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In vitro Shoot Multiplication in *Bruguera cylindrica* W & A.

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Abstract

Bruguiera cylindrica W & A. (Rhizophoraceae) is an important rare mangrove plant. This plant has various medicinal properties and environmental importance. They are very important in protecting the coasts from cyclones. This mangrove plant needs to be conserved so that the future generation may benefit by this plant. The main aim of the study is the mass production of the important rare mangrove plant, *Bruguiera cylindrica* through micropropagation methods because the survival rate of seedlings is naturally very low.The present work suggests protocols for the large-scale multiplication through shoot multiplication. The explants were cultured on MS (Murashige and Skoog) medium fortified in different concentrations of benzyl adenine (BA) and supplemented with different combinations of hormones for shoot multiplication. Different concentrations of benzyl adenine (BA) and combination of BA and Naphthalene acetic acid (NAA), Kinetin, activated charcoal and sodium chloride. Encapsulation in the form of synthetic seeds is also done as part of the study.

Keywords: Micropropagation, *Bruguiera cylindrica*, Activated charcoal, Encapsulation

1. Introduction

Bruguiera cylindrica W&A is an important mangrove plant belonging to the family Rhizophoraceae. It grows in mangrove swamps in south east Asia. It is a small tree growing up to 20 meters tall but often grows as a bush. It is found in south east asia with the range extending from India and SriLanka through Malaysia, the Philippines, Indonesia and Papua New Guinea to Australia. In traditional medicine the skin of the fruit is used to stop bleeding and the leaves are used to lower blood pressure. The timber of *Bruguiera cylindrica* is dense, reddish and strong and is used in constructions. Extracts are made from the pneumatophores which are used in the manufacture of perfume. The parts of the tree are eaten. Two extracts of *Bruguiera cylindrica* and *Exocaria agallocha* showed significant anti-TMV activity. Tannins which were commercially important plant products have been studied for their seasonal changes in 14 mangroves species and tannins ranges from 2.41 to 21.42 mg/g dry weight (Kathiresan *et al*., 1990). They are very important in protecting the coasts from cyclones. The mangrove plