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Evaluation of biomolecular activities during induction of *in vivo* multiple shoot regeneration in *hypocotyls* of some *Rhizophoraceae* mangroves

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The biochemical changes influencing shoot regeneration in mangroves, viz. *Bruguiera parviflora* (Roxb) Wt. & Arn. Ex Griff. , *Kandelia candel* (L.) Druce. and *Rhizophora apiculata* Bl. was studied by analysing antioxidant enzyme activities during induction of *in vivo* multiple shoot regeneration. The impact of NaCl and proline on enzyme behavior was evaluated during multiple shootings in treated and control hypocotyls. Peroxidase (POX) activity in *K. candel* increased by 2.58 and 2.80 fold as compared to *B. parviflora* and *R. apiculata*, respectively, after 5 days of NaCl treatment. Exogenous application of NaCl also enhanced catalase (CAT) activity considerably in *K. candel*. Similarly, superoxide dismutase (SOD) activity in *K. candel* increased 1.6 and 3.7 fold as compared to *B. parviflora* and *R. apiculata*, respectively. Proline application, however, inhibited the enzyme activity in all three species. Antioxidant enzymes may be used as possible markers for evaluating species-specific ability for induction of *in vivo* multiple shoot regeneration in Rhizophoraceae mangroves.

Key words: *Bruguiera,* catalase (CAT), *Kandelia,* peroxidase (POX), proline, *Rhizophora,* superoxide dismutase (SOD).

INTRODUCTION

Mangroves represent intertidal forest communities of tropical and sub-tropical region having great ecological and economic significance in providing forestry and fishery products to a large human population, protecting coastal zones from erosion, storms and also in supplying food and shelter for a large number of fishes. However, destruction of mangrove forests for creating agricultural lands and loss of habitat due to industrial pollution has resulted in a gradual depletion of this valuable and vulnerable unique resource worldwide. Most of the mangrove species of Odisha coast are also threatened by human intervention, that is, encroachment upon the land for cultivation and shrimp culture, for exploitation of timber, fuel and fodder and for several other uses (Basak et al., 2000; Upadhyay et al., 2002). Mangrove reforestation by seed or propagule planting is a common and conventional way to regenerate new individuals especially for tree mangroves belonging to the family Rhizophoraceae. However, habitat destruction and reduced population of mature and seed-producing plants limit the scope of conventional afforestation through propagule planting.

In vivo multiple shoot regeneration technique may be an alternative way to clonal multiplication and conservation of valuable indigenous Rhizophoraceae mangroves

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Abbreviations: SOD, Superoxide dismutase; **BP,** *Bruguiera parviflora;* **RA,** *Rhizophora apiculata*; **KC,** *Kandelia candel*; **POX,** peroxidase; **NBT,** nitroblue tetrazolium.

(Basak and Das, 2002). Greater demand and extensive use of propagules of *Rhizophora, Kandelia* and *Bruguiera* spp. especially for reforestation also contribute to their rapid depletion from primary habitats. For endangered mangroves, it is, therefore, felt essential to standardize technique for vigorous and low cost planting material production through alternative methods such as multiple shoot regeneration through decapitation of hypocotyls. It has been proved that decapitation induces numerous juvenile shoots from upper region of hypocotyls of *Bruguiera gymnorrhiza* giving rise to explants suitable for stem-cutting/air layer to propagate Rhizophoraceae mangroves vegetatively. *In vitro* multiple shoots are also developed from 'callus' regenerated near and upon cut surface of the hypocotyls explants of *B. gymnorrhiza* (Satuwong et al., 1995). However, understanding the biochemical changes associated with *in vivo* regeneration of multiple shoots especially in Rhizophoraceae mangroves is rather poor.

The salt tolerant behavior of plant has been explained through changes in antioxidant enzymes particularly at time of salt stress in mangroves (Dasgupta et al., 2012). Superoxide dismutase (SOD) catalyzes the conversion of superoxide to hydrogen peroxide and oxygen. Since understanding about salt stress effect on active oxygen metabolism in mangrove plants is insufficient, the current work examined species specific antioxidant enzymes behaviour during induction of multiple shoot regeneration in hypocotyls of three Rhizophoraceae mangroves.

