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Studies of Non-Enzymatic and Growth Changes in Two Vegetatively Propagated Mangrove Species i.e. *Excoecaria agallocha* and *Cerbera manghas* at NaCl Stress During Hardening

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Abstract: ***The experiment was conducted for both morphological and non enzymatic parameters observation in two vegetatively propagated back mangrove species i.e. Excoecaria agallocha and Cerbera manghas during hardened against different concentration of NaCl under shade-net house conditions. Variation was observed during this experimental study. Both these species survived during salt stress for period of 28 days experiments. But the growth status of C. manghas was negligible or affected in comparison E. agallocha at higher concentration of salt stress. Again E. agallocha showed more content of total Phenolic, total Flavonoid, Proline and Reducing power activity at high salinity than C. manghas. While, IC_{50} of DPPH scavenging of E. agallocha was comparatively lower and show more scavenging power of free radicals. So, E. agallocha is more adaptive in different salinity regime than C. manghas. The baseline data may facilitate further course of research on successful re-establishment of other salt-sensitive mangrove species in the wild.***

Keywords: E. agallocha, C. manghas, NaCl treatments, hardening, morphological growth, non-enzymatic assay

1. Introduction

Excoecaria agallocha and *Cerbera manghas* are non vivipary back mangrove tree species having both ecological and economical importance. Natural regeneration of these species is very difficult due to predation of seeds and high salinity. Plants have certain mechanisms to adapt in environmental stress conditions (Faical et al. 2009; Rahnama and Ebrahimzadeh 2004). Antioxidants protect the plant from free radicals during oxidative stress conditions (Ozsoy et al. 2008). Free radical scavenging and reducing power capacity can determine antioxidant potential (Banerjee et al. 2008). Many secondary metabolites in plants like phenols, flavonoids etc. have major role against stress conditions (Ayaz et al. 2000).

Again the salt tolerant plant requires compatible solutes within the cytosol and organelles for osmotic adjustment (Rhodes and Hanson 1993). Osmoprotectants can also stabilize proteins and membranes. The effect of salt stress can be reduced by the accumulation of proline (Saxena et al. 2013; Matysik et al. 2002). In different plant species, proline synthesis in salinity condition can be considered as a stress indicator (Giridarakumar et al. 2003; Tiwari et al. 2010). In certain plants, glycine betaine become accumulates at salt stress condition and maintains osmotic equilibrium (Subbarao et al. 2001; Giridarakumar et al. 2003; Rhodes and Hanson 1993). The biochemical function of osmoprotectants is the scavenging of ROS (Bohnert and Jensen 1996). The aim of the present study was to examine the salt tolerant behaviour of vegetatively propagated back mangrove species *E. agallocha* and *C. manghas* at different concentration of salt stress

2. Materials and Methods

2.1 Planting materials and its macro-propagation

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; 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. (Figure 1 a, b & c)The *C. manghas* were vegetatively propagated with standard methods from stock plants, which were available in the nursery of RPRC. The rooted plantlets were transferred to the polybags and then they were kept under shade-net house and allowed to grow for a period of two months (Figure 2 a, b & c).

The experiment was set up for hardened plantlets in 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 28th day (Figure 1d & 2d) .

2.2 Total phenolic and Flavonoid content

Total phenolic contents were determined by method of Singleton and Rossi, (1965) with an absorbance of 765nm and was expressed as Gallic acid equivalents (GAE) in milligrams per gram leaf sample and calculated against Gallic acid as standard.

Total flavonoid contents were determined by method of Bao *et al.* 2005 with an absorbance of 510nm and were calculated using quercetin as standard and expressed as

milligram of quercetin equivalent (QE) per gram leaf sample.

2.3 DPPH radical scavenging assay

This assay was carried out by method Chan *et al.* 2007 and the absorbance was taken at 517 nm. 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.

Reducing power assay

This type of assay was carried out by method of Oyaizu *et al.* 1986 and the Fe^{2+}can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The absorbance was measured at 700 nm and was expressed as Ascorbic acid equivalents (AAE) in mg per gram leaf sample.

Proline analysis

Proline analysis was done by method of Bates *et al.* 1973 and the absorbance was taken at 520 nm and was calculated by using L-proline as standard and expressed as milligrams per gram leaf tissue.

