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Micropropagation and Phenolic exudation protocol for *Excoecaria agallocha*-an important mangrove

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ABSTRACT

Objective: To develop a standard micropropagation protocol for an important vulnerable mangrove *Excoecaria agallocha*. **Methods:** Collection of explants, surface sterilization, phenolic exudation and medium was standardized. Shoot induction, shoot multiplication and rooting were carried out in MMS medium supplemented with BAP, Kinetin, Zeatin, 2ip, NAA, IAA and IBA. Hardening was carried out after root well established. **Results:** The best phenolic exudation removal was resulted in 4 g/L activated charcoal. The maximum shoot induction response showed in MMS medium and better shoot induction was performed in the concentration of BAP (3.9 μmol) and NAA (1.34 μmol). Rooting induction was performed high range at 5.02 μmol of IAA. Well rooted micro-shoots were hardened and acclimatized. **Conclusions:** From the present investigation, it can be concluded that a standard micropropagation protocol was developed for an important vulnerable *mangrove* species.

1. Introduction

Mangroves are halophytic woody plants that serve as protection against cyclone, Tsunami and a source of energy for based coastal food chain. In addition, it is used for drugs, dyes, tannins and also medicine. Mangroves are derived from eighty families of tree and shrubs in habituating shoreline and estuaries in tropical and subtropical coastal regions[1]. However, mangrove habitats are ecologically important as they function as natural nutrient filters and recyclers, aid in floodwater mitigation and help protect coastal areas from seawater intrusion. Mangrove habitat has been under serve destruction worldwide at alarming levels and reclamation[2]. Such levels of destruction and habitat fragmentation raise concerns about conservation of diversity on mangrove. To augment conservation, management efforts in germinate and particularly for unique genotype has to be made[1].

Excoecaria agallocha (*E. agallocha*) L. (Euphorbiaceae) is a vulnerable mangrove tree; also called milky mangrove and highly tolerant to adverse environmental conditions[3]. *E. agallocha* is used for the treatment of ulcer and aphrodisiac. The extract of these plants is also for rheumatism, paralysis, cutaneous infection. *E. agallocha* is used as purgative and in treatment of epilepsy, dermatitis, leprosy, toothache. *E. agallocha* having active compounds like excoecariatoxins, fluratoxin, glycerides of fatty acid, lipids and waxes; phorbol, esters, polyhenols, polysaccharides, saponins, steroids, tannins, triterpenes[3]. The urgency for conservation of *E. agallocha* is mainly due to the cutting of trees for fuel by the local people. In addition, low seed production and low seed germination[4]. Most of mangrove has seed germination problem caused by embryo dormancy, seed coat impermeability by water. It has been suggested that the germination paucity might be related to climate changes, because seed viability due to water deficit stress. Taken together, the insufficient seed production and low germination have been negative influence on reproductive yield and consequently on population value and pharmacologically important species[3].

Germination experiments with seed of *E. agallocha* subjected to different scarification treatments were unsuccessful due to the low seed viability. Therefore

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micropropagation may be alternative means to conserve this unique species. The main objected this study was to develop a micropropagation protocol for *E. agallocha*. Various plant cultured medium, anti-browning agents and plant growth regulators (PGRs) were tried. The bud materials (axillary and apical) collected from adult plants for the whole study. Emphasis was made on achieving the most satisfactory suitable off-spring through micropropagation.

2. Materials and methods

Shoot tips and nodes were excised from the growing tips of adult trees of *E. agallocha* form Pichavaram mangrove Reserve forest, Tamil nadu and used as explants. Shoot tips and nodal segments (not longer than 2 cm) were excised from the young shoots. The explants were washed in tap water for 5 min followed by 10 percent Tween 20 (Liquid detergent; Himedia, India) for 5 min then surface sterilized with 70% (v/v) ethanol for 2 min and rinsed 3 times by sterile distilled water and then treated with one percent of methyl N-(1H-benzimidazol-2-yl) carbamate (Bavistin).Then the explants were treated with 20% sodium tungstate and sodium carbonate solution to remove phenolic compounds from the explants and washed with 0.1% $HgCl_2$ for 2 min and then washed thrice with sterile water. These explants were placed in different medium along with the various concentrations of activated charcoal, citric acid and ascorbic acid (Table 1) to exudates.