MATERIALS AND METHODS

Healthy and mature propagules of Rhizophoraceae mangroves viz. *Bruguiera parviflora* (Roxb.) Wt. & Arn. Ex Griff. (BP), *R. apiculata* Bl. (RA) and *K. candel* (L.) Druce. (KC) were collected during October, 2010 from Mahanadi mangrove wetland in mid-region of the Odisha coast, India (20° 18'-20° 32' N latitude and 86° 41'-86° 48' E longitude).

Induction of multiple shoots

The hypocotyls of all three studied species having uniform length (18 to 20 cm for BP, 30 to 32 cm for RA and 33 to 35 cm for KC) and diameter (0.4 to 0.5 cm for BP, 1.4 to 1.5 cm for RA and 0.8 to 1.0 cm for KC) were decapitated in the collar region (meristematic junction between plumule and hypocotyls) and grown in polybags containing a mixture of garden soil and sand (1:1). The plant materials were kept under mist system in green house conditions.

Treatments

The decapitated hypocotyls were treated with 200 ppm NaCl (T1), 100 ppm proline (T2) and a combination of NaCl (200 ppm) plus proline (100 ppm) for 10 days. In order to analyze changes in antioxidant enzyme activities during multiple shoot regeneration, samples were collected at different time intervals, that is, at Day 0, Day 5 and Day 10.

Extraction of antioxidative enzymes and their assays

The fresh decapitated part of the hypocotyls (test sample, 500 mg each) was homogenized with pre-chilled mortar and pestle in ice-cold 0.1 M phosphate buffer (pH 6.5). The crude homogenate was centrifuged at 10,000 rpm at 4°C. The supernatant so obtained was used for analysis of the enzymes viz. peroxidase (POX) and super oxide dismutase (SOD).

Peroxidase (POX) assay

To determine the POX 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 of test sample extract were 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 was recorded at 530 nm in 1 min intervals up to 10 min in a spectrophotometer (Model Specord 50. Split-beam, Analytik Jena, Germany) following the method of Quesada et al. (1992). The enzyme activities were expressed in terms of an average increment in absorbance per minute.

Superoxide dismutase (SOD) assay

SOD activity was estimated by monitoring the inhibition of photochemical 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 absorbance was read at 560 nm against the non-irradiated blank. The enzyme activities were expressed in terms of amount of an enzyme causing half the maximum inhibition of NBT reduction.

Catalase (CAT)

The freshly decapitated part of the hypocotyls (500 mg each) was homogenized in a mortar and pestle using 50 mM sodium phosphate buffer (pH 7.0). The homogenate was then centrifuged at 10,000 rpm at 4°C for 20 min. The supernatant was used as source of enzyme. The CAT activity was assayed from the rate of H_2O_2 decomposition measured through the decrease of absorbance at 240 nm, following the procedure of Bergmeyer (1983). The reaction mixture contained 2.0 ml of 50 mM sodium phosphate buffer (pH 7.0), 1.0 ml of H_2O_2 (30 %) and 0.01 ml enzyme extract. The enzyme activities were expressed in terms of an amount of enzyme which decomposes 1 µmol of H_2O_2 per minute.

Statistical analysis

The experiments were set with three replications (10 hypocotyls per replication), and descriptive statistics was used to analyze and organize the resulting data using Graph Pad Prism (5.0 Version)