Glycine betaine (GB) analysis

GB analysis was done by method of Greive *et al.* 1983 and the absorbance was taken at 365 nm and was calculated by using GB as standard and expressed as milligrams per gram leaf tissue.

2.4 Statistic analysis

The biochemical data obtained in this experiment were presented as mean values of triplicate for non-enzymatic observations and the difference between control and treatments were analysed using two ways ANOVA (Graph Pad Prism, Version 7).

Table 1: Percentage (%) of increase in morphological parameters in both E. agallocha (EA) and C. manghas (CM) after 28days of NaCl exposure during hardening

Treatments	Height in cm		collar perimeter in cm		No. of Branches		No. of Leaves	
	EA	CM	EA	CM	EA	CM	EA	CM
T0	2.66	6.22	7.8	7.84	21.05	6.66	12.12	2.97
T1	2.98	11.11	10.56	13.04	35.7	18.18	14.81	23.91
T2	5.95	10.46	10.14	16.27	37.5	**30**	22.09	27.77
T3	**9.16**	**12.1**	12.03	**18.6**	**38.46**	25	22.95	**31.52**
T4	6.75	4.54	**12.59**	10	33.33	0	**23.91**	4.7
T5	5.8	0.82	10.37	4.44	18.18	0	10.97	-5.42

Figure 1: (a) Rearing of wildlings in the nursery of RPRC at initial stage, (b) Growth of wildlings in nursery of RPRC, (c) Rooted *E. agallocha* through stem cuttings, (d) Salt acclimatized vegetatively propagated *E. agallocha*.

Figure 2: (a) Stock plants of *C. manghas* in nursery of RPRC Premises, (b) Survived stem cuttings in the plastic pots (c) Collection of rooted stem cuttings for transformation (d) Salt acclimatization of propagated stem cuttings in the nursery of RPRC premises.

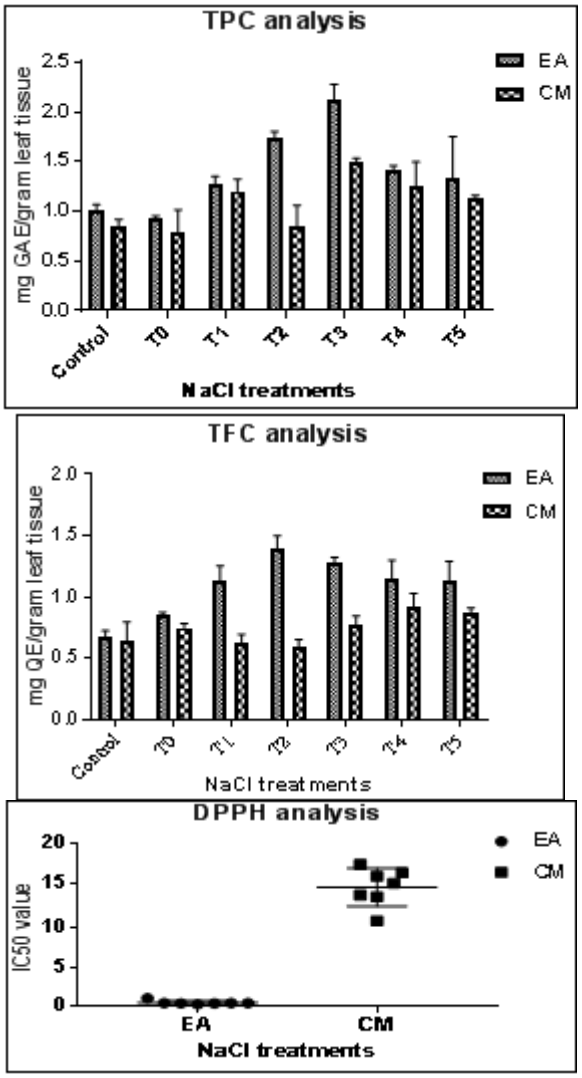

Figure 3: TPC (total phenolic content),TFC (total flavonoid content) and DPPH analysis in between EA (Excoecaria agallocha) and CM (Cerbera manghas) at different concentration of salt.

Abbreviation: GAE= Gallic acid equivalent; QE= Quecertin equivalent; IC_{50}= 50% inhibition capacity;;T0 = Control, T1 = 100mM, T2 =200mM, T3 = 300mM, T4 = 400mM and T5= 500mM NaCl**.** The data represent mean ± SD of three replicates.

