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Bio-Molecular Studies in NaCl Induced Vegetatively Propagated *Excoecaria agallocha* L. during Hardening

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A B S T R A C T

Effects of salinity (NaCl) on protein content and antioxidant activity of vegetatively propagated plantlets of *Excoecaria agallocha* were evaluated during hardening period. The vegetatively propagated *E. agallocha* plantlets were treated with different concentration of salinity i.e. 0, 100, 200, 300, 400 and 500 mM NaCl for 28 days where bio-molecular parameters were observed at zero, 7th, 14th, 21st and 28th day of NaCl treatments. Three distinct and thick protein/polypeptide bands at 43, 20.1 and 14.3 kDa were detected in both with or without salt treated saplings during qualitative analysis of *E. agallocha* leaf protein. This indicates *E. agallocha* can survive wide salinity range. The lowest value of IC_{50} value in 200mM NaCl (at 21st day) and highest value of total phenolic content in 300mM (at 14th day), total flavonoid content in 200mMNaCl (at 28^{th}day), reducing power in 200mM NaCl (at 21^{st}day), proline content in 300 mM NaCl (at 14th day) and glycine betaine content in 400mM NaCl (at 14th day) could be the biochemical marker. This research work can provide preliminary data on salt tolerant behavior of vegetatively propagated *E. agallocha* plantlets during hardening period; which can facilitate further course of research on its field transfer.

Introduction

Excoecaria agallocha L. is a small non viviparous back mangrove tree species belongs to family Euphorbiaceae and found in coastal region of Odisha. This mangrove species have both ecological and economical value. This plant is also distributed in a number of other countries of temperate and tropical Asia, Australia and South western Pacific (GRIN database 2008). Continuous habitat loss, anthropogenic disturbances and salinity changes are the great obstacles for the regeneration (naturally) of this species. Now, there is great demand of artificial regeneration of mangroves followed by its establishment in

the wild. Soil salinity causes extremely unfavorable conditions and affects the productivity of plants. In environmental stresses (salt stresses), plants have developed different biochemical and physiological mechanisms for tolerance (Faical *et al.,* 2009; Rahnama and Ebrahimzadeh 2004). The production of Reactive oxygen species and its quenching become imbalanced due to increased salt stress and cause oxidative damage (Parvaiz *et al.,* 2008; Spychalla *et al.,* 1990). Antioxidants possess the ability to protect the plant from the oxidative stress (Ozsoy *et al.,* 2008). Antioxidant potential can be determined through the free radical scavenging and reducing power capacity (Banerjee *et al.,* 2008). Many secondary metabolites (phenols, flavonoids) in plants act against different types of stress conditions (Ayaz *et al.,* 2008).

Again the salt tolerant plant requires compatible solutes to accumulate in the cytosol and organelles where osmotic adjustment and osmoprotection is taking place (Rhodes and Hanson 1993). Osmoprotectants can also raise osmotic pressure in the cytoplasm and stabilize proteins and membranes at salt stress. Proline accumulation helps in alleviation of plants against salt stress (Matysik *et al.,* 2002; Saxena *et al.,* 2013). Increased levels of proline contribute to the turgor maintenance of cells and considered as a stress indicator in several plant species at salt stress (Giridarakumar *et al.,* 2003; Tiwari *et al.,* 2010). Glycine betaine is one of the quaternary ammonium compounds that accumulate in certain plants when exposed to environmental stresses, such as salinity. It could play a vital role in maintaining intracellular osmotic equilibrium in salt stress (Giridarakumar *et al.,* 2003; Rhodes and Hanson 1993; Subbarao *et al.,* 2001). The biochemical function of osmoprotectants is the scavenging of free radicals (Bohnert and Jensen 1996).The aim of the present study was to examine the salt tolerant behavior of vegetatively propagated back mangrove species *Excoecaria agallocha* at different stage of salt stress through the measurements of osmolytes and in *vitro* antioxidant activity.

Materials and Methods

Planting materials and experimental set up

Excoecaria agallocha was the targeted species for the experiment. The *E. agallocha* wildlings were collected from the Odisha coast and grown in shade-net house of RPRC. By using standard methods (Basak *et al.,* 1995, 2000; Basak and Mahapatra 2009; Eganathan *et al.,* 2000), the hardened *E. agallocha* wildlings were vegetatively propagated through stem cuttings and were allowed to grow in polybags (8”×6”) and kept under shade-net house condition for two months.

The experiment was set up for hardened plantlets under shade-net house of the institutional premises where rooted cuttings were allowed to grow with six different NaCl treatments i.e. Control (T0, zero salinity), 100mM (T1), 200mM (T2), 300mM (T3), 400mM (T4) and 500mM (T5) treated up to 28 days with an interval of one week.

Quantitative and qualitative 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. Protein was estimated by standard procedure (Lowry *et al.,* 1951). Gel electrophoresis (SDS PAGE) was carried out for qualitative analysis of proteins (Laemmli 1970).

Non enzymatic analysis

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 antioxidants i.e. total phenolic contents (Singleton and Rossi 1965), total flavonoid contents (Bao *et al.,* 2005) and reducing power (Oyaizu *et al.,* 1986) respectively. Total phenol content, total Flavonoid content and reducing power were expressed as Gallic acid equivalents (GAE), quercetin equivalent (QE) and ascorbic acid equivalents (AAE) respectively.

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. 1ml of sample extract was mixed with 2ml of DPPH solution (0.003 gram DPPH/50ml methanol). The mixture was shaken vigorously and allows standing in dark for 30 minutes. The absorbance was taken at 517 nm (Chan *et al.,* 2007). The scavenging assay was represented by IC_{50} value. The IC_{50} was the minimum concentration of sample needed to scavenge the half of the DPPH solution.

Proline analysis

0.5 g plant tissue was taken and homogenized in 5 ml of 3% sulphosalycylic acid using pre washed mortar and pestle. Filter the homogenate through Whatman No. 1 filter paper and collect filtrate will be used for the estimation of proline content. Proline was measured as described by Bates *et al.,* (1973). The total proline content was calculated by using L-proline as standard and expressed as milligrams per gram leaf tissue.

Glycine betaine analysis

0.5 g dry plant leaf 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. Glycine betaine of leaf sample was estimated by standard procedure (Greive *et al.,* 1983). Reference standards of Glycinebetaine were prepared in 2 N sulphuric acids and the procedure for sample estimation was followed.

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).

Results and Discussion

Highest protein contents was recorded (20.85±0.15 mg/g) at 21st day of 300 mM NaCl treated plantlets (T3) i.e. (Figure 1). The total protein content decreased at higher concentrations (beyond 400 mM) of NaCl. In

Sesuvium portulacastrum, protein content become increased with increasing NaCl concentration up to an optimal level and then decreased (Venkatesalu *et al.,* 1994). Proteases (both acidic and alkaline) under high salinity cause decrease in protein content and increase in free amino acids content in *Bruguiera parvifora* (Parida *et al.,* 2004).

The highest value of the protein content at 21st day of 300 mM NaCl treated *E. agallocha* plantlets (T3) could be the protein bio-molecular marker. On the basis of qualitative analysis, three distinct protein bands were appeared at 43, 20.1 and 14.3 kDa respectively (Figure 2) in each lane of both control and salt treated protein leaf sample of *E. agallocha*.

Maximum total Phenolic content (2.375±0.714 mg/g) was shown at 14th day in 300mM treated (T3) plantlets (Table 1). The total content increases considerably up to 300mmNaCl and then decreases at 7th, 14th and 28th day respectively (Table 1). Phenolic content increases considerably in plants at increased salinity but decreases at higher concentration of salt (Agastian *et al.,* 2000). Phenolics can also be synthesized in *Bruguiera parviflora* leaves with increased salinity (Parida *et al.,* 2002). Plant phenolics are biogenetically arising from the shikimate-phenylpropanoids-flavonoids pathways; which are needed for several plant metabolisms (Lattanzio *et al.,* 2006).

Phenolics are aromatic compounds produced by plants provide protection against stress and have important roles in synthesis of lignin and pigments (Bhattacharya *et al.,* 2010). In this study, 300mM NaCl is suitable for this mangrove species and act as phenolic bio-molecular marker at 14th day (*E. agallocha*). Total Flavonoid content (TFC) showed major variation at 14th and 21st days of salinity treatments. The Flavonoid content was recorded maximum i.e. 1.4±0.1 mg/g in 200mM NaCl (T2) treated plantlets at 28th day (Table 1). The content increases considerably at 7th and 28th day in 300 and 200mM NaCl treated saplings (Table 1). Salt stress i.e. 50 and 100 mM NaCl significantly increases Flavonoid content in barley (Ali and Abbas 2003). The level of Flavonoid content increases in the leaf tissue along with increase in salinity was noticed in *Simarouba glauca* (Rajamane and Gaikwad 2014). The Flavonoids can also protect mangroves from UV radiation by reducing singlet oxygen level (Agati *et al.,* 2007). Flavonoids are secondary metabolites and serve as free radicals scavengers. In this study, the highest value indicates the flavonoid bio-molecular marker. On the basis of IC_{50} value at different day period of the experiment, the minimum IC_{50} value i.e. 0.12 mg/ml was recorded in 200mM NaCl (T2) treated plantlets respectively at 21st day (Table 1). DPPH assay evaluate the total antioxidants potential against free radicals (Huang et al., 2005; Koleva et al., 2002).

The capacity of biological reagents to scavenge the DPPH radical can be expressed as its magnitude of antioxidation ability. The ability of the radical scavenger depends on disappearance of the DPPH (Deng et al., 2011). DPPH radical scavenging activity of callus cultures of *Salvadora persica* increased gradually when grown on increasing concentrations of NaCl (Sharma and Ramawat 2013). In this study, the lowest value of IC_{50} is the free radical scavenging bio-molecular markers. The maximum value of reducing power i.e. 4.326±0.27 mg/g was recorded in 21st day of 200mM NaCl (T2) treated plantlets (Table 1). This generally depends on the presence of reductones; which can reduce oxidized intermediates of lipid peroxidation processes by donating electrons and react with free radicals and then convert them into more stable metabolites (Rajamanikandan *et al.,* 2011).

Table.1 Total phenolic contents, total Flavonoid contents, DPPH scavenging capacity, Reducing power, proline contents and Glycine betaine (GB) contents of *Excoecaria agallocha* plantlets at different stages of salt stress