Shoot bud development from explant and culture conditions Surface sterilized explants with at least one axillary or terminal bud were placed in culture tubes with different PGR, combinations. The three different culture media were tried to culture of *E. agallocha* explants *viz.*, Murashige and Skoog medium[5], Modified Murashige and Skoog medium[6] and Woody Plant Medium[7], with various concentrations of BAP and NAA to estimate the suitable culture medium for *E. agallocha* (Table 2). Of the three medium MMS medium was found to induce the maximum growth in *E. agallocha*. Hence these medium was used throughout the experiment. The medium consisted of $MgSO_4$ (185 mg/L), KNO_3, $KHPO_4$ (85 mg/L) and $CaCl_2$ (440 mg/L) with the omission of NH_4NO_3 and supplemented with 30 g/L of sucrose, 8 g/L agar and pH was adjusted to 5.8. Treatment included seven replications, one shoot was inoculated in culture tube with 10 mL of medium and 450 explants were used for the study. Culture chamber conditions for all experiments were 16 h photo-period (Cool white lamps, Phillips Master LD 36, photosynthetic photo lux density 90 μmol m-2s-1) and 24/19 ℃ day/night temperature, respectively[8]. The number of explants developed lateral shoots and the length of the shoot and the physiological state of the shoot after 5 weeks were determined. MMS medium with 1.4, 3.9, 4.8, 5.4 and 7.4 μmol of BAP and 1.34 μmol of NAA (Table 1) were used for this study.

In the second experiment, the optimal concentration of BAP, NAA and IAA was estimated by using various concentration of these growth regulating chemicals (Table 2). Stem segments were vertically placed each test tube for all multiplication treatments, nondestructive observation were performed after 4 and 6 week cultures. Each experiment was repeated three times and number of shoot, shoot length of explant were observed.

2.1. Rooting treatments and plant green house establishment

Fifty elongated micro-shoots of 4th week old were placed in MMS medium for root induction with 0.5 to 2.0 mg/L of IBA, NAA and IAA and cultured for 2 week. The shoots was transferred to root expression medium consisting of Modified Murashige and Skoog (Half Strength macronutrient) in PGRs free medium. The in vitro rooting experiment was set up in a complete randomized block design with three blocks with 5 replicates number of shoots rooted, number of roots per shoot and mean root length. *In vitro* rooted plantlets were removed from culture media and agar was washed from roots, and transplanted in to small plastic pots containing a peat-pertlite mixture 1:1 (w/v) covered with transposable polythene bags to maintain a high relative humidity. The potted plantlets were kept in greenhouse 24-26 ℃ during the day and 18-20 ℃ during the night. Ventilation of the plantlet was increased after 7 days by increasing size of the holes made in the polythene cover. After 3 week the polythene cover was removed. After 5 week of acclimatization individual plants were transferred to a plastic pot (diameter 9 cm) containing the same growing mixture and transferred to the shade net of 70% shade. Air temperature was in the shade net 25-28 ℃ during the day and 20-22 ℃ during the night.

3. Results

Tissue browning is a constant drawback that renders tissue culture work difficult in this species. The presence of phenolic compounds and high ployphenol oxidase activity cause explants browning affects vegetative propagation and limits morphogenic response. The treatments used during explant excision allowed tissues to stay green during the first two subcultures. Explants maintained on a medium without antioxidants or adsorbent compounds released brown-black exudates into medium. Except for activated charcoal, the browning percentage was inversely related to the compounds concentration tested, ascorbic acid and citric acid. Among the anti-browning agents, activated charcoal at 4 g/L, citric acid at 100 mL/L and ascorbic acid at 100 mg/L had significantly higher percentage of anti-browning of nodal ex-plants than the shoot tip explants at 30 days. Adding of activated charcoal 4 g/L was significantly lesser browning of both types of explants (Figure 1). In the present study shoot induction was carried out from node

Table 1.
Effect of anti-browning agents by removing phenolic compounds in basal medium.