3. Results and Discussion

Morphological changes were appeared in both the back mangrove species in different salt conditions. In *E. agallocha*, the maximum height (9% increase, at 300mMNaCl), collar perimeter (13% increase, 400mMNaCl), Number of Branches (38.5% increase, 300mMNaCl) and Number of Leaves (24% increase, 400mMNaCl) were measured. In *C. manghas*, the maximum height (12% increase, at 300mMNaCl), collar perimeter (18.6% increase, 300mMNaCl), Number of Branches (30% increase, 200mMNaCl) and Number of Leaves (31% increase, 300mMNaCl) were measured (Table 1). The stem diameter, number of branches and number of leaves increased in 250 mMNaCl treated *Aegiceras corniculatum* L. (Mohanty *et al.* 2013). This study revealed that the suitable range of salinity for these two species. But the growth of morphological characters of *C. manghas* was drastically affected at higher salinity.

Non Enzymatic Parameters

The total phenolic content in both *E. agallocha* and *C. manghas* become increases up to 300mM NaCl (T3) and then decreases but still the content remain high in comparison to control. The maximum value was 2.125±0.15 mg GAE/gram leaf tissue for *E. agallocha* (T3) and 1.5±0.035 mg GAE/gram leaf tissue for *C. manghas* at 28th day of NaCl exposure. (Figure 3). Phenolic content increases considerably in plants at increased salinity and decreased at high salinity (Agastian *et al.* 2000). In *Bruguiera parviflora*, phenolics were synthesized with increase in salinity (Parida *et al.* 2002). In this study, total phenolic content in both *E. agallocha* and *C. manghas* increased up to 300mM NaCl and then decreases considerably. Again, the maximum value

of phenolic content of *E. agallocha* was greater than the maximum value of *C. manghas* at 28th day of NaCl exposure.

The total flavonoid content in *E. agallocha* become increases up to 200mM NaCl (T2) with the maximum value 1.4±0.1 mg QE/g leaf tissue and then decreases but still the content remain high in comparison to control; while total flavonoid content in *C. manghas* show variation with maximum value of 0.9 ±0.106 mg QE/g leaf tissue in 400 mM NaCl (T4) at 28th day of NaCl exposure. (Figure 4). Salt stress (50 and 100 mM NaCl) significantly increases Flavonoid content in barley (Ali and Abbas 2003). Flavonoids reduced singlet oxygen and protect mangroves from UV radiation (Agati *et al.* 2007). In this experiment, the total flavonoid content in *E. agallocha* increased up to 200mM NaCl and then decreases considerably while total flavonoid content in *C. manghas* showed variation. Again, the maximum value of flavonoid content in *E. agallocha* was greater than the maximum value of *C. manghas* at 28th day of NaCl exposure.

DPPH assay evaluate the total antioxidants potential against free radicals (Koleva *et al.* 2002; Huang *et al.* 2005). The IC_{50} value for DPPH scavenging in *E. agallocha* become decreases up to 500mM NaCl (T5) with the minimum value 0.19 mg/ml; while IC_{50} value in *C. manghas* show variation with minimum value of 13.8 mg/ml in 300 mM NaCl (T3) at 28th day of NaCl exposure (Figure 5). In this study, the IC_{50} value for DPPH scavenging in *E. agallocha* decreased up to 500mM NaCl; while IC_{50} value in *C. manghas* showed variation in 300 mM NaCl (T3) at 28th day of NaCl exposure. Again, minimum value of IC_{50} of *E. agallocha* was smaller than minimum IC_{50} value *C. manghas* at 28th day of NaCl exposure.

The reducing power activity in *E. agallocha* and *C. manghas* become increases up to 300mM NaCl (T3) with the maximum value of 4.06±0.53 and 0.973±0.2 AAE mg/g leaf tissue respectively at 28th day of NaCl exposure and then decreases. But still the content remains high in comparison to control (Figure 6). In this study, the maximum reducing power value of *E. agallocha* is greater than *C. manghas* respectively at 28th day of NaCl exposure. Here Fe^{3+}/ferricyanide complex become reduced in to the ferrous form; which can be a significant indicator of its antioxidant capacity and depends on the presence of reductones (Rajamanikandan *et al.* 2011).

Proline content in *E. agallocha* and *C. manghas* become increases up to 400mM NaCl (T4) with the maximum value of 1.34±0.13 and 0.254±0.002 mg/g dry leaf tissue respectively at 28th day of NaCl exposure and then decreases. But still the content remains high than control (Figure 7). Salt stress causes increase in proline accumulation in the leaves of many plant species (Aziz *et al.* 1999 ; Hernandez *et al.* 2000). Proline accumulates in larger amounts in salt stressed plants than other amino acids and osmotically very active (Ashraf 1994; Mansour 1998). In this study, proline content in *E. agallocha* and *C. manghas* increased up to 400mM NaCl and then decreased. Besides this, the maximum value of proline content in *E. agallocha* was greater than the maximum value of *C. manghas*.

The glycine betaine content in *C. manghas* become increases up to 300mM NaCl (T3) with the maximum value 0.77±0.268 mg/g leaf tissue and then decreases but still the content remain high in comparison to control; while glycine betaine content in *E. agallocha* show variation with maximum value of 0.12±0.04 mg/g leaf tissue in 500 mM NaCl (T5) at 28th day of NaCl exposure (Figure 8). Glycine betaine (GB) under salt stress was found to be high in some salt tolerant species (Jagendorf and Takabe 2001). In some mangroves, GB protects photosynthetic machinery like *Avicennia marina* (Ashihara *et al.* 1997). GB is compatible solute and protects plants photosynthetic machinery and also found in some mangroves such as *Avicennia marina* (Ashihara *et al.* 1997).

4. Conclusions

From the above finding, we concluded that both *E. agallocha* and *C. manghas* can survive in different range of salinity. But *E. agallocha* have more adaptability than *C. manghas* due to highest total Phenolic content, the Flavonoid content, reducing power, Proline content and lowest value of IC_{50}. Besides this the growth of *C. manghas* became reduced at higher concentration of salinity. This indicates *C. manghas* more sensitive to salt stress than E. agallocha. So the vegetative propagation *E. agallocha* followed by salt acclimatization and its reintroduction in denuded area could be more fruitful than *C. manghas*.

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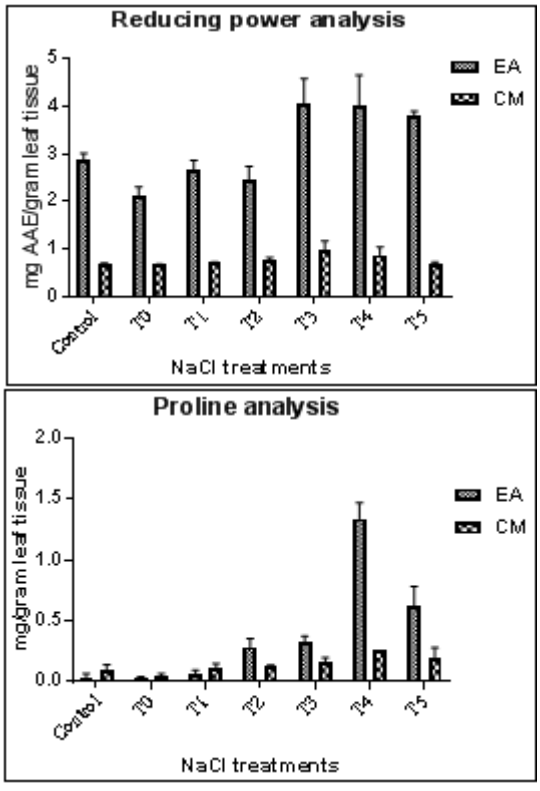

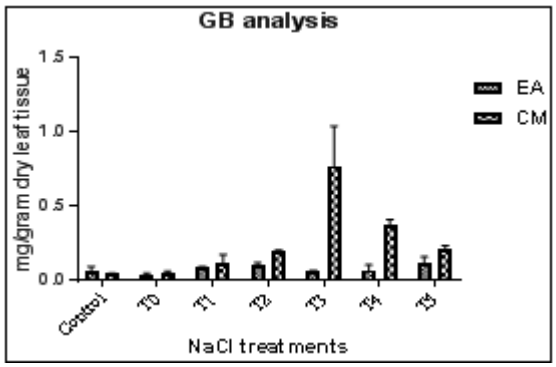

Figure 4: Reducing power, Proline and GB (Glycine betaine) analysis in between EA (Excoecaria agallocha) and CM (Cerbera manghas) at different concentration of salt.

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

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