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Micropropagation for the conservation of *Rhizophora mucronata* Lam.

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ABSTRACT

The deterioration of mangrove forests has caused ecological imbalance. So, it is needed to restore and safeguard the ecological prosperity. The present study describes a reproductive protocol for the revival of such mangroves to maintain ecological wealth. *Rhizophora mucronata* Lam. commonly called "loop root mangrove" support to restore mangrove habitats. MS medium amended with different phytohormones including Indole-3-acetic acid (IAA), Benzyl adenine (BA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) either alone or in combinations were used to study schizogenesis, rhizogenesis and synthetic seed production. Seeds of *Rhizophora mucronata* cultured on MS medium fortified with BA along with NaCl and activated charcoal (AC) are found to be effective for shoot and root multiplication. Combination of BA, 2,4-D, AC and NaCl is responsible for callogenesis. Synseeds were produced by encapsulating *in vitro* buds in calcium alginate beads. *In vitro* raised plantlets were rooted in IAA and hardened with Hoagland's solution. Hardened plantlets were transferred to pots with sterile sand, 10 g NaCl and farmyard manure.

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1. Introduction

Mangrove ecosystems afford important wetland communities of plants and animals (Nagelkerken *et al*., 2008). It consists of unique species of trees and shrubs along coastal, estuarine and riverine areas in tropical and subtropical latitudes (Twilley *et al*., 1996). They assist coastal stabilization and dispense nursery areas for economically important fishes and crustaceans in the tropics and sub-tropics (Tomlinson 1986). They secure shorelines by slowing erosion and provide natural barriers, protecting coastal communities from increased storm surge, flooding and hurricanes. The mangroves played a vital role in coastal protection against cyclones, typhoons and tidal waves (Sandilyan and Kathiresan, 2015). The mangroves help the socio-economic development of the coastal communities by supplying 'seeds' for agriculture industries as well as providing the traditional source of medicines, honey, firewood, fodder and timber. Mangrove forests are extremely productive ecosystem providing critical services that benefit all of us. The nations that receive the prominent economic benefits from mangroves include the USA, China, India and Mexico (Menendez *et al*.,2020). Mangrove plants have great potential to adapt climatic changes, incidence of solar ultraviolet-B-radiations (Rahman, 1990; Moorthy and Kathireshan, 1996), and the rise in sea level. Mangroves are endangered by hostile habitat and human abuse. The fast destruction and degradation of the mangroves causes coastal erosion and fall in fisheries management, so its conservation is to be needed. *In vitro* propagation method can be adopted for the conservation of mangrove ecosystem. An attempt was made to study the regeneration and growth performance in two species, *Rhizophora mucronata* and *R. apiculata* (Kulkarni and Bhosale, 1991).

Rhizophora mucronata Lam. belongs to the family Rhizophoraceae which is inhabited in estuaries, tidal creeks and flat coastal areas (Schwarzbach and Ricklefs, 2000). The plant needed low salinity for their optimum growth (Patel *et al*., 2010). Natural propagation of the species is retarded due to low survival rate and crab attack of its seedlings (Duke, 1983). *Rhizophora mucronata* has long been traditionally used for the treatment of elephantiasis, haematoma, hepatitis, ulcers and febrifuge (Bandaranayake 2002; Ravikumar *et al*., 2005). In ethnomedicine, *Rhizophora mucronata* bark is also mentioned for its anti-diarrheal properties (Harrison, 1977). Ethanolic extract of leaves shows anti-inflammatory and free radical scavenging activity (Ray *et al*., 2016). Quinizarin derived from methanolic extracts of *Rhizophora mucronata* significantly showed antimicrobial, antioxidant and cytotoxic activity (Sachithanandam *et al*., 2022). Biosynthesized silver nanoparticle from the species had probable antibacterial activity (Abdi *et al*., 2019). The *Rhizophora mucronata* leaves polysaccharide showed antibiofilm activity against the gram positive and gram-negative pathogens (Jha *et al*., 2022). Ningsih *et al*., (2020) reported that L-glutamic acid was the utmost amino acid present in the leaves of *Rhizophora mucronata*. The plant is well nourished with the area having annual day time temperature 20-28 ^{0}c and annual rainfall in the range of 1000-3000 mm. They need sunny position for finest growth (De Deurwaerder, 2012).