Days of exposure	NaCl Treatment	Total Phenol contents (mg GAE/g leaf tissue)	Total Flavonoid contents (mg QE/g leaf tissue)	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	1.016±0.052	0.675±0.05	0.72	2.89±0.13	0.037±0.03	0.066±0.03
7th Day	T0	0.941±0.137	0.616±0.052	0.78	2.99±0.179	0.115±0.06	0.053±0.023
	T1	1.216±0.08	0.74±0.08	1.04	2.7±0.052	0.28±0.2	0.056±0.021
	T2	1.316±0.097	0.816±0.076	1.06	2.68±0.064	0.91±0.8	0.081±0.038
	T3	1.475±0.294	0.845±0.068	1.02	2.07±0.041	0.605±0.38	0.104±0.0144
	T4	1.454±0.083	0.725±0.175	1.05	1.75±0.09	0.44±0.4	0.104±0.031
	T5	0.85±0.108	0.633±0.062	1.06	3.87±0.14	0.565±0.24	0.078±0.006
14th Day	T0	1.387±0.206	0.716±0.087	0.37	3.22±0.12	0.21±0.09	0.112±0.01
	T1	1.941±0.256	1.2±0.23	0.72	3.23±0.14	1.21±0.42	0.14±0.02
	T2	2.183±0.484	1.108±0.24	0.57	3.74±0.03	1.485±0.14	0.16±0.06
	T3	2.375±0.714	0.94±0.038	0.73	3.52±0.07	1.685±0.32	0.18±0.02
	T4	1.983±0.226	1.058±0.038	0.65	3.46±0.11	1.525±0.35	0.188±0.01
	T5	1.858±0.604	0.816±0.101	0.15	3.71±0.161	1.25±0.11	0.18±0.052
21st Day	T0	0.966±0.08	0.862±0.021	0.54	3.81±0.064	0.033±0.03	0.04±0.02
	T1	1.85±0.198	1.25±0.28	0.22	4.08±0.28	0.065±0.03	0.029±0.016
	T2	1.8±0.066	1.133±0.038	0.12	4.326±0.27	0.105±0.03	0.044±0.021
	T3	1.825±0.09	1.35±0.025	0.45	1.82±0.15	0.321±0.05	0.014±0.002
	T4	0.991±0.062	0.895±0.026	0.33	2.96±0.75	1.29±0.35	0.02±0.006
	T5	1±0.025	0.937±0.033	0.46	2.88±0.08	0.2±0.008	0.025±0.012
28th Day	T0	0.929±0.026	0.85±0.025	0.28	2.14±0.17	0.025±0.017	0.038±0.014
	T1	1.27±0.081	1.133±0.12	0.31	2.66±0.21	0.065±0.03	0.09±0.006
	T2	1.741±0.062	1.4±0.1	0.31	2.47±0.27	0.285±0.07	0.1±0.02
	T3	2.125±0.15	1.275±0.05	0.3	4.06±0.53	0.325±0.05	0.064±0.004
	T4	1.42±0.04	1.15±0.15	0.26	4.02±0.64	1.34±0.13	0.068±0.039
	T5	1.341±0.407	1.133±0.16	0.19	3.8±0.1	0.625±0.16	0.12±0.04

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

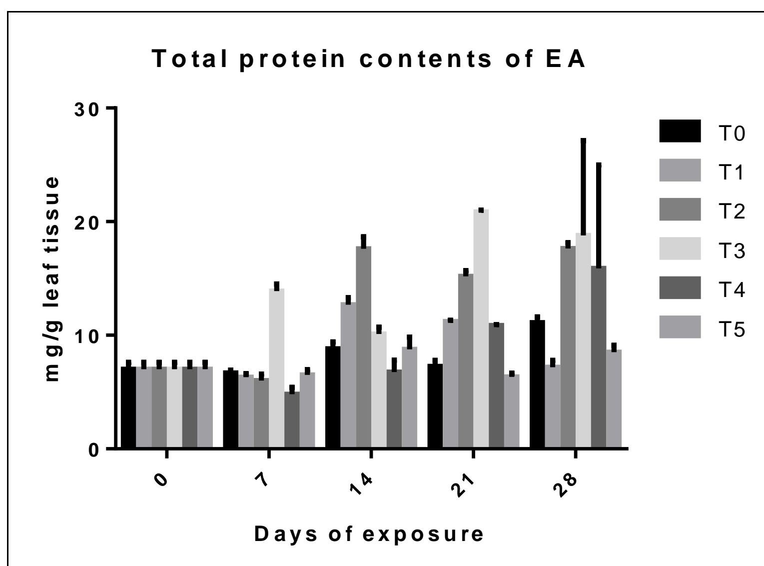

Figure.1 Quantitative analysis of total proteins in *E. agallocha* leaf sample during hardening at different salt concentration