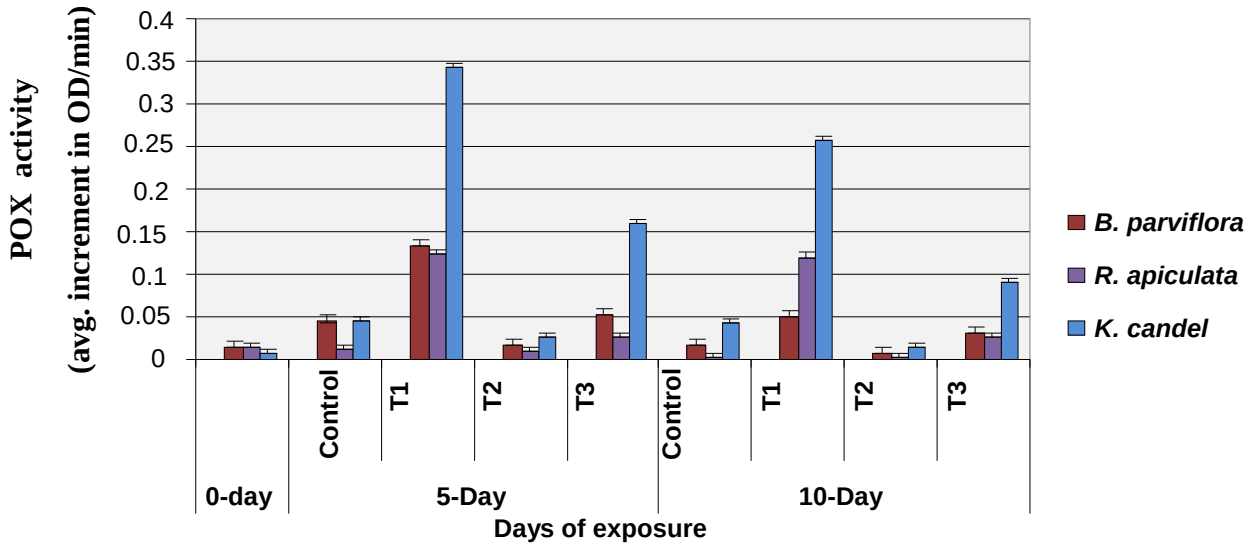

Figure 1. Peroxidase (POX) activity (in terms of an average increment in optical density per minute) during multiple shoot development in three mangrove species. T1, NaCl (200 ppm); T2, proline (100 ppm); T3, NaCl (200 ppm) + proline (100 ppm).

RESULTS

Antioxidant enzymes and their assays

Changes in peroxidase activity

Peroxidase enzyme activity was measured both in decapitated control and treated hypocotyls during the 10 days of decapitation (Figure 1). Highest activity was noted on day 5 when hypocotyls were treated with only 200 ppm NaCl (T1) in all studied species (Figure 1). The peroxidase activity got lowered in samples either treated with only proline (T2) or in combination with NaCl (T3). Maximum activity was observed in *K. candel* followed by *B. parviflora,* and *R. apiculata*, respectively. Peroxidase activity in *K. candel*, *R. apiculata, B. parviflora* increased 7.6, 1.04 and 3.0 times, respectively, after day 5 of saline treatment as compared to control.

Changes in catalase activity

Changes in the activity of catalase enzyme during multiple shoot regeneration in the studied species is shown in Figure 2. As seen in the case of peroxidase, maximum catalase activity was also recorded in *K. candel* on day 5, on application of NaCl (T1). The magnitude of enzyme activity gradually tapered off during subsequent period of multiple shoot regeneration. In all species, Catalase activity was minimal, when samples were administered only proline**.** Catalase activity was up by 2.2 time after Day-5 in *K. candel* compared to control.

Changes in superoxide dismutase activity

The superoxide dismutase enzymatic activity was most prominent in the saline condition (T1) and minimal with proline treated samples irrespective of species (Figure 3). Interestingly, the enzyme activity became maximum on day 5 after which it started to decline. As with peroxidase and catalase, the superoxide dismutase activity was also highest for *K. candel* amongst all studied species. The saline stress induced four time increase in the enzyme activity in *K. candel* on day 5 treatment with NaCl.

DISCUSSION

Efficient *in vivo* shoot regeneration in hypocotyls of mangrove Rhizophoraceae, that is, *K. candel, B. parviflora*, and *R. apiculata* was affected by exogenous application of sodium chloride (NaCl) and proline. An important consequence of salinity stress is the generation of excessive reactive oxygen radicals/molecule (ROS) (Xiong and Zhu, 2002). Tian et al. (2003) observed that peroxide radicals, hydrogen peroxide, and antioxidant enzymes influence organogenesis during *in vitro* culture of shoot buds from strawberry callus. The activities of POX and CAT decreased during 0 to 6 weeks culture period and gradually increased in the later stage of shoot bud formation (7 to 8 weeks of culture). The CAT activity, however, continuously declined during the culture period. Our results revealed that, POX activity in *K. candel* increased by 2.58 fold after day 5 of treatment as compared to *B. parviflora* and 2.80 fold as compared to *R. apiculata*. The POX acti-