Explants	Period days	Control %±SD	Activated charcoal (g/L) *percentage±SD			Ascorbic acid (mg/L) percentage±SD			Citric acid (mg/L) percentage±SD		
			2	4	6	50	100	150	50	100	150
Node	15	18.0±0.2	96.0±0.4	98.0±0.3	96.0±0.3	97±0.3	96.0±0.2	92.0±0.2	92.0±0.6	93.0±0.2	96.0±0.5
	30	8.0±0.1	92.0±0.6	86.0±0.2	92.0±0.2	84±0.3	90.0±0.1	88.0±0.1	72.0±0.2	64.0±0.2	60.0±0.6
Shoot tip	15	32.0±0.4	92.0±0.5	98.0±0.1	93.0±0.5	84±0.4	86.0±0.2	82.0±0.5	88.0±0.2	90.0±0.3	87.0±0.3
	30	10.0±0.2	84.0±0.2	83.0±0.5	74.0±0.1	72±0.1	82.0±0.3	79.0±0.3	54.0±0.2	62.0±0.4	58.0±0.1

*Results are presented in percentage of explant not browning. Browning percentage = Number of browning explants / Number of explants 100.

Table2.
Response of shoot induction in MMS medium with various combination of NAA and BAP.

Growth regulators (μmol)		Shoot initiation per explant (%)		
		Weeks in culture		Shoot length (mm)
NAA	BAP	6	7	6
0	0.0	38.0±0.2	44.0±0.6	28.0±0.1
0	1.4	46.0±0.6	53.0±0.7	25.0±0.3
0	3.9	52.0±0.4	61.0±0.4	25.0±0.4
0	4.8	49.0±0.6	51.0±0.6	28.0±0.2
0	5.4	49.0±0.2	46.0±0.2	26.0±0.2
0	7.4	40.0±0.1	40.0±0.3	29.0±0.3
1.34	0.0	38.0±0.6	42.0±0.5	26.0±0.1
1.34	1.4	49.0±0.6	50.0±0.1	25.0±0.4
1.34	3.9	76.0±0.2	82.0±0.6	31.0±0.2
1.34	4.8	56.0±0.6	59.0±0.1	26.0±0.1
1.34	5.4	39.0±0.2	41.0±0.3	27.0±0.4
1.34	7.4	51.0±0.6	49.0±0.6	29.0±0.3

Table 3.
Effect of growth regulators on shoot multiplication of *E. agallocha*.

Growth regulators (μmol)		Shoots per explant (number)		
		Weeks in culture		Shoot length (mm)
Auxins (1.34)	Cytokinin (3.9)	10	11	10
0	0	1.5±0.2	1.5±0.1	7.9±0.2
IAA	Zeatin	1.5±0.6	1.9±0.6	7.1±0.6
IAA	Kinetin	1.4±0.1	1.6±0.7	9.2±0.5
IAA	2iP	1.4±0.3	1.4±0.5	14.0±0.1
IAA	BAP	2.3±0.4	3.2±0.6	10.0±0.6
IBA	BAP	1.4±0.5	1.4±0.1	7.3±0.6
IBA	BAP	1.5±0.2	1.4±0.3	8.0±0.3

Table 4.
Effect of root induction in MMS medium with various concentration of auxins.

Growth regulators (μmol / L)			% Response mean±SD	Mean No. of roots mean±SD	Mean root length (cm) mean±SD
IBA	IAA	NAA			
1.47	–	–	18.9±1.2	1.8±0.3	1.6±0.2
2.85	–	–	38.6±1.6	2.9±0.9	2.4±0.4
4.23	–	–	61.6±1.8	4.6±0.4	3.4±0.4
5.61	–	–	59.6±0.9	3.4±0.9	1.8±0.3
–	1.74	–	28.6±2.1	2.2±0.4	2.4±0.3
–	3.38	–	69.1±1.2	4.4±0.6	3.1±0.3
–	5.02	–	86.0±0.9	5.9±0.6	3.8±0.5
–	6.60	–	72.9±1.6	4.2±0.5	3.2±0.5
–	–	1.26	10.6±1.4	1.2±0.6	1.1±0.1
–	–	2.53	18.9±1.2	1.6±0.4	1.8±1.0
–	–	3.80	38.6±0.9	2.6±0.9	2.6±0.6
–	–	5.07	26.8±1.2	2.1±0.4	2.1±0.3

and shoot tip explants in various concentrations of BAP and NAA combinations. Among the various concentrations 3.9 μmol of BAP and 1.34 μmol of NAA were expressed a well response than other combinations (Table 2) and they promoted the more number of shoot induction per explants (3.8) (Figure 2, 3). The best result in terms of shoot length was

also obtained from the medium augmented with 1.34 μmol NAA and 3.9 μmol BAP, although the effectiveness of this treatment did not differ significantly from that of the medium deprived of growth regulators and from that of the other media containing BAP alone (4.8 or 7.4 μmol) or combination with NAA (0.12 μmol NAA and 3.9 μmol BAP) (Table 2). The combination of 1.34 μmol NAA and 3.9 μmol BAP produced favorable results in terms of numbers and length of axillary shoots were produced. The equal molar concentrations of cytokinins (kinetin, 2ip and zeatin) and auxins (IAA and IBA) were used for the multiplication of shoots (Table 3). After 4 weeks of culture, axillary shoot formation was inferior at

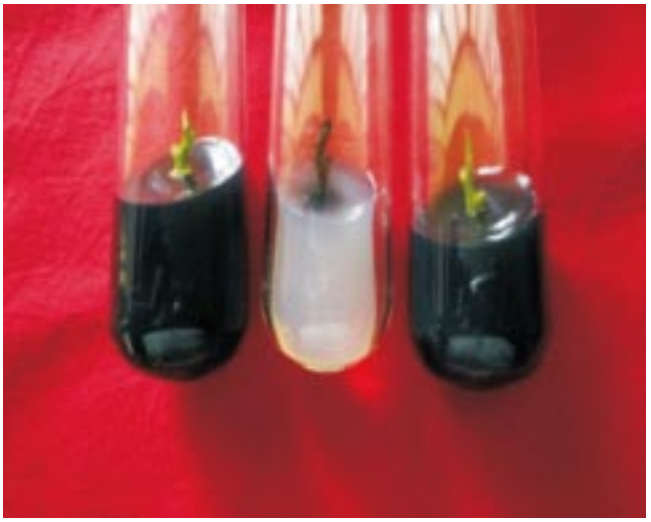

Figure 1. Comparison of phenolic exudation.

Figure 2. Shoot initiation.

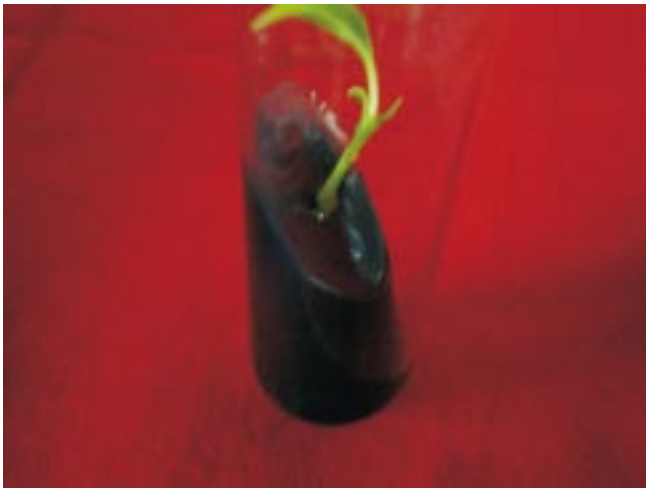

Figure 3. Shoot elongation.

Figure 4. Rooting.

Figure 5. Up rooted micro shoots.

Figure 6. Hardening.

absence of PGRs medium when compared to the medium with presences of PGRs, whereas after 11 week the culture was comparatively higher in to the combinations of IAA along with zeatin and IAA along with BAP. The number of shoots produced after 11 week using 1.34 μmol IAA in combination with 3.9 μmol BAP was significantly higher than that obtained on the media containing kinetin, 2ip and zeatin.

The MMS basal medium supplemented with combinations of BAP (3.9 μmol) and IAA (1.34 μmol) promoted the maximum axillary shoot production than other combination of growth regulator (Table 3). Among the auxins tests the IAA combination with BAP was the significant effective in terms of number of axillary shoots produced and the shoot length was also did not significantly higher than the other combinations of kinetin and zeatin (Table 4). The shoot length in the medium supplemented with 2ip (3.9 μmol) and IAA (1.34 μmol) was significantly higher (14 mm) than

Figure 7. Potting.

Figure 8. Field acclimatization.

those exposed by the other cytokinins. Although, medium supplemented with slightly higher IAA, shoot length was not significantly different from that produced with other auxins. Hence, in the present investigation on *E. agallocha* revealed that IAA at 1.34 μmol in combination with BAP at 3.9 μmol induces a higher rate of shoot induction.

Isolated *in vitro* raised shootlets were excised and subjected to different rooting treatments containing MMS root expression medium augmented with NAA, IBA and NAA combinations (Table 4). Adventitious roots were induced directly from the shoot base without intervening callus phase in all media. However, the significant frequency (86% ±0.9%) of root formation, number of roots with length of (3.8 ±0.51) cm was observed on MMS medium supplemented with 5.02 μmol of IAA (Table 4). Rooting frequency was increased gradually and reached maximum percentage after 11 weeks of root culture.

The *in vitro* raised plants of *E. agallocha* were removed and washed with sterile distilled water subjected to hardening on sterile vermiculate and sand (3:1) containing adequate moisture, the rooted micro-shoots were found to acclimatize (Figure 6). A polythene covering was effective in maintain the relative humidity, and is necessary for hardening the tissue culture raised plantlets. Hardened plantlets were transferred to pots containing soil, garden yard manure and red earth at 1:1:1 ratio under the green house (Figure 7). These acclimatized plantlets were introduced into mangrove forest (Figure 8).

4. Discussion

The uses of activated charcoal was oxidized the phenolic compounds in the culture medium which might released by both explants[9], also obtained in ascorbic acid[8]. Antioxidants protect explants by decreasing the redox potential of phenols in the culture medium and this was achieved by reverting quinines that were formed by oxidation of phenolic compounds produced in damaged tissue or by competing with free radicals and removing them from the reaction[10]. With activated charcoal, hydrogen bonds absorb polyphenols, reducing their synthesis and thereby preventing the browning of explants. Synergistic effect of cytokinin and auxins in axillary shoot inductions had been also reported in Wattakaka volubilis[11], in rootstocks selections of *Cymbidium*[12], and in *Avicennia marina1*. The same result was supported by the Faisal *et al*[13]. The result was also similar that of Naseem and Mohamed[14]. multiplications and the result was supported

by Faisal *et al* with 1.34 μmol IAA and 3.55 μmol BAP[13]. The auxins minimal concentration will break the bud dormancy and enhance the axillary bud induction[13].

Thus, research on adventitious root formation is highly important from the practical point of view. Hence, adventitious root production in isolated micro cuttings of *E. agallocha* was achieved in the presence of various auxins (IAA, IBA and NAA) in MMS medium. Exogenous auxins are often used in a number of plant species to promote in vitro rooting of *in vitro* produced microshoots[15]. Overall, Presence of auxins in medium exhibited better rhizogenesis. There are other reports of IAA being effective in stimulation adventitious such as Quraishi and Mishra[16,17]. The equal molar concentrations IAA and IBA was induced maximum rooting response and the similar results were reported in Garcinia indica and Lessertia fructescens[18,19].

In summary from the study clearly describes an affordable and reproducible method for the production of medicinally important and vulnerable mangrove. In other words, the work denotes the successful and rapid production of *E. agallocha* species. Prolonging the culture vigor and avoiding somaclonal variation through autotrophic micropropagation should be considered, at least woody species, as they often need prolonged and complex culture maintenance conditions.

Conflict of interest statement

We declare that we have no conflict of interest.

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