

A MICROPROPAGATION PROTOCOL FOR A CRITICALLY ENDANGERED MANGROVE EXCOECARIA AGALLOCHA L

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Abstract

Excoecaria agallocha L. is a critically endangered mangrove tree from the Pichavaram mangrove reserve forest, the Tamil Nadu Coastal area. It is distributed on the seashore and the edge-mangrove. In order to reduce the decrease in number of these Mediterranean mixed stand, unsupervised forest management practices have drastically been reduced. In addition, the deforestation of the mangrove area, along with a low seed germination rate further endanger this species. In this study we developed a protocol for the micropropagation of adult *Excoecaria agallocha*. Microcuttings were obtained from lateral and apical twigs of mature plants and used as explants. Microcuttings with axillary buds were grown on different media, plant growth regulators and phenolic exudation substances. The axillary shoots produced on uncontaminated explants were excised, segmented and recultured in the same medium, to increase the stock of shoot cultures. The Modified Murashige and Skoog (MMS) medium, augmented with different concentrations of *N*⁶- benzyl adenine (BAP) and Naphthalene acetic acid (NAA), either alone, or in combinations, as a potential medium for shoot multiplication by nodal segments, was tested. In the following experiment, equal molar concentrations of four cytokonins [BAP, Kinetin and 2- isopentenyladenine (2iP)] in combination with equal molar concentrations of three auxins [NAA, Indole acetic acid (IAA) and indole-3- butyric] were used to test the rate of axillary shoot proliferation, induced on MMS agar medium supplemented with 3.9 μM BAP and 1.34 μM NAA after 6 weeks in culture. Different auxins (NAA, IBA and IAA) were to determine the optimum conditions for in vitro rooting of microshoots. The best results were accomplished with NAA 5.41 μM (89% rooting) and with IBA at 2.85 or 5.71 μM (86% and 86.5% rooting, respectively).

Keywords: Conservation; Mangrove; Microshoots; Micropropagation; Rooting;
Critically endangered species

Introduction

Mangroves are halophytic woody plants that serve as protection against cyclone, Tsunami and are a source of energy for coastal inhabitants. It is also used for drugs, dyes, tannins and also for medicine. Mangroves are derived from eighty families of trees and shrubs growing on the 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 [2]. The mangrove habitat has been under severe destruction worldwide at alarming levels [3]. Such levels of destruction and habitat fragmentation raise concerns about the conservation of mangrove diversity. To augment conservation, management efforts to germinate and especially for unique genotypes, has to be made [1].

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Excoecaria agallocha L. (Euphorbiaceae) a mangrove tree, also called milky mangrove, highly tolerant to adverse environmental conditions [4]. *E. agallocha* is used for the treatment of ulcer and as an aphrodisiac. The extract of this plants is also used for rheumatism, paralysis, cutaneous infections [5]. *E. agallocha* is used as a purgative and in the treatment of epilepsy, dermatitis, haematuria, leprosy, toothaches. *E. agallocha*, has active compounds, such as excoecariotoxins, fluratoxin, glycerides of fatty acid, lipids and waxes, phorbol, esters, polyhenols, polysaccharides, saponins, steroids, tannins, triterpenes [6]. The urgency to conserve *E. agallocha* is mainly due to the unsupervised cutting of trees, for fuel and various actions done by people. Moreover, low germination and seed production [7, 8] raise concerns, as most of mangroves have seed germination problems, caused by embryo dormancy and seed coat impermeability to water. It has been suggested that the germination paucity might be related to climate changes, because seed viability decreases due to water deficit stress [9]. Taken together, the insufficient seed production and low germination have been a negative influence on the reproductive yield and consequently on population value of this pharmacologically important species [6].

Germination experiments with seeds of *E. agallocha* subjected to different scarification treatments were unsuccessful, due to low seed viability [9]. Therefore, micropropagation may be an alternative means to conserve this unique species. The main aim of this study was to develop a micropropagation protocol for *Excoecaria agallocha*. Various plant culture mediums, plant growth regulators (PGRs) and anti-browning agents were tried. Bud samples (axillary and apical) were collected from adult plants for the whole study. We focused on achieving the most satisfactory and suitable off-spring, through micropropagation.

Materials and Methods

Shoot tips and nodes were excised from the growing tips of adult trees of *E. agallocha* from the Pichavaram mangrove Reserve forest, Tamil nadu and used as explants. Explants were transported to the laboratory at 4°C. Shoot tips and nodal segments (not longer than 1 cm) were excised from the young shoots. The explants were washed under tap water for 5 min followed by a 10 percent Tween 20 (Liquid detergent; Himedia, India) bath for 5 min, then the surface was sterilized with 70% (v/v) ethanol for 2 min and rinsed 3 times with sterile distilled water and then treated with one percent of Methyl N-(1H-benzimidazol-2-yl) carbamate. Then the explants were treated with 20% sodium tungstate and sodium carbonate solution to remove phenolic compounds from the explants [10] and washed with 0.1 HgCl₂ for 2 min and then washed thrice with sterile water. Those explants were placed in different mediums along with various concentrations of activated charcoal, Citric acid and Ascorbic acid, to exudate.

Shoot bud development from explant and culture conditions

Decontaminated 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 on *E. agallocha* explants viz., Murashige and Skoog medium [11], Modified Murashige and Skoog medium [12] and Woody Plant Medium [13] with various concentrations of BAP and NAA, to estimate the suitable culture medium for *E. agallocha*. Out of the three mediums, the MMS medium was found to induce the maximum growth in *E. agallocha*. Hence, this medium was used throughout the experiment. The medium consisted of MgSO₄ (185 mg/l), KNO₃, KHPO₄ (85mg/l) and CaCl₂ (440 mg/l), with the omission of NH₄NO₃, and supplemented with 30 g/l of sucrose, 8g/l agar and the pH was adjusted to 5.8. Treatments consisted of seven replications. One shoot was inoculated in a 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°C day/night temperature, respectively [14]. The number of explants that developed lateral shoots and the length of the shoots and their physiological state after 5 weeks were recorded.

MMS mediums with 1.4, 3.9, 4.8, 5.4 and 7.4 μM of BAP and 1.34 μM of NAA were used for this study.

In the second experiment, the optimal concentration of BAP, NAA and IAA was estimated by using various concentrations of those growth regulating chemicals. Stem segments were vertically placed in each test tube for all multiplication treatments. Nondestructive observation were performed after 4 and 6 weeks of culture. Each experiment was repeated three times and the number of shoots the length of the explant were observed.

Rooting treatments and plant green house establishment

Fifty 4 week old microshoots were placed for elongation in a half-strength MMS medium for root induction with 0.5 to 2.0 mg/l of IBA, NAA and IAA and cultured for 2 week. The culture condition were 19°C with 16 photoperiod [15] following the root induction treatment. The shoots were transferred to root expression medium consisting of Modified Murashige and Skoog (Half Strength macronutrient) in PGRs free medium. The *in vitro* rooting experiment (Fig. 1) was set up in a complete randomized block design with three blocks with 5 replicated number of shoots rooted, number of roots per shoot and mean root length. The data was subjected to analysis of variance (ANOVA) and the least-significant- difference was ($P \leq 0.01$) level.

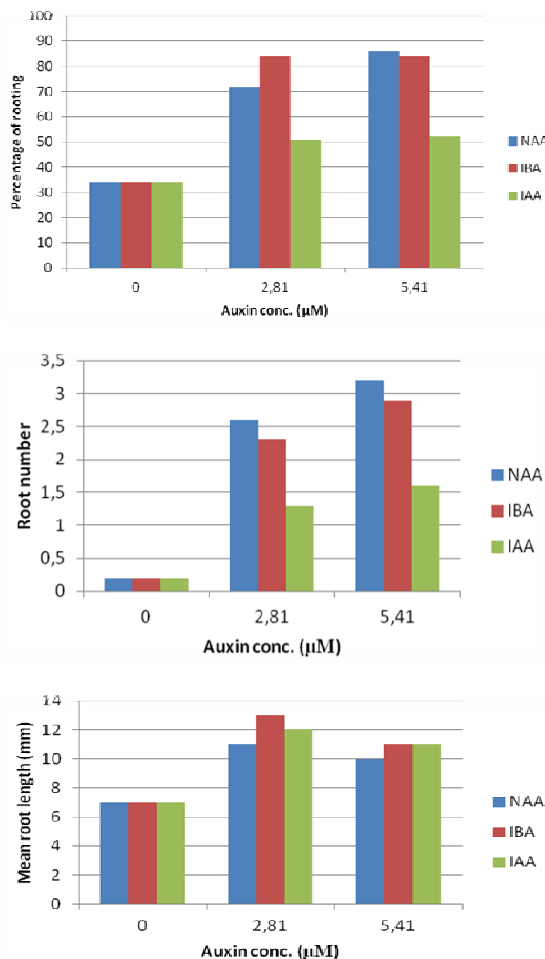


Fig. 1. The effect of different auxins on *in vitro* rooting of *Excoecaria agallocha* L. axillary shoots after a 4-wk culture. Mean separation was performed by Fisher's protected least significant difference ($P \leq 0.01$). Columns with different letters are significantly different from each other.