The plant is in least concern category of IUCN redlist (Alamsjah and Fauzullmron, 2021). Anthropogenic activities viz. land reclamation, pollution, construction of buildings lead the species to the brim of extinction. Owing to its medicinal, ecological and economic importance, conservation of *Rhizophora mucronata* is necessary. *In vitro* propagation through rapid multiplication of *Rhizophora mucronata* is one of the solutions to overcome such adverse effect. *In vitro* propagation is an alternative mean of propagation that can be engaged in mass production of plantlets in a relatively shorter period which provide the plant material capable of producing secondary metabolites. The present study aims to standardise *in vitro* propagation protocol with eight replicates and repeated thrice for the species, *Rhizophora mucronata* through shoot multiplication, organogenesis and synthetic seed production.

2. Materials and Methods

Rhizophora mucronata Lam. Collected from Ayiramthengu, of Kollam district was established in the green house of F.M.N. College, Kollam as a source of explant. The plant was grown for 6 months in the green house and healthy explants were taken from the established mother plant. Farmyard manure along with 10g salt/ pot was added for the growth of mother plant to provide the natural environment.

Healthy and disease-free seed, shoot tips and nodal segments with eight sets were selected as explants irrespective of season by direct observation. The explants were rigorously washed in running tap water for 10-15 minutes and treated with 10% labolene for 20 minutes in a conical flask. The conical flask bearing explant were kept under running tap water for 40 minutes. The explants were treated with 0.1% mercuric chloride solution inside the laminar air flow cabinet. Then it was washed with double distilled autoclaved water for 3 minutes. The surface sterilized shoot tip, nodal segments and seeds were used for inoculation. The explants were inoculated into MS medium (Murashige and Skoog, 1962) with different phytohormones such as Indole-3-acetic acid (IAA) Benzyl adenine (BA) and 2, 4-Dichlorophenoxy acetic acid (2, 4-D) along with NaCl and activated charcoal (AC) to absorb exudates from explants and to provide natural environment. Observations were taken after 30 days of inoculation and the data was statistically analysed with the standard error of the mean.

Rooted plantlets were transferred to autoclaved vermiculite in plastic cups supplemented with Hoagland's solution (Epstein, 1972) for nourishment and were kept at 25±1^{0}C with a light intensity of 3000 lux at 12/12 hours photoperiod. Plantlets with newly sprouted leaves were transplanted to pots containing10g NaCl, autoclaved sand and farmyard manure and were watered regularly.

In vitro derived shoot buds were washed in sterile distilled water and were transferred to 2.5% w/v solution of $CaCl_2$ after dipping it in sodium alginate for bead formation. The solution was decanted and collected beads were repeatedly washed in sterile double distilled water. Synthetic seeds were stored in sealed sterile petri plates. Series of 1-5 % sodium alginate were used for studying its germination frequency (Ipekci and Gozukirmizi, 2003)

3. Results and Discussion

Tissue culture technique is used to develop enormous genetically identical plants from a single mother plant by a process called micropropagation. The method provides an advantage over other as it can be used to evolve disease free plants by using mother plant meristems (apical and axillary) as explants.

The present study describes a reproducible protocol for rapid clonal propagation of *Rhizophora mucronata*. The cytokinin BA is found to be best for the shoot proliferation as well as rooting of *Rhizophora mucronata.* Lower concentration of BA (2-3 mg/l), 500 mg/l of AC and 500 mg/l NaCl were found to be most suitable growth regulator combination for shoots and seeds. Manjula *et al*., (1999) reported a low level of BA in *in vitro* propagation of *Ocimum basillicum* through axillary shoot proliferation. Seeds cultured on MS medium containing both BA and IAA showed meagre response. Seeds of *Rhizophora mucronata* cultured on MS medium fortified with 3 mg/l BA along with 500 mg/l AC and 500 mg/l NaCl showed good response (table1) for rhizogenesis (Fig.1C). Benson *et al*., (2020) also observed similar type of rhizogenesis in *Sonneratia alba.* After rooting, plantlets were transferred to vermiculate or to autoclaved soil and nourished with Hoagland's solution (Fig. 1E). The plantlets survived well in the pot containing autoclaved soil along with 10 g of NaCl. The hardened plantlets were transferred to marshy lands of Kollam (Fig. 1F).

Presence of auxin along with cytokinin stimulate callus induction. Among them 2,4-D enhances callusing in *Rhizophora mucronata.* With the increasing concentration of 2,4-D intensity of callus also increased (Fig.1D). The MS medium augmented with NaCl, AC and 2,4-D, in combination with BA also induced callus formation. Shoot tip cultured on 2 mg/l BA, 2 mg/l 2, 4- D, 500 mg/l AC and 500 mg/l NaCl produced white friable callus. Nodal segments cultured on MS medium amended with 1 mg/l 2, 4-D, 500 mg/l AC and 500 mg/l NaCl produced milky white callus. In *Rhizophora mucronata* exudation was very high and it imparts brownish colour to the medium. This exudation was checked by adding AC in the medium. A gradual increase in the rate of germination of seeds and decrease in exudation were noticed from lower concentration to higher concentration of AC. Ogita *et al*., (2004) reported such a decrease in exudations in higher concentrations of AC in *Kandelia candel*. Synseeds were produced by encapsulation of *in vitro* buds in calcium alginate beads and is germinated in agar gelled MS basal medium. The synseeds showed low germination frequency on low concentration of sodium alginate whereas a high germination frequency (86.7%) was observed on 4% sodium alginate. Ipekci and Gozukirmizi (2003) also observed maximum germination frequency in *Paulownia elongata* when the concentration of sodium alginate is elevated. Synseeds retained thier viability for 3 months when stored at 4^{0}C.

