8±0.622	11.4	0.672±0.036	0.081±0.009	0.09±0.014
	T3	2.82±0.311	8.4	1.02±0.009	0.15±0.062	0.36±0.056
	T4	2.88±0.537	6.6	0.897±0.05	0.093±0.018	0.24±0.056
	T5	2.32±0.169	7.05	0.789±0.027	0.074±0.002	0.21±0.014
14th Day	T0	1.42±0.014	15	0.709±0.006	0.072±0.033	0.0875±0.014
	T1	1.74±0.028	11.7	0.636±0.012	0.084±0.022	0.0775±0.014
	T2	2.54±0.028	10.35	0.794±0.043	0.084±0.011	0.0675±0.028
	T3	2.26±0.028	10.08	0.876±0.024	0.141±0.007	0.2025±0.028
	T4	1.96±0.028	12.3	0.719±0.027	0.023±0.007	0.1425±0.07
	T5	1.02±0.028	12.9	0.716±0.013	0.043±0.012	0.1275±0.056
21st Day	T0	1.34±0.028	18.6	0.724±0.031	0.157±0.004	0.031±0.001
	T1	1.62±0.028	12.9	0.668±0.018	0.155±0.004	0.13±0.014
	T2	1.7±0.028	9.9	0.723±0.019	0.155±0.001	0.195±0.007
	T3	1.36±0.028	6.6	0.575±0.004	0.137±0.012	0.48±0.141
	T4	1.22±0.056	13.5	0.664±0.003	0.114±0.002	0.34±0.028
	T5	0.98±0.014	14.7	0.561±0.013	0.186±0.039	0.34±0.056
28th Day	T0	1.04±0.042	16.65	0.676±0.014	0.041±0.026	0.05±0.014
	T1	1.43±0.056	15	0.727±0.005	0.116±0.033	0.12±0.056
	T2	1.48±0.056	15.6	0.783±0.049	0.13±0.005	0.198±0.008
	T3	0.64±0.113	13.8	0.973±0.2	0.165±0.032	0.77±0.268
	T4	0.54±0.056	15.9	0.882±0.171	0.254±0.002	0.37±0.042
	T5	0.5±0.113	13.95	0.705±0.025	0.193±0.086	0.215±0.0212

Abbreviation: T0 = Control, T1 = 100mM, T2 =200mM, T3 = 300mM, T4 = 400mM and T5= 500mM NaCl**.** The data represent mean ± SD of three replicates.



Abbreviation: T0 = Control, T1 = 100mM, T2 =200mM, T3 = 300mM, T4 = 400mM and T5= 500mM NaCl**.** The data represent mean ± SD of three replicates; M= Medium range protein marker; kDa= Kilodalton.

Fig 1: Gel electrophoresis (SDS-PAGE) of *Cerbera manghas* protein at different concentration of salt stress.

3. Results and Discussion

In *Cerbera* manghas, there is variation in total protein contents at different stages of salt stress. The protein content increases up to 400mM (at 7th day) and 200mM NaCl (at 14th, 21st and 28th day respectively) and then decreases. The protein content was recorded maximum (2.88 ± 0.5374 mg/g) at 7th day of 400 mM NaCl treated plantlets (T4) i.e. (Table 1). In *Cerbera manghas* mangrove species, the variation of total protein contents was clearly found at different stages of salt stress. The protein content increases up to 200mM NaCl at 14th, 21st and 28th day respectively. 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 [4]. However, in this study, the total protein content decreased further at higher concentrations (beyond 300 mM) of NaCl at 14th, 21st and 28th day respectively. 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 [18]. Besides this, the highest value of the protein content at 7th day of 400 mM NaCl treated *C. manghas* plantlets (T4) i.e. (Figure 1) was the protein bio-molecular marker at that specific day. On the basis of SDS-PAGE analysis, two peptide bands i.e. 20.1 (thick) and 43 kDa (thin) in *C. manghas* were found in both control and salt treated leaf sample. This could be the marker peptide for this species.

Antioxidant capacity was evaluated by DPPH scavenging and reducing power activities. The IC_{50} value for DPPH scavenging become decreases up to 400mM (at 7th day) and up to 300mM NaCl (at 14th, 21st and 28thday respectively). The minimum percentage increase in IC_{50} (17.94% increase) was measured at 300mM (T3) NaCl treated plantlets with a period of 28 days (Table 1). On the basis of IC_{50} value at different day period of the experiment, the minimum IC_{50} value i.e. 6.6 mg/ml was recorded in 21st and 7th day of 300 and 400mM NaCl treated plantlets respectively. The lowest IC_{50} is the highest DPPH scavenging. DPPH radical Scavenging activity of callus cultures of *Salvadora persica* increased gradually when grown on increasing concentrations of sodium chloride [19]. DPPH assay is one of the most easy, rapid, sensitive, and reliable ways to evaluate the ability of antioxidants to scavenge free radicals, which are known to be a major factor in the biological damage caused by oxidative stress [20, 21].

