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A comparative assessment of NaCl induced protein and antioxidant enzymes during hardening of two vegetatively propagated back mangrove species

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Abstract
NaCl stress application on plants including mangroves has been expanded over the last decade to know the salt tolerant behaviour. In the present study, both *Excoecaria agallocha* and *Cerbera manghas*, were vegetatively propagated and hardened against different concentration of NaCl i.e. control, 100, 200, 300, 400 and 500mM NaCl (salt acclimatization) for a period of 28days under shade-net house for the analysis of protein content and enzymatic activity. Both the species increased their protein content up to 200-300mM NaCl stress and then decreased at higher salinity. Besides this, *E. agallocha* leaves showed highest protein content in comparison to *C. manghas*. The specific enzymatic activity of the antioxidant enzymes i.e. POX, CAT and SOD increased at higher salt stress with higher activity in *C. manghas* leaves for this short period of NaCl exposure and determine its sensitiveness to NaCl and may not acclimatized against long term exposure NaCl stress due to its less protein contents. Again re-establishment of salt acclimatized vegetatively propagated *E. agallocha* saplings can be easier than *C. manghas.*

Keywords: nacl stress, protein, peroxidase (POX), Catalase (CAT), Superoxide dismutase (SOD), hardening

1. Introduction
Both *Excoecaria agallocha* (family- Euphorbiaceae) and *Cerbera manghas* (family-Apocyanaceae) are non viviparous and latex bearing back mangrove tree species. This mangrove species have both ecological and economical value. Continuous habitat loss, anthropogenic disturbances and changes in soil/water salinity are the great obstacles for the natural regeneration of this species. Besides this, salinity creates extremely unfavourable conditions and affects plant productivity. In response to various environmental stresses such as salt stresses, plants have developed different physiological and biochemical mechanisms to adapt or to tolerate stress (Faical *et al.,* 2009; Rahnama and Ebrahimzadeh 2004) [1, 2]. In general, the protein content increased with increasing concentration up to an optimal level. Agastian *et al.,* (2000) [3] reported that soluble protein increases at low salinity and decreases at high salinity in mulberry. Beyond the optimum level, the protein content decreased in *Phalaria arundinacea* (Maeda *et al.,* 1995) [4] *Sesuvium portulacastrum* (Venkatesalu *et al.,* 1994) [5]. Extremely decreased protein content of leaves was reported due to salinity in a non-secreting mangrove species *Bruguiera parviflora* (Parida *et al.,* 2002) [6]. Soluble protein contents of leaves decrease in response to salinity (Parida *et al.,* 2002, Gadallah, 1999) [6,7]. The protein content increases in halophytes with increasing concentrations of salt such as *Atriplex satidea* (Flowers and Dalmond, 1992) [8], and *Helochola setulosa* (Joshi *et al.,* 2002) [9].
Antioxidant enzymes i.e. SOD, POX and CAT activities are closely related to salt tolerance of many plants as reported in various researches (Rahnama and Ebrahimzadeh, 2004, AzevedoNeto *et al.,* 2006, Koca *et al.,* 2007) [2,10,11]. In plant cell, antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) have been considered as a defensive team, whose combined purpose is to protect cells from oxidative damage (Mittler, 2002) [12]. The ROS concentration in the tissues directly exposed to salt are strongly influenced by the coordinated action of different antioxidative enzymes (Munns and Tester, 2008) [13]. The stem cutting techniques for mangrove species like *Bruguiera parviflora, Cynometra iripa, Excoecaria agallocha, Heritiera fomes* and *Thespesia populnea* were standardized successfully (Basak *et al.,* 1995) [14].The aim of the present study was to examine the salt tolerant behaviour of vegetatively propagated back mangrove species *Excoecaria agallocha* and *Cerbera manghas* at different concentration of salt stress.

2. Materials and Methods
2.1 Source of the planting materials
The *E. agallocha* wildlings were collected from the Odisha coast and grown in shade-net house of RPRC and vegetatively propagated by using standard methods (Basak *et al.,* 1995, 2000; Basak & Mahapatra, 2009; Eganathan *et al.,* 2000) [14, 15, 16, 17]; while, *C. manghas* were vegetatively propagated from the stock plants available in the nursery of RPRC through stem cuttings (Basak and Mahapatra, 2009) [16]. Then the plantlets of both species were kept under shade-net house and allowed to grow for a period of two months.

2.2 Experimental condition
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 28th day.

2.3 Protein extraction and estimation

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 (Lowry *et al.*, 1951) [18]. 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. 0.1N NaOH was prepared by mixing 0.4 gram of NaOH in 100ml of distilled water. Reagent-A (2% Na_2CO_3 solution) was prepared by mixing 2 gram of Na_2CO_3 in 100ml of 0.1N NaOH. Reagent-B1 (20% of sodium potassium tartarate solution) was prepared by mixing 2 gram of sodium potassium tartarate in 100ml solution. Reagent-B2 (10% of copper sulphate solution) was prepared by mixing 1 gram of sodium potassium tartarate in 100ml solution. 100 ml of reagent-C was prepared with the mixture of 96ml of reagent-A, 2ml of reagent-B1 and 2ml of reagent-B2.