Conventional propagation methods of mangroves are hindered by its poor seed viability, insect attack as well as poor survival rate of the seedlings. *In vitro* propagation method is one of the best methods adopted to overcome such challenges for the conservation. The restoration and conservation of such mangrove ecosystems is an asset which support humans and animals in the present and future. Thus, the present investigation established an efficient protocol for the rapid clonal propagation of the species thereby, enable secondary metabolite enhancements which provide the basis of active drug ingredients in medicine.

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Table 1. Effect of different phytohormones on shoot multiplication, organogenesis & rhizogenesis.

Hormonal concentration (mg/l)			Activated Charcoal (mg/l)	NaCl (mg/l)	Explant	No.ofShoots (Mean±SE)	No.of Roots (Mean±SE)	Intensity of Callus	Colour of Callus
2,4-D	**BA**	**IAA**							
0.5	-	-	500	500	Node	-	-	+	Milky white
1	-	-	500	500	Node	-	-	+++	Milky white
2	2		500	500	Shoot tip	-	-	++	White friable
-	-	-	1000	-	Shoot tip	1.14±0.33	-	-	-
-	-	-	250	-	Seeds	0.71±0.17	-	-	-
-	-	-	500	-	Seeds	1.4±0.28	-	-	-
-	-	-	750	-	Seeds	1±0.30	-	-	-
-	-	-	1000	-	Seeds	2.28±0.27	-	-	-
-	-	-	-	250	Seeds	0	-	-	-
-	-	-	-	500	Seeds	1.28±0.27	-	-	-
-	-	-	-	750	Seeds	0	-	-	-
-	-	-	-	1000	Seeds	0	-	-	-
-	-	-	250	250	Seeds	1.28±0.18	-	-	-
-	-	-	500	500	Seeds	3±0.30	-	-	-
-	-	-	750	250	Seeds	1±0.26	-	-	-
-	-	-	1000	500	Seeds	1.71±0.17	-	-	-
-	1	-	-	-	Seeds	0	-	-	-
-	2	-	-	-	Seeds	0	-	-	-
-	3	-	-	-	Seeds	0.57±0.20	-	-	-
-	4	-	-	-	Seeds	1.4±0.29	-	-	-
-	1	-	-	-	Shoot tip	0	-	-	-
-	2	-	-	-	Shoot tip	0.71±0.18	-	-	-
-	3	-	-	-	Shoot tip	0	-	-	-
-	4	-	-	-	Shoot tip	0	-	-	-
-	1	1	-	-	Seeds	0	-	-	-
-	2	2	-	-	Seeds	0	-	-	-
-	3	3	-	-	Seeds	0.71±0.18	-	-	-
-	4	4	-	-	Seeds	0	-	-	-
-	5	5	-	-	Seeds	0	-	-	-
-	2	-	1000	-	Seeds	0	-	-	-
-	3	-	1000	-	Seeds	2±0.43	-	-	-
-	4	-	1000	-	Seeds	0.85±0.33	-	-	-
-	5	-	1000	-	Seeds	0.71±0.18	-	-	-
-	2	-	1000	-	Shoot tip	1.71±0.18	-	-	-
-	3	-	1000	-	Shoot tip	0	-	-	-
-	4	-	1000	-	Shoot tip	0	-	-	-
-	5	-	1000	-	Shoot tip	0	-	-	-
-	1	-	500	500	Shoot tip	-	-	-	-
-	2	-	500	500	Shoot tip	-	-	-	-
-	3	-	500	500	Shoot tip	0.71±0.18	-	-	-
-	4	-	500	500	Shoot tip	0.42±0.20	-	-	-
-	1	-	500	500	Seeds	-	-	-	-
-	2	-	500	500	Seeds	-	-	-	-
-	3	-	500	500	Seeds	0.85±0.14	3.42±0.29	-	-
-	4	-	500	500	Seeds	0.57±0.20	1.2±0.20	-	-
-	5	-	500	500	Seeds	0.71±0.18	0.85±0.14	-	-

+, ++, +++ indicate intensity of callusing

Table 2. Germination frequencies of encapsulated synthetic seeds of *Rhizophora mucronata* on 2.5% Calcium chloride and varying concentration of Sodium alginate on MS medium fortified with BA, NaCl and AC.