The in vitro rooted plantlets were removed from culture media and the agar was washed from roots, then the plants were transplanted into small plastic pots containing a peat-perlite mixture 1:1 (w/v) covered with transposable polythene bags to maintain a high relative humidity. The potted plantlets were kept in greenhouses (24-26°C) during the day and 18-20°C during the night. Ventilation of the plantlet was increased after 7 days by increasing the size of the holes made in the polythene cover. After 3 weeks the polythene cover was removed. After 5 weeks of acclimation individual plants were transferred to a plastic pot (diameter - 9 cm) containing the same growing mixture and then transferred to the shade net of 70% shade. Air temperature was 25-28°C in the shade net during the day and 18-22°C during the night.

Results and Discussions

Tissue browning is a constant drawback that renders tissue culture work difficult for this species. The presence of phenolic compounds and the high polyphenol 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 (30d). Explants maintained in a medium without antioxidants or adsorbent compounds released brown-black exudates into the medium. Except for where activated charcoal was present, the browning percentage was inversely related to the tested compounds concentration of ascorbic acid and citric acid (Fig. 2). Among the anti-browning agents, activated charcoal at 4 g/l, citric acid at 100 m/l and ascorbic acid at 100 mg/l had a significantly higher percentage of anti-browning of nodal explants than the shoot tip explants after 30 days. Adding of activated charcoal 4 g/l caused significantly lesser browning of both types of explants. The use of activated charcoal was to oxidize the phenolic compounds in the culture medium that might be released by both explants [16, 17], as also obtained in ascorbic acid [18]. Antioxidants protect explants by decreasing the redox potential of phenols in the culture medium and that was achieved by reverting the quinines that were formed by the oxidation of the phenolic compounds produced in damaged tissue, or by competing with free radicals and removing them from the reaction [19]. With activated charcoal, hydrogen bonds absorb polyphenols, reducing their synthesis and thereby preventing the browning of explants.

In the present study shoot induction was carried out from nodes and shoot tips of explants in various concentrations of BAP and NAA combinations. Among the various concentrations, 3.9 μ M of BAP and 1.34 μ M of NAA received a better response than other combinations (Table 1) and they promoted a higher number of shoot inductions per explants (3.8) (Fig. 3).

Table 1. Shoot multiplication response of *Excoecaria agallocha* L to MMS mediums with various combinations of NAA and BAP

Growth regulators (μ M)		Shoots per explant (number)		Shoot length(mm)
NAA	BAP	Weeks in Culture		
		4	5	5
0	0	1.2e	1.4e	8.0abc
0	1.4	1.7bc	1.9bc	5.0d
0	3.9	1.9	2.0b	5.0d
0	4.8	1.7ab	1.8bcd	8.0abc
0	5.4	1.7bc	1.7bcde	6.0cd
0	7.4	1.5cd	1.5de	9.0ab
1.34	0	1.4de	1.6cde	6.0cd
1.34	1.4	1.7bc	1.8bcd	5.0d
1.34	3.9	2.2a	3.8a	11.0a
1.34	4.8	1.3de	1.7bcd	6.0cd
1.34	5.4	1.4de	1.5de	7.0bcd
1.34	7.4	1.7bc	1.7bcde	9.0ab

In each column, average values followed by the same letters are not significantly different than the $P < 0.01$ level indicated by Fisher's protected least significant different test



Fig. 2. Axillary shoot development



Fig. 3. Shoot elongation

A synergistic effect of cytokinin and auxins in axillary shoot inductions was also reported in *Rosa roxburghii* [20], in rootstocks selections of *Pirus communis* and *Pirus betulfolia* [21], in *Eucalyptus grandis* [22], and in *Mytus communis* [23]. The best result, in terms of shoot length, was also obtained from the medium augmented with 1.34 μ M NAA and 3.9 μ M BAP, (Fig. 4), although the effectiveness of thts 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 μ M) or combination with NAA (0.12 NAA and 3.9 μ M BAP) (Table 1). The combination of 1.34 μ M NAA and 3.9 μ M BAP produced favorable results in terms of numbers and length of axillary shoots. Equal molar concentrations of cytokinins (kinetin, 2ip and zeatin) and auxins (IAA and IBA) were used for the multiplication of shoots (Table 2). After 4 weeks of culture, axillary shoot formation was inferior where PGRs were absent from the medium, compared to the medium with PGRs, whereas after 11 weeks 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 weeks using 1.34 μ M IAA in combination with 3.9 μ M BAP was significantly higher than that obtained in the media containing kinetin, 2ip and zeatin.