The maximum value reducing power (1.02±0.009 mg AAE/gram leaf tissue) was recorded in 7th day of 300mM NaCl (T3) treated plantlets. The reducing power (RP) capacity of *C. manghas* increases continuously from zero to 300mM (at 7th, 14thand 28thday) NaCl treated plantlets and then decreases (Table.1). A reducing power is an indicative of reducing agent having the availability of atoms (reductants) which can donate electron and react with free radicals and then convert them into more stable metabolites and terminate the radical chain reaction [22].

The proline (Pr) content of *C. manghas* increases up to 300mM (at 7th and 14th day) and 400mM (at 28th day) NaCl treated plantlets and then decreases (Table.1). In *C. manghas*, the maximum value of Proline (0.254±0.002 mg/ gram fresh leaf) was recorded in 28th day of 400mM (T4) NaCl treated plantlets. Stress (including salt) causes increase in proline contents in the leaves of *Lycopersicon esculentum* [23] and other plant species [24]. Proline accumulates in larger amounts in salt stressed plants along with other osmolytes which is osmotically very active, contributes to membrane stability and pacifies the effect of NaCl on cell membrane disruption [25].

In *C. manghas*, the maximum value of Glycine betaine (GB) i.e. 0.77±0.268 mg/gram fry leaf tissue was recorded in 28th day of 300mM (T3) NaCl treated plantlets (Table 1). Glycine betaine acts as defensive molecules in higher plants at extreme conditions of salt, drought, temperature or light stress [26, 27]. GB might play an important role in enhancing plant tolerance to some abiotic stresses such as salt, drought, and extreme temperatures [28]. GB is the most common compatible solute accumulates in some mangroves such *as Avicenia marina*during NaCl stress [29]. Glycine betaine preserves thylakoid and plasma membrane integrity at salt stress [30]. Proline and glycine betaine can reduce membrane injury, improved K+ uptake and growth. These metabolites also increased chlorophyll contents [31]. Again, it is evident that cell membrane protection under salinity stress can be provided by GB or Proline and thus leading to increased salt tolerance in plants [32].

4. Conclusion

In the present investigation, 20.1 and 43 kDa peptide could be the bio-molecular peptide markers for this species with or without treatment of salinity. Both DPPH scavenging and reducing power activity of *C. manghas* were identified as marker antioxidant in 300mMNaCl treated plantlets at 21st and 7th day respectively; while glycine betaine and proline acted as marker metabolites at 28th day in response to the salt stress expressed during 300 mM and 400 mM NaCl treatment plantlets respectively. The increase in biochemical activity up to 300 to 400mM NaCl indicates the better survival of this back-mangrove species in that range of salinity.

5. Acknowledgement
The authors acknowledge the financial support provided by the Forest and Environment Department, Govt. of Odisha under State Plan Budget of Regional Plant Resource Centre, Bhubaneswar, Odisha.

6. References

1. Ved DK, Kinhal GA, Ravikumar K, Sankar RV, Sumathi R, Mahapatra AK, *et al*. Conservation Assessment & Management Prioritisation for Medicinal Plants of Orissa. A synthesis of Regional expertise in medicinal plants Taxonomy & Distribution through a workshop held at Bhubaneswar during 7th-10th October 2007. Regional Plant Resource Centre, Bhubaneswar & FRLHT, Bangalore, 2008, pp45.
2. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry. 2010; 48:909-930.
3. Maeda Y, Takemoto K, Aso S, Takenago H. Relationship between salt tolerance and contents of cations and free amino acids in reed canary grass (*Phalaris arundinace* L.) grown on soil perfused with cattle urine. Journal of Japanese society of Grassland Science. 1995; 41:60-6.
4. Venkatesalu V, Rajkumar R, Chellappan KP. Growth and mineral distribution of *Sesuvium portulacastrum* L.a salt marsh halophyte under sodium chloride stress. Communications in Soil Science Plant Analysis. 1994; 25:2797-2805.
5. Szabados L, Savoure´ A. Proline: a multifunctional amino acid. Trends in Plant Science, 2010; 15:89-97.
6. Munns R, Tester M. Mechanisms of salinity tolerance. Annual Review of Plant Biology, 2008; 59:651-681.
7. Banerjee D, Chakrabarti S, Hazra AK, Banerjee S, Ray J, Mukherjee B. Antioxidant activity and total phenolics of some mangroves in Sunderbans. African Journal of Biotechnology, 2008; 7:805-810.
8. Kalaivani T, Mathew L. Free radical scavenging activity from leaves of *Acacia nilotica* (L.) Wild. ex Delile, an Indian medicinal tree. Food and Chemical Toxicology, 2010; 48:298-305.
9. Oyedemi SO, Afolayan AJ. In vitro and in vivo antioxidant activity of aqueous leaves extract of *Leonotis leonurus* (L.) R. Br. International Journal of Pharmacology, 2011; 7:248-256.
10. Basak UC, Mahapatra AK. Conservation and propagation of some rare or endangered or threatened (RET) tree species of India. Regional Plant Resource Centre, Bhubaneswar, 2008, pp 07.
11. Basak UC, Mahapatra AK. Vegetative propagation of mangroves for reintroduction in the wild. Regional Plant Resource Centre, Bhubaneswar, 2009.
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin Phenol reagent. Journal of Biological Chemistry, 1951; 193:265-275.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970; 227:680-685.
14. Chan EWC, Lim YY, Omar M. Antioxidant and antibacterial activity of leaves of Etlingera species (Zingiberaceae) in Peninsular Malaysia. Food Chemistry, 2007; 104:1586-1593.
15. Oyaizu M. Studies on product on browning reaction prepared from glucose amine. Japanese Journal of Nutrition, 1986; 44:307-315.
16. Bates LS, Waldren RP, Teare ID. Rapid determination of the free proline of water stress studies. Plant Soil, 1973; 39:205-207.
17. Greive CM, Grattan SR. Rapid assay for determination of water-soluble quaternary amino compounds. Plant Soil, 1983; 70:303-307.
18. Parida AK, Das AB, Mohanty P. Defense potentials to NaCl in a mangrove, *Bruguiera parviflora*: differential changes of isoforms of some antioxidative enzymes. Journal of Plant Physiology, 2004; 161:531-542.
19. Sharma V, Ramawat KG. Salinity-induced modulation of growth and antioxidant activity in the callus cultures of miswak (*Salvadora persica*). 3-Biotech, Springer. 2013; 3:11-17.
20. Koleva II, Van Beek TA, Linssen JPH, De Groot A, Evstatieva LN. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochemical Analysis, 2002; 13:8-17.
21. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. Journal of Agricultural and Food Chemistry, 2005; 53:1841-1856.
22. Rajamanikandan S, Sindhu T, Durgapriya D, Sophia D, Ragavendran P, Gopalakrishnan VK, *et al*. Radical Scavenging and Antioxidant Activity of Ethanolic Extract of *Mollugo nudicaulis* by In vitro Assays. Indian Journal of Pharmaceutical Education and Research, 2011; 45:310-316.
23. Aziz A, Martin-Tanguy J, Larher F. Salt stress-induced proline accumulation and changes in tyramine and polyamine levels are linked to ionic adjustment in tomato leaf discs. Plant Science, 1999; 145:83-91.
24. Lee TM, Liu CH. Correlation of decreased calcium contents with proline accumulation in the marine green macroalga *Ulva fasciata* exposed to elevated NaCl contents in seawater. Journal of Experimental Botany, 1999; 50:1855-1862.
25. Ashraf M. Organic substances responsible for salt tolerance in *Eruca sativa*. Biologia Plantarum, 1994; 36:255-259.
26. Holmstrom KO, Somersalo S, Mandal A, Palva TE, Welin B. Improvement tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. Journal of Experimental Botany, 2000; 51:177-185.
27. Sakamoto H, Murata N. Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. Journal of Experimental Botany, 2000; 51:81-88.
28. Quan R, Shang M, Zhang H, Zhao Y, Zhang J. Engineering of enhanced glycine betaine synthesis improves drought tolerance in maize. Plant Biotechnology Journal, 2004; 2:477-486.

29. Ashihara H, Adachi K, Otawa M, Yasumoto E, Fukushima Y, Kato M, *et al*. Compatible solutes and inorganic ions in the mangrove plant *Avicennia marina* and their effects on the activities of enzymes. Zeitschrift fur Naturforschung, 1997; 52:433-440.
30. Rhodes D, Hanson AD. Quaternary ammonium and tertiary sulfonium in higher plants. Annual Review of Plant Physiology Plant Molecular Biology, 1993; 44:357-384.
31. Gadallah MAA. Effects of proline and glycinebetaine on *Vicia faba* responses to salt stress. Biologia Plantarum, 1999; 42:249-257.
32. Mansour MMF. Protection of plasma membrane of onion epidermal cells by glycinebetaine and proline against NaCl stress. Plant Physiology and Biochemistry, 1998; 36:767-772.