No. of encapsulated seeds	Concentration of sodium alginate	% of Germination (Mean±SE)
50	1%	26.7±0.14
50	2%	40±0.08
50	3%	53.4±0.04
50	4%	86.7±0.06
50	5%	72.4±0.24

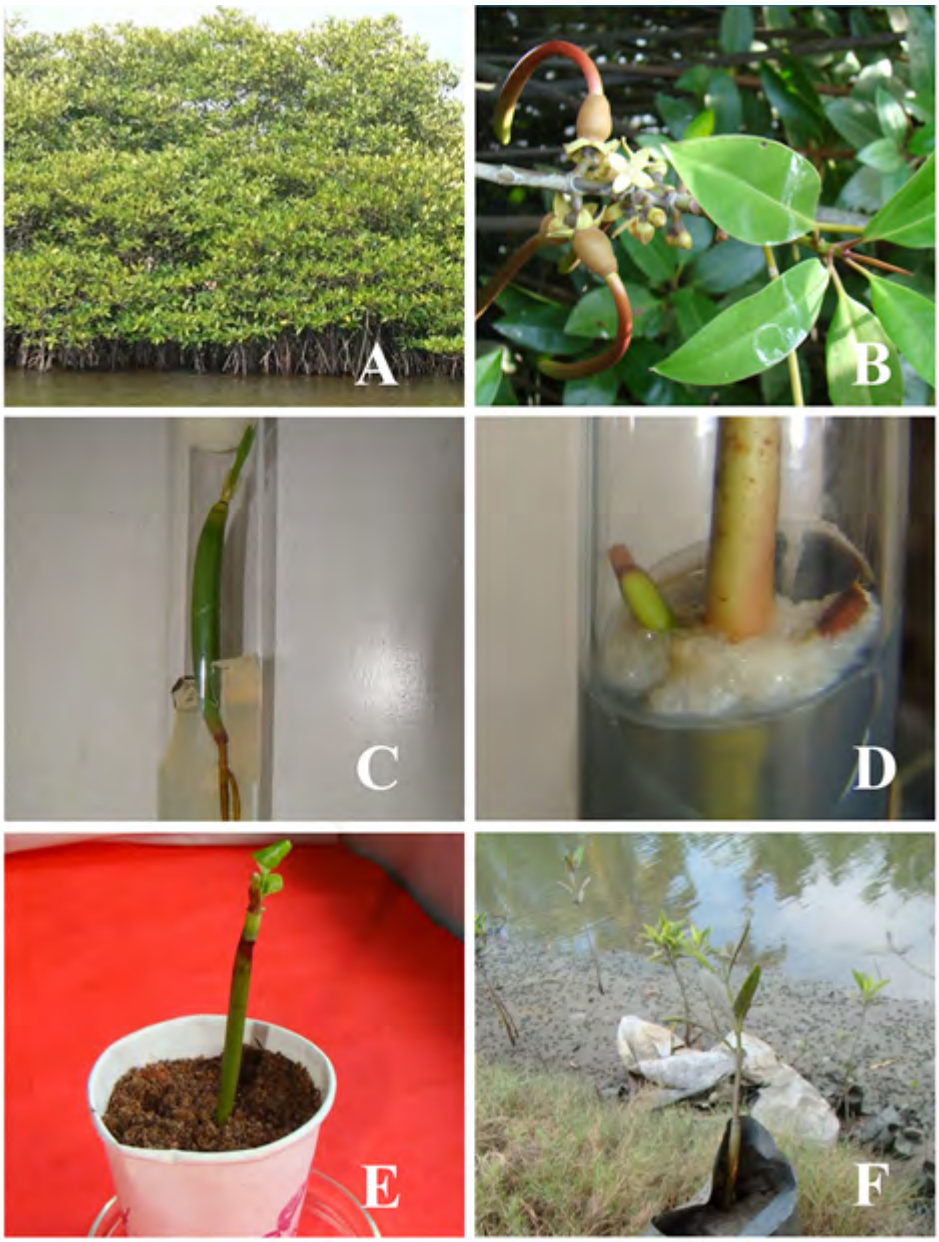

Fig. 1. (A-B) Habitat of *Rhizophora mucronata*, (C) shooting and rooting, (D) white friable callus, (E) Hardened *in vitro* cultured plantlet of Rhizophora mucronata, (F) establishment of hardened plantlet on marshy places

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