Effect of auxin derivatives on morphological and isoenzyme pattern of enzymatic antioxidant peroxidase (POX) of "blinding eye mangrove" *Excoecaria agallocha.* L stem cuttings

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Abstract

In our investigation, the effect of auxin on rooting and sprouting behavior of stem cutting of Excoecaria *agallocha* has been studied. Initially stem cuttings were pretreated to remove the phenol content in the cutting and then they stem were subjected to hormonal treatment with auxins derivatives as IBA 2000 ppm, IPA 2000 ppm, NAA 2000 ppm, and IBA+NAA combination 2000 ppm. The root length and their number, rooting and sprouting percentage, number of leaves per cutting, leaf area, and photosynthetic pigments were analyzed on days 40, 50, and 60 after planting (DAP). Also the isoenzyme pattern of peroxidase of root and leaf were analyzed. Among the auxin treatments, IBA 2000ppm vastly enhanced rooting and sprouting behavior followed by IPA, IBA+NAA, and NAA of *Excoecaria agallocha.* The isoenzyme analysis for peroxidase clearly showed that peroxidase (POX) highly supported both root initiation and elongation processes in *Excoecaria agallocha* L.

Keywords: Excoecaria *agallocha*; Mangrove; Stem cutting; Auxins, Peroxidase isoenzymes

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Introduction

Mangrove forests are among the world's most productive ecosystems, widely distributed in the inter tidal zone of the tropical and subtropical coastlines of the globe (Tomlinson, 1986; Hogarth, 1999). Mangrove forests are generally considered as even-aged forests, primarily developing after a disturbance or colonization of mud flats and such uneven-aged mangrove forest is represented by the diameter distribution of both trees and saplings (Saenger 2002; Trettin et al., 2016). Taxonomically, mangroves constitute diverse angiosperm plants exhibiting a set of physiological adaptations (Tomlinson 1986), protecting the coastal zone from natural calamities such as cyclones, tidal thrust, and tsunamis and also providing a wide variety of goods and services used by coastal people (Zhang et al., 2006).

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The importance of mangroves for coastal community and humans has been well documented throughout the tropics (Sandilyan and Kathiresan, 2012). Mangroves prevent encroachment by the seas by checking soil erosion and thereby stabilizing the shoreline. Mangrove habitat has been under severe destruction worldwide at alarming levels and reclamation (Kathiresan K, Bingham BL, 2001). While mangrove ecosystems are unique habitats of the coastal wetland systems, they are facing intense pressure due to wanton destruction by humans for various developmental needs. Such levels of destruction and habitat fragmentation raise concerns about conservation of diversity on mangrove. The present work was carried out in Pichavaram mangrove. Pichavaram is situated in the south east coast of Tamil nadu, India. The mangroves of Pichavaram are threatened largely due to several natural and anthropogenic pressures. Grazing is one of the reasons for degradation of mangroves in the peripheral area of Pichavaram (Selvam et al., 2001).

Excoecaria agallocha L. (Euphorbiaceae) known as “milk mangrove” grows in sandy soil or in the drier harder sandy mud near the terrestrial fringes of mangrove vegetation. All parts of the plant release white latex, causes temporary blindness if enters in the eyes and hence, so called as “blinding tree”. It has been traditionally used to treat sores and the smoke of its bark has been used to treat leprosy (Ghani, 2003). Clinical trials carried out on this plant have shown its potential anti-HIV, anticancer, antibacterial, and antiviral properties (Peter and Sivasothi, 1999). The vegetative propagation of *Excoecaria agallocha* is necessary because this species is threatened by human intervention i.e., encroachment upon the land for cultivation and shrimp culture, for exploitation of timber, fuel, and fodder, and for other uses. Generally, *Excoecaria agallocha* reproduces through seeds. Sudden depletion of the growing stock post-dispersal predation on seeds by crabs, poor flowering, and seed setting necessitates vegetative propagation of these species for re-establishment in degraded forest areas (Das, *et al.,* 1997). In *Excoecaria agallocha* adventitious root formation is extremely difficult. But it can be done with the help of plant growth regulator application. Among various plant growth substances auxins play a central role in the process of root formation (Davis et al., 1989; De Klerk et al., 1999). They induce root initials (Nordstrom et al., 1991; Nag et al., 2001) and influence the growth of the newly formed roots in the expressive phase of root development (Bellamine et al., 1998). Auxin along with peroxidase plays a major role in root initiation and elongation. Many studies on adventitious root formation have suggested a fundamental role of peroxidase. The role of peroxidase in plant growth can be clearly studied by isoenzyme pattern. Numerous papers have reported that auxin induces changes in peroxidase isoenzymes during adventitious rooting (Chen et al., 2002a).