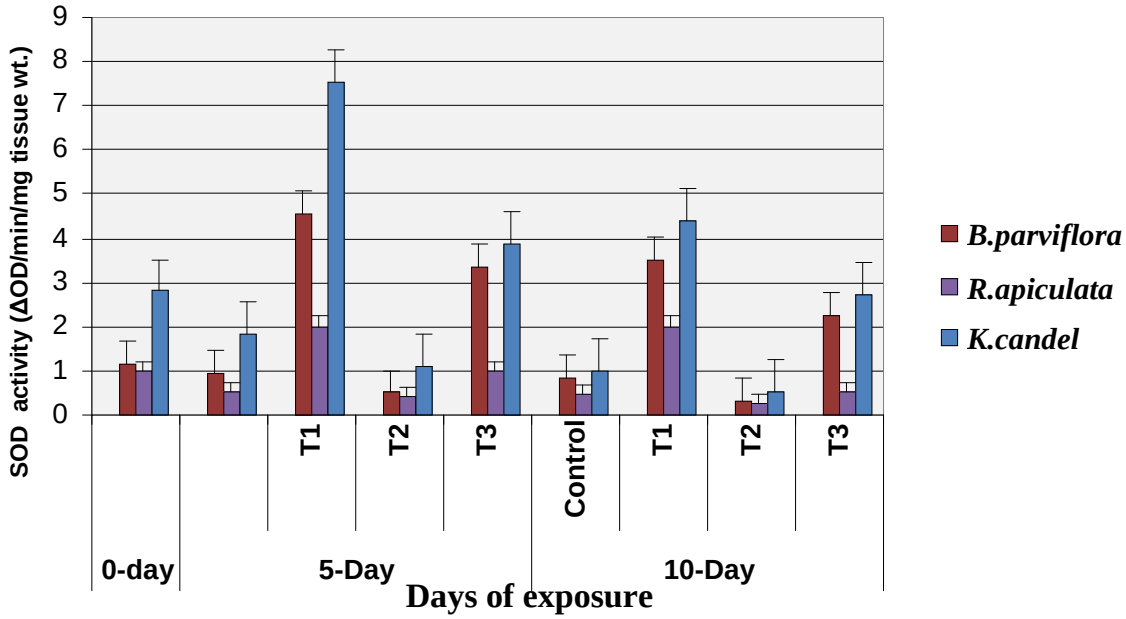

Figure 2. Catalase (CAT) activity (in terms of an amount of enzyme which decomposes 1 µmol of H_2O_2 per minute) during multiple shoot development in three mangrove species). T1, NaCl (200ppm); T2, proline (100ppm); T3, NaCl (200ppm) + proline (100ppm).