Table 2. Analysis of variations for shoot multiplication in MMS with various combination of IAA and 2iP

Source	F value		
	Shoots per explant (number)	Shoot length (mm)	
	Weeks in culture		
	4	6	6
BAP conc.	113.40*	38.10*	47.74*
NAA conc.	0.10 ^{ns}	16.36*	3.10 ^{ns}
BAP conc. \times NAA conc.	26.60*	9.86*	34.01*

^{ns} Non significant * Significant at $P \leq 0.01$

The MMS basal medium supplemented with combinations of BAP (3.9 μ M) and IAA (1.34 μ M) promoted the maximum axillary shoot production (Table 2). The same result was reported by the Rao *et al.* [24]. Among the auxin tests, the IAA combination with BAP was significantly more effective in terms of number of axillary shoots produced and shoot length was also higher than that of other combinations of kinetin and zeatin (Table 4). The result was also similar to that of Ahmed and Mohamed, [25]. The shoot length in the medium supplemented with 2ip (3.9 μ M) and IAA (1.34 μ M) was significantly higher (14 mm) than that obtained with other cytokinins. Nevertheless, in the medium supplemented with slightly higher IAA, shoot length was not significantly different from that produced with other auxins. Hence, the present investigation on *E. agallocha* revealed that IAA at 1.34 μ M, in combination with BAP at 3.9 μ M induced a higher rate of shoot induction, multiplications and the same result was reported by Rao *et al.* [24] with 1.34 μ M IAA and 3.55 μ M BAP.

***In vitro* rooting**

Isolated *in vitro* raised shoot-lets were excised and subjected to different rooting treatments containing an MMS root expression medium, augmented with NAA, IBA and NAA combinations (Table 4). Adventitious roots were induced directly from the shoot base without any callus phase in all the media. Thus, the research on adventitious root formation is highly important from a practical point of view. Adventitious root production in isolated micro cuttings of *E. agallocha* was achieved in the presence of various auxins (IAA, IBA and NAA) in MMS mediums. Exogenous auxins are often used with a number of plant species to promote *in vitro* rooting of *in vitro* produced microshoots [26]. Overall, the presence of auxins 1.5 mg/l and 2.0 mg/l in the medium exhibited better rhizogenesis. However, a significant frequency ($86 \pm 0.9\%$) of root formation, number (5.9 ± 0.61) of roots with proper length (3.8 ± 0.51) was observed in MMS medium supplemented with $5.02 \mu\text{M}$ of IAA (Table 4; Fig. 5). Rooting frequency was increased gradually and it reached a maximum percentage after 11 weeks of root culture. There are other reports of IAA being effective in stimulating adventitiousness, such as that of Fogaca and Fett- Neto [27], Zhang et al. [28]. Figure 6 reveals that equal molar concentrations IAA and IBA induced maximum rooting response and similar results were reported for *Garcinia indica* [29] and *Rauvolfia tetraphylla* [30]. *In vitro* well rooted plants were uprooted from the test tubes, transferred for hardening to green houses covered with holed polythene covers (Fig. 7), then transferred to pots (Fig. 8) and finally, the plants were transferred to the Picharavaram mangrove forest. We recorded a 86% rate of survival.



Fig. 4. Plantlet with *in vitro* regenerated root system.



Fig. 5. Root multiplied plantlet



Fig. 6. Hardened plant



Fig. 7. Hardened plant



Fig. 8. *Excoecaria agallocha* introduced in a mangrove field.

Conclusion

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

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Abbreviations

- NAA - Naphthalene acetic acid
- MS - Murashige and Skoog Medium (1962)
- MMS - Modified Murashige and Skoog medium (2008)
- Bap - 6- Benzylamino purine
- KIN - Kinetin
- WPM - Woody Plant Medium (1981)
- PGR - Plant Growth Regulators

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