Keeping all these in view, auxin derivatives such as Indole-3-butyric acid (IBA), Napthylacetic acid (NAA), and Indole-3-pyruvic acid (IPA) have been chosen for the present study. The study dealt with the effect of auxins (IBA, NAA, and IPA) on rooting and sprouting of the stem cutting of *Excoecaria agallocha.* Moreover, the isoenzyme pattern of peroxidase in both leaf and root of *Excoecaria agallocha* was also studied.

Materials and Methods

Plant materials and hormonal treatment

Healthy and uniform stem cuttings (10-15 cm in length) of *Excoecaria agallocha* were collected from Pichavaram in Chidambaram, Cuddalore district, Tamilnadu, India. Initially stem cuttings were pretreated with sodium carbonate and sodium tungstate for 5-10 mins and the treated cuttings were washed two to three times with distilled water. The stem cutting that was devoid of phenol was dipped for 30 mins in IBA 2000 ppm, IPA 2000 ppm, NAA 2000 ppm, and IBA+NAA combination 2000 ppm. After hormonal treatment, plants were transferred to plastic tray containing coarse sand and soil in 1:1 ratio and the tray was kept in the mist chamber at 32/26 °C (maximum and minimum) and relative humidity (RH) varied between 60-75 percent during the experimental period. The observations on the number of cuttings rooted, number of roots and sprouts produced on each cutting, and their

Table 1
Effect of auxin on the rooting of *E. agallocha*

Treatment (ppm)	40th DAP			50th DAP			60th DAP		
	RL	RN	R%	RL	RN	R%	RL	RN	R%
Control	7.0± 0.189	18.0± 0.486	20.0	8.0± 0.240	17.0± 0.510	10.0	10.5± 0.357	20± 0.680	30.0
IBA 2000	17.0± 0.459	36.0± 0.978	60.0	21.0± 0.630	46.0± 1.380	60.0	29.0± 0.298	56.0± 1.901	80.0
IPA 2000	16.0± 0.432	28.0± 0.756	60.0	18.5± 0.555	28.0± 0840	50.0	25.0± 0.850	37.0± 1.258	60.0
NAA 2000	10.0± 0.270	24.0± 0.648	30.0	11.5± 0.345	23.0± 0.690	20.0	18.0± 0.612	28.0± 0.952	40.0
IBA+NAA 20000	8.5± 0.229	19.0± 0.513	40.0	12.0± 0.360	21.0± 0.630	30.0	20.0± 0.680	29.0± 0.986	50.0

RN - Rooting number, RL - Root length, R% - Rooting percentage, DAP-Days after planting

length, fresh weigh, and dry weigh were recorded on each treatment 40, 50 and 60 days after planting (DAS).

Pigment composition

Chlorophyll was extracted and estimated according to the method followed by Arnon (1949). Xanthophyll contents were estimated by the method explained by Neogy et al. (2001) and the results were expressed in mg g^{-1} fresh weight.

Extraction for enzyme activity

One gram of plant tissue was homogenized in 1 ml of an ice-cold solution containing 100 mM phosphate buffer (pH 7.8), 1 mM EDTA, and 0.5% (v/v) Triton X – 100. The homogenate was then centrifuged for 30 mins at 18,000 rpm. The eluent was stored at -20˚C for subsequent analysis of peroxidase isoenzyme.

Isoenzyme analysis

Isoenzymes were separated using 7.5% separating and 5% stacking polyacrylamide native gels. Electrophoresis was carried out at 4 °C under non-denaturing conditions as described by Laemmli (1970). Following electrophoretic separation, the gel was stained for peroxidase isoenzymes. The gel was incubated in the staining solution for a few minutes till the clear band appeared. After clear bands appeared the gel was washed with distilled water and photographed immediately. The staining solution was prepared

Fig. I. Effect of auxin on growth of *Excoecaria agallocha*: 1: Control, 2: IBA (2000), 3: IPA (2000), 4: NAA (2000), 5: IBA+NAA (2000)

by dissolving 500 mg of benzidine in 0.5 ml of ethanol, and 5 ml of acetic acid and 95 ml of distilled water was added to it. The contents were mixed thoroughly and filtered through filter paper. Finally, 250 µl of hydrogen peroxide was added before staining.

Statistical Analysis

Each treatment was analyzed with at least four replicates and a standard deviation (SD) was calculated and data were expressed as ± SD of four replications.

Results

Results showed that the application of IBA, IPA, NAA, and IBA+NAA combination resulted in the best root and shoot growth in stem cutting. Tables 1 and 2 show significant differences with respect to the percent of rooting, root number,

Table 2
Effect of auxin on the sprouting of *E. agallocha*

Treatment (ppm)	40th DAP			50th DAP			60th DAP		
	Sprouting %	No. of leaves	Leaf area	Sprouting %	No. of leaves	Leaf area	Sprouting %	No. of leaves	Leaf area
Control	10.0	9.0± 0.243	2.1± 0.056	30.0	12.0± 0.360	2.6± 0.078	20.0	10.0± 0.340	3.0± 0.102
IBA 2000	50.0	20.0± 0.540	9.3± 0.251	70.0	30.0± 0.900	12.3± 0.369	80.0	35.0± 1.190	14.9± 0.506
IPA 2000	40.0	17.0± 0.459	6.9± 0.186	50.0	21.0± 0.630	7.3± 0.219	70.0	25.0± 0.850	9.8± 0.333
NAA 2000	20.0	12.0± 0.324	5.8± 0.156	20.0	18.0± 0.540	7.0± 0.210	30.0	19.0± 0.646	8.3± 0.282
IBA+NAA 20000	40.0	18.0± 0.486	7.3± 0.197	40.0	20.5± 0.615	86.0± 0.258	50.0	23.0± 0.782	10.5± 0.357

Table 3
Effect of auxin on the fresh and dry weight (leaf and root) of *E. agallocha* L.

8.5	40th DAP				50th DAP				60th DAP			
	Leaf		Root		Leaf		Root		Leaf		Root	
	FW	DW	FW	DW	FW	DW	FW	DW	FW	DW	FW	DW
Control	1.05± 0.028	0.85± 0.022	2.99± 0.080	0.99± 0.026	2.02± 0.060	0.97± 0.029	2.80± 0.084	0.93± 0.027	2.10± 0.071	1.33± 0.043	2.82± 0.095	1.0± 0.034
IBA2000	3.37± 0.090	1.33± 0.036	4.04± 0.109	1.34± 0.036	5.96± 0.173	1.60± 0.048	6.60± 0.198	2.20± 0.066	6.45± 0.219	2.63± 0.089	6.79± 0.230	2.46± 0.083
1PA2000	3.10± 0.083	1.03± 0.027	5.20± 0.140	1.73± 0.046	5.19± 0.155	1.70± 0.051	5.63± 0.168	1.87± 0.056	5.46± 0.141	1.70± 0.056	5.95± 0.202	2.08± 0.070
NAA2000	2.35± 0.063	0.78± 0.021	5.16± 0.139	1.72± 0.036	4.09± 0.122	1.03± 0.030	5.10± 0.153	1.70± 0.051	4.85± 0.164	1.65± 0.056	5.26± 0.178	1.95± 0.066
1BA+NAA 2000	2.49± 0.067	1.19± 0.032	4.93± 0.133	1.64± 0.044	4.91± 0.147	1.65± 0.049	5.25± 0.157	1.72± 0.051	5.19± 0.176	2.48± 0.084	5.41± 0.183	1.86± 0.063

root length, percent of sprouting, number of leaves, and leaf area in auxin treatments at different concentrations.

The root length of *E. agallocha* increased with the age of the plant. It increased significantly compared with the control in all treatments. IBA 2000 ppm treatment highly increased the root length when compared to plants treated with IPA 2000 ppm, IBA+NAA 2000 ppm combination, and NAA 2000 ppm. IBA 2000 ppm treatment also highly increased the number of roots compared to the other treatments and the control. The highest rooting rate (80%) on 60th day was recorded in the cuttings treated with IBA 2000 ppm followed by IPA 2000 ppm and other treatments.

Different sprouting rates were obtained for the cuttings treated with different auxin (Table 2). The cuttings treated with IBA at 2000 ppm had a sprouting rate of (80%) which was significantly higher than that of control and other treatments. IBA treatment increased the number of leaves per plants followed by IPA, IBA+NAA, and NAA. The leaf area increased with the age in the control and treated plants. IBA 2000 ppm highly increased the leaf area when compared to other treatments and control.

The fresh and dry weights of roots and leaves per cutting showed an increasing trend with the age of the treated and control cuttings (Table 3). Among the different auxin-treated plants, IBA significantly increased fresh and dry weights of both roots and leaves when compared with other treatments and control.

Auxin-treated plants typically appeared dark and greener and this has been correlated with an increase of the chlorophyll content in *E. agallocha*. Hormonal treatment increased the xanthopyll content to a larger extent when compared to the control. Among different auxin treatments, IBA concentration showed increased

photosynthetic pigments at all stages of growth (Fig. II).

The isoenzyme pattern of peroxidase showed single peroxidase band in leaves treated with IBA, IPA, NAA, and IBA+NAA combination, exhibiting high intensity of band in NAA treatment with relative mobility value (Rm) of 0.62. While in roots two peroxidase bands were found in all auxin treatments as well as in control. In contrast, the NAA-treated roots alone showed a significantly increased intensity of band with Rm value 0.57 (Fig. III).

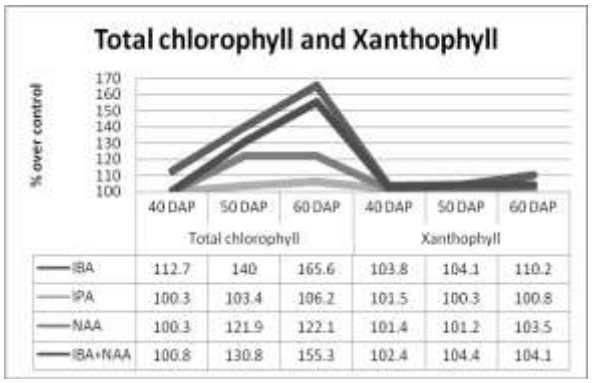

Figure II. Effect of auxin on total chlorophyll and xanthophyll of *Excoecaria agallocha*; DAP: days after planting

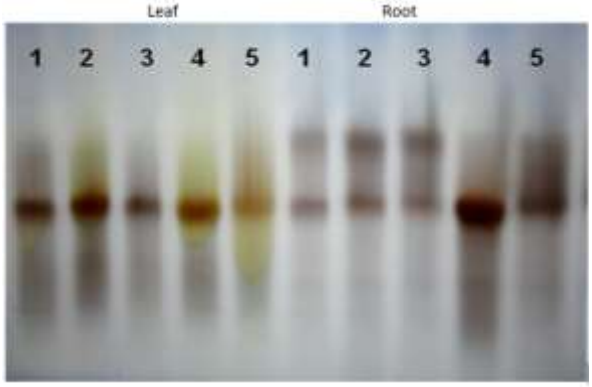

Fig. III. Effect of auxin on isoenzyme pattern of peroxidase; 1: Control, 2: IBA, 3: IPA, 4: NAA, 5: IBA+NAA

Discussion

Results showed a significant influence of auxin derivatives on increasing the rooting and sprouting of *Excoecaria agallocha* (Fig. I). Auxin has been shown to regulate different aspects of plant growth and development by affecting numerous processes including cell division, cell elongation, and differentiation (Woodward and Bartel, 2005). Also, the induction of rooting by auxin application has been reported by many researchers in other plant species (Davies 1996; Blakely et al., 1988). Exogenous application of IBA to the base of cuttings positively impacted rooting percentage, rooting number per cutting, and rooting length in our study. Similar result was observed in *Cynometra iripa, Heritiera fomes* (Basak et al., 1995). Moreover, the rooting in cutting of another mangrove *Avicennia alba* showed similar type of response (Reddy et al., 1994). In soybean hypocotyls auxins (IBA, IAA, and NAA) effectively promoted the rooting (Chie-hung Chou et al., 2010). The differential root regenerating ability of different auxins individually or in combination might depend on their respective capacity to synthesize protein essential for the regeneration and elongation of roots (Ghost 1974; Basak et al., 1999).

Our finding also suggests the pretreatment of IBA could be very effective in enhancing the sprouting of *Excoecaria agallocha.* Similar results were also reported in *Jatropha curcas* (Sunita Koclhar et al., 2008). Different concentrations of auxin were significantly different over control with regard to the number of leaves developed per cutting. The results reported by Chalapathi et al. (2000) in *Stevia* followed similar trends. The possible reason for such increase may be due to the activation of shoot growth by overcoming ABA levels of buds which probably increased the number of nodes that lead to the development of more leaves. Generally, auxin plays a significant role in the elongation of petiole, midrib, and major lateral veins of the leaves.

Leaf production was increased in auxin-treated plants. Similar results were reported in *Cotinus coggygria* (Pacholczak et al., 2005). The interesting observation was that the shoots were formed much earlier than roots in *E. agallocha*. Shoots were formed earlier due to reserve carbohydrates and started producing auxins, which moved downward and accumulated in the lower portion of the cuttings. When the concentration reached a threshold value, endogenous auxins at the extreme basal end started to become metabolized and signaled the process of root initiation.

Increased fresh and dry weight of root and leaf per cutting was recorded in the cuttings

treated with IBA followed by other treatments. Similar effect has also been observed in *Psoralea corylifolia* (Faisal and Anis, 2006) and Azadirachta *indica* and *Pongamia pinnata* (Palanisamy, et al., 1988).

Hormonal treatment significantly increased the chlorophyll and xanthophyll contents in *E. agallocha* (Fig. II). Among different auxin treatments IBA showed more increase in photosynthetic pigment contents. IBA increased the vegetative growth and pigment concentration in maize (Kaya et al., 2006). A similar result was also observed in grapevine cuttings. In the present study, the increased photosynthetic content in leaves increased in IBA-treated cutting that might have altered the synthesis and translocation of assimilates (Kaur et al., 2002). Moreover, the exogenously IBA probably serves as a stable storage form of IAA because IBA can be converted back to IAA and thus can be slowly released when required by the plant (Normanly, 2010; Woodward and Bartel, 2005).

In auxin-treated *E. agallocha* stem cutting the isoenzyme pattern of peroxidase showed single peroxidase band in leaves while root showed two peroxidase bands in all auxin treatments and in control (Fig. III). A similar result was also observed in *Jatropha curcas* cutting, in which two peroxidase bands were observed at the phase of root elongation in auxin treatment (Sunita et al., 2008). In soybean hypocotyls, the activity of anionic POX and cationic POX was significantly suppressed by exogenous auxins at the inductive phase. The anionic POXs are most significantly increased in IBA-treated tissues as compared with control (Chien-Hug Chou et al., 2010). The role of peroxidase in the rooting of poplar cuttings has been pointed out earlier by Gunes (2000). The present study suggests that peroxidase help with auxin catabolism and in triggering the root initiation process. A similar role can also be played by IAA-oxidase but IAA oxidase plays a part only for triggering and initiating the roots/root primordial; peroxidase involved both root initiation and elongation processes.

Conclusion

The present study revealed a significant influence of IBA, IPA, NAA, and IBA+NAA combination on increasing the rooting and shooting of *E. agallocha.* The result of isoenzyme analysis clearly showed the role of peroxidase during rooting initiation and elongation.

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