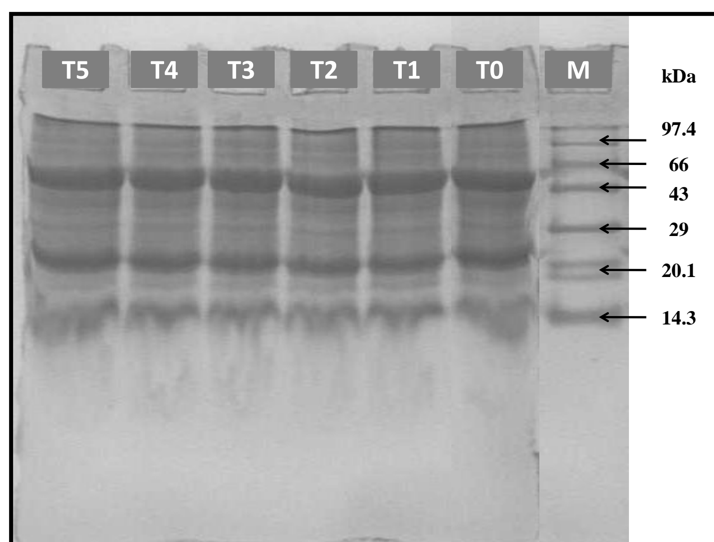

Figure.2 Qualitative (SDS-PAGE) analysis of proteins in *E. agallocha* leaf sample during hardening at different salt concentration

In this study, the maximum value is the reducing power (antioxidant potential) bio-molecular markers. The maximum value of Proline content (1.685 ± 0.32 mg//g leaf tissue) was recorded in 14th day of 300mM NaCl (T3) treated plantlets (Table 1). In *E. agallocha,* the percentage of synthesized proline content increased continuously from zero to 400mM (T4) NaCl treated plantlets with maximum increased and then decreased up to 500mM (T5) NaCl treated plantlets after 28 days of experiment. At 21st, 28th day, proline content increased up to 400mM NaCl and then decreases. At 14th day, proline content increases up to 300mM NaCl and then decreases (Table 1). Free proline accumulation determines salt tolerance potentials between the two cultivars of Foxtail millet (*Setaria italica* L.) seedlings with different salt sensitivity (Veeranagamallaiah

et al., 2007). Stress (including salt) causes increase in proline contents in the leaves of many plant species (Aziz and Khan 2001; Lee and Liu 1999). Here, the maximum values act as the proline bio-molecular markers.

Similarly, the maximum value of Glycine betaine (GB) content (0.188±0.01 mg/g dry leaf tissue) was recorded in 14th day of 400mM NaCl (T4) treated plantlets (Table 1). In salt tolerant species, accumulation of glycine betaine under salt stress was high (Jagendorf and Takabe 2001). GB protects photosynthetic machinery in case of some mangroves such *as Avicennia marina* (Ashihara *et al.,* 1997). 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 (Holmstrom *et al.,* 2000; Sakamoto *et al.,* 2000). GB is the most common compatible solute; which protects plants photosynthetic machinery and also found in some mangroves such *as Avicennia marina* (Ashihara *et al.,* 1997). Glycine betaine can preserves thylakoid and plasma membrane integrity at salinity stress (Rhodes and Hanson 1993). Here, the maximum value acts as the Glycine-betaine bio-molecular markers.

Three distinct and thick protein/polypeptide bands were detected at different concentration of salt stress and control during qualitative analysis of *E. agallocha* leaf protein. No changes appear in peptide in between control and salt treated sapling leaves during hardening with different concentration of NaCl. This indicates that the vegetatively propagated *E. agallocha* species survived in varied range of salinity during four weeks of hardening. The highest value of total phenolic content, total flavonoid content, reducing power, proline and glycine betaine and lowest value of IC_{50} could be the biochemical marker at respective salt stress in *E. agallocha*. Vegetative propagation *E. agallocha* followed by salt acclimatization and its reintroduction in denuded area may be could be practices.

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