2.4 Gel electrophoresis

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. Leaf samples were macerated in a mortar-pestle, and to this was added 5 ml of extraction buffer The mixture was centrifuged at 10000 rpm for 20 min. Supernatants were used as samples. Running buffer was prepared from 30.3 g Tris base, 144.0 g glycine and 10.0 g SDS. Completely dissolve in about 800 ml distilled H_2O and then more distilled H_2O up to 1 liter. SDS protein sample buffer 1.25 ml 1 M TrisHCl, (pH 6.8), 4.0 ml 10% (w/v) SDS, 2.0 ml glycerol, 0.5 ml 0.5 M EDTA, 4 mg bromophenol blue, 0.2 ml beta-mercaptoethanol (14.3 M). Bring the volume to 10 ml with distilled H_2O (Laemmli, 1970) [19].

2.5 Peroxidase

Enzyme extract was prepared by grinding 0.5 g of leaf sample in chilled pestle and mortar by adding 5 ml of 0.1M sodium 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 (Quesada *et al.*, 1992) [20]. The specific enzyme activity was expressed in terms of U/mg protein.

2.6 Catalase

Catalase enzyme extract was prepared from 0.2 g of leaf sample with the application 4.0 ml of 0.1M sodium 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) [21]. The specific enzyme activity was expressed in terms of U/mg protein.

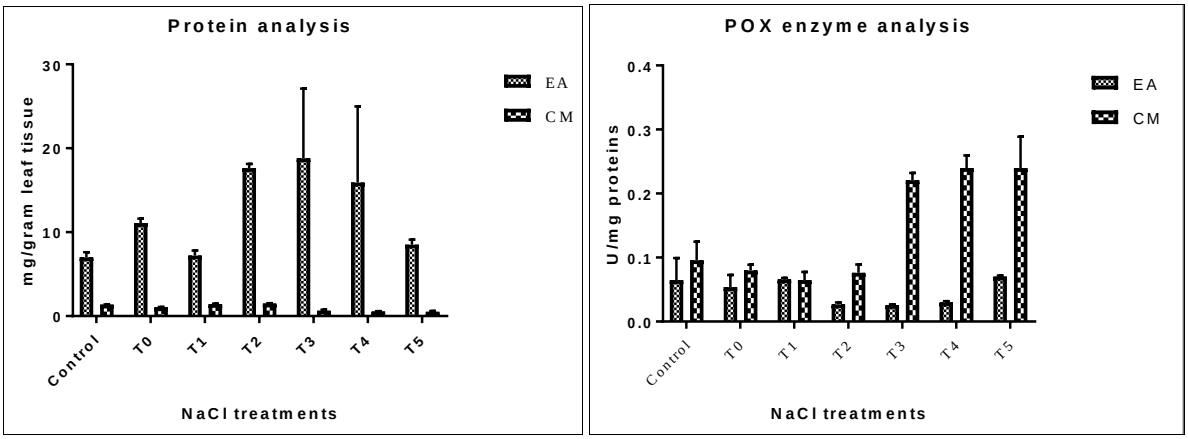
2.7 Superoxide dismutase

The extract for SOD enzyme assay was prepared by grinding 0.2 g of leaf sample in 5.0 ml of 0.1M sodium 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 nitrobluetetrazolium (NBT) in a reaction mixture containing 50 mM 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) [22]. The specific enzyme activity was expressed in terms of U/mg protein.

2.8 Statistic analysis

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

3. Results



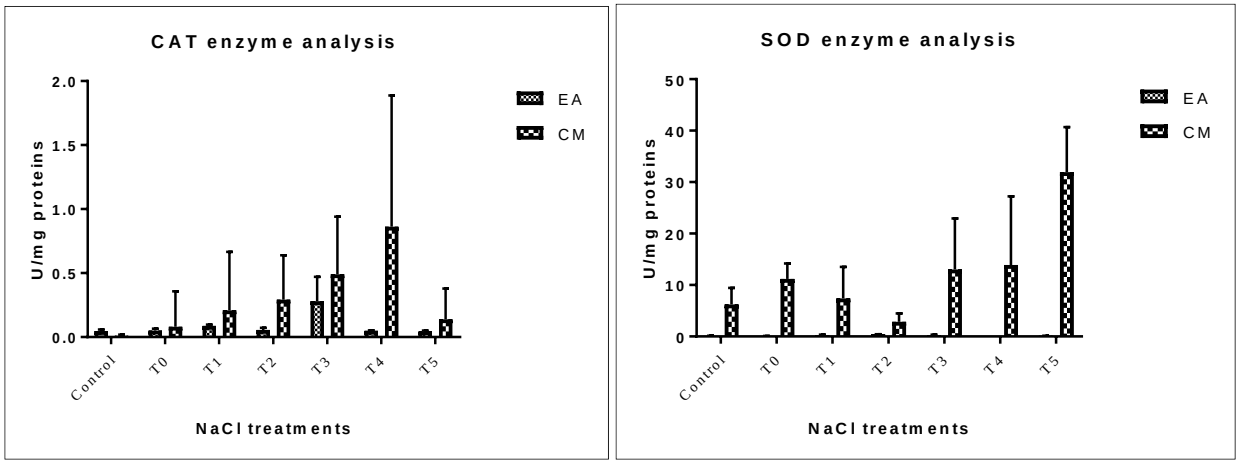


Fig. 1: Protein (a) and Specific enzyme (b, c and d) analysis of vegetatively propagated *Excoecaria agallocha* (EA) and *Cerbera manghas* (CM) saplings at different concentration of salt stress during hardening of 28days.
Abbreviation: POX= Peroxidase; CAT= Catalase; SOD= Superoxide dismutase; T0 = Control, T1 = 100mM, T2 =200mM, T3 = 300mM, T4 = 400mM and T5= 500mM NaCl. The data represent mean ± SD of three replicates.

3.1 Quantitative and Qualitative analysis of proteins

The protein content in *E. agallocha* become increases up to 300mM NaCl (T3) with the value of 18.8±8.32 mg/g leaf tissue and then decreases but still the content remain high in comparison to control; while the protein in *C. manghas* become increase up to 200mM NaCl (T2) with the value of 1.48±0.056 mg/g leaf tissue at 28th day exposure to NaCl and then decreases drastically even below the value of control [Fig. 1(a)]. The day factor, treatment factor and Interaction (day × treatment) were significant. According to multiple comparisons test, the comparison of T3 of *E. agallocha* with T2 treatment of *C. manghas* at 28th day was highly significant with P value <0.0001.Two numbers of distinct bands were appeared in each lane at 43 and 20.1 kDa region in both *E. agallocha* and *C. manghas*. In *E. agallocha*, the bands are thicker than *C. manghas* [Fig. 2].

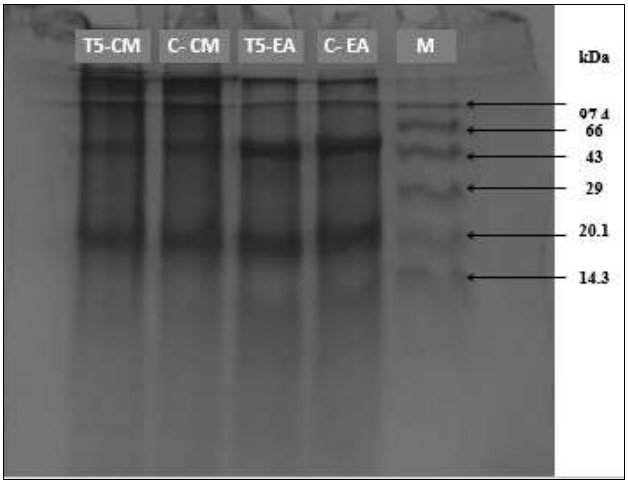


Fig. 2: Gel electrophoresis of *E. agallocha* (EA) and *C. manghas* (CM) leaf sample (20µl sample) after 28 days of NaCl exposure at different concentration.
Abbreviation: M=Marker; C= Control; T5= 500mMNaCl; kDa= Kilo dalton

3.2 Antioxidant enzymes

The peroxidase activity (POX) in both *E. agallocha* and *C. manghas* show variation. The maximum value of POX was recorded 0.07±0.0017 in 500mM NaCl (T5) in *E. agallocha* and 0.24±0.01961 (400mM NaCl, T4) and 0.24±0.049 (500mM NaCl, T5) in *C. manghas* respectively at 28th day of NaCl exposure [Fig. 1(b)]. The day factor, treatment factor and Interaction (day × treatment) were significant. According to multiple comparisons test, the comparison of T5 of *E. agallocha* with T4 and T5 treatment of *C. manghas* at 28th day was highly significant with P value <0.0001.

The Catalase activity (CAT) in *C. manghas* become increases up to 400mM NaCl (T4) with the maximum value 0.862±1.025 leaf tissue and then decreases but still the content remain high in comparison to control; while CAT activity in *E. agallocha* show variation with maximum value of 0.281±0.189 in 300 mM NaCl (T3) at 28th day of NaCl exposure [Fig. 1(c)]. The day factor, treatment factor and Interaction (day × treatment) were not significant. The Superoxide dismutase enzyme (SOD) activity in both *E. agallocha* and *C. manghas* showed variation. The maximum SOD activities were recorded 0.425±0.03 at 200mM NaCl (T2) in *E. agallocha* and 31.94±8.755 at 500mM NaCl (T5) in *C. manghas* respectively at 28th day of NaCl exposure [Fig. 1(d)]. The day factor, treatment factor and Interaction (day × treatment) were significant. According to multiple comparisons test, the comparison of control, T1, T2, T3, T4 and T5 of *E. agallocha* with T5 treatment of *C. manghas* at 28th day were highly significant with P value <0.0001.

4. Discussion

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) [5]. However, in this study, the total protein content decreased further at higher concentrations (beyond 300 mM in *E. agallocha* and 200 mM in *C. manghas*) of NaCl (Fig. 1(a)]. This was in agreement with the findings of Parida *et al.*, (2004) [23]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. Besides this, the maximum value of protein content in *E. agallocha* is much greater than the protein content of *C. manghas* [Fig. 1(a)]**.** On the basis of qualitative analysis, two distinct protein bands were appeared at 43 and 20.1 kDa respectively in each lane due to run of 20µl protein leaf sample of both *E. agallocha* and *C. manghas* mangrove species in Gel [Fig.

2]. On the basis of comparison, less amount of proteins are synthesized in *C. manghas* in comparison to *E. agallocha*. Again, protein bands in *E. agallocha* were more prominent than *C. manghas.*

Peroxidase enzyme activity might be useful for adaptation required prevention of peroxidation of membrane lipids (Kalir *et al.,* 1984) [24]. In *Aegiceras corniculatum,* peroxidase activity was found increased along with increasing salt concentration (Manikandan *et al.,* 2004) [25]. Increase in POX activity demonstrates the accumulation of H_2O_2 in salt stress condition (Jiang *et al.,* 2001) [26]. A rise in the peroxidase activity with salinity has also been verified in *Morus alba* (Sudhakar *et al.,* 2001) [27]. The specific enzyme activity of peroxidase (POX) in both *E. agallocha* and *C. manghas* show variation and become higher at 500mM NaCl stress. Again, the maximum specific enzyme activity of POX in *E. agallocha* was comparatively less than *C. manghas* at 28th day of NaCl exposure [Fig. 1(b)].

The Catalase activity decreased with increasing concentration of NaCl in *Phaseolus radiatus* (Saha *et al.,* 1999) [28]. CAT, which is involved in the degradation of hydrogen peroxide into water and oxygen, is the most effective antioxidant enzymes in preventing oxidative damage (Willekens *et al.,* 1995) [29]. The changes in CAT activity may depend on the species, the development and metabolic state of the plant, as well as on the duration and intensity of the stress (Chaparzadeh *et al.,* 2004) [30]. In this study, the specific enzyme activity of catalase (CAT) in *C. manghas* become increased up to 400mM NaCl (T4) and then decreases. This finding is an agreement with increase of catalase enzyme activity up to optimum level in *Ipomoea pes-caprae* (Venkatesan *et al.,* 1999) [31]. CAT activity in *E. agallocha* showed maximum activity at 300mM (T3) NaCl exposure. Besides this, the maximum specific enzyme activity of CAT in *E. agallocha* was comparatively less than *C. manghas* after 28 days of NaCl exposure [Fig. 1(c)].

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) [32]. Many studies found a positive correlation between salt stress and the abundance of SOD in plants cells (Badawi *et al.,* 2004, Cavalcanti *et al.,* 2007) [33,34]. SOD activity increased in comparison with control under stress condition in different cultivars. Others showed that salt treatment significantly increases the SOD activity. Increase in Reactive Oxygen Species, especially O_2^-, causes to increase in SOD, because this enzyme scavenges O_2^- (Esfandiari *et al.,* 2007) [35]. The specific enzyme activity of Superoxide dismutase enzyme (SOD) in both *E. agallocha* and *C. manghas* showed variation and show maximum activity at 200mM (T2) and 500mM (T5) NaCl stress respectively. The maximum specific enzyme activity of SOD in *E. agallocha* was widely less than *C. manghas* at 28th day of NaCl exposure [Fig. 1(d)].

5. Conclusion

We found maximum protein contents, POX, CAT and SOD activity were recorded at 300, 500, 300 and 200mM NaCl stress in *E. agallocha* and at 200, 500, 400 and 500mM NaCl stress in *C. manghas* respectively. From the above finding, we concluded that both *Excoecaria agallocha* and *Cerbera manghas* can survive in different range of salinity. But *E. agallocha* can sustain better in higher salinity for long term exposure to the salt stress. This is due to its comparatively high protein contents. Again *C. manghas* shows higher specific antioxidant enzyme activity in short period of NaCl exposure and determine its sensitiveness against salt stress. 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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