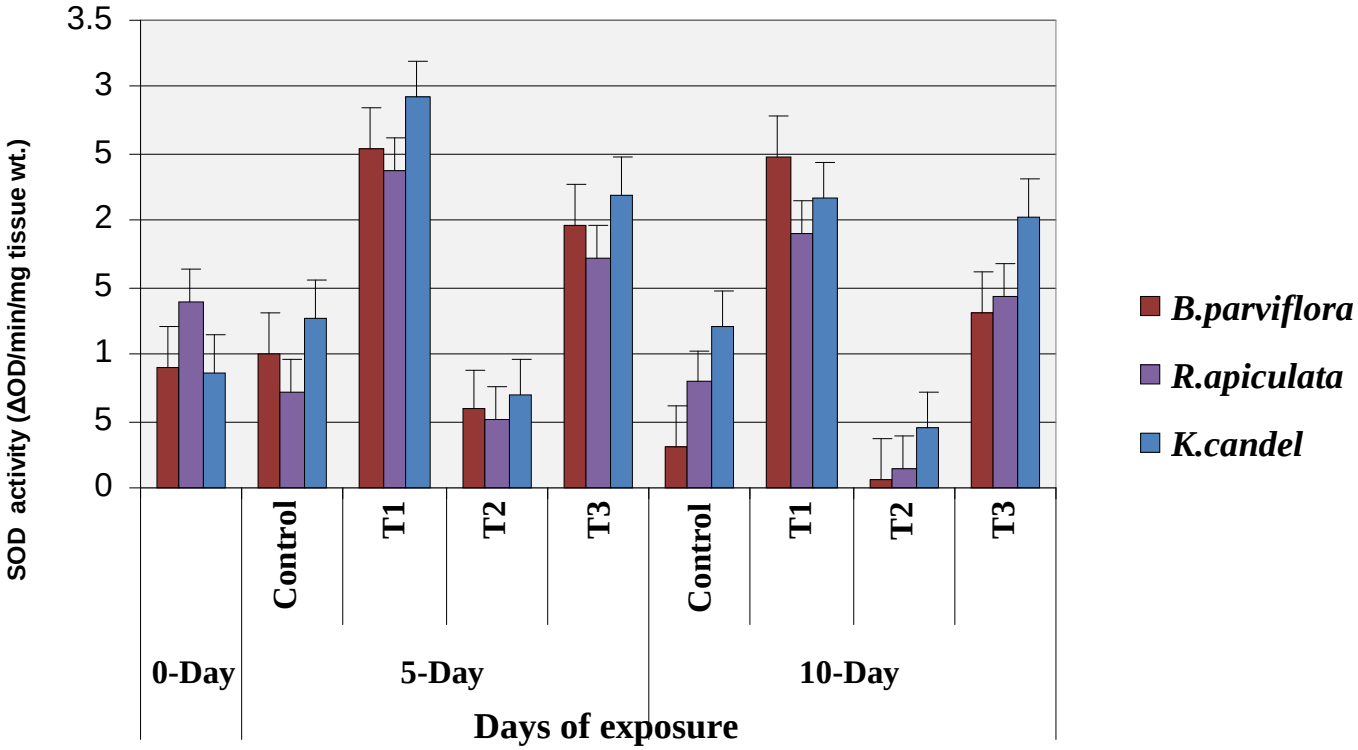

Figure 3. Superoxide dismutase (SOD) activity (in terms of amount of an enzyme causing half the maximum inhibition of NBT (nitroblue tetrazolium) during multiple shoot development in three mangrove species. T1, NaCl (200 ppm); T2, proline (100 ppm); T3, NaCl (200 ppm) + proline (100 ppm).

vity in the treated samples measured 7.6 fold higher activity over the control in *K. candel*. The POX, CAT and SOD activities increased at early stage, that is, upto day 5 and declined after day 10 of decapitation. The results further suggest that NaCl enhances CAT activity in *K. candel* by 1.148 fold and 1.072 fold as compared to *B. parviflora, R. apiculata* respectively after day 5 of treatment. The CAT activity increased by 2.29 fold over the control in *K. candel*. Takemura et al (2000) demonstrated that the activities of antioxidant enzyme, SOD and CAT could increase five to eight times soon after the plants were transferred from water to high salinity in 10 days. In the studied species, the SOD activity in *K. candel* increased 1.6 fold compared to *B. parviflora* and 3.7 fold compared to *R. apiculata*. But with the treatment of proline, the enzyme activity declined in all the three species. Our result demonstrated that the activity of POX, CAT and SOD increased after applying NaCl at Day-5 of decapitation and gradually decreased in the Day-10 of decapitation. The POX, CAT and SOD showed an immediate induction upon exposure to NaCl solution. Similar activity has also been reported in salt-treated *Pistacia vera L.* by Hosein (2012).

The present study indicates that *in vivo* multiple shoot regeneration from hypocotyls of Rhizophoraceae mangroves was influenced by salinity and proline leading to expression of species-specific differential antioxidant enzyme activities. The specific enzymes viz. peroxidase, catalase and superoxide dismutase may be used as indicator to depict pathway of shoot organogenesis in *B. parviflora*, *K. candel* and *R. apiculata.* It may also be concluded that further studies on 'genetic regulation' on the above scores may open new avenue for evaluating species-specific respond concerning multiple shoot regeneration of the above mangrove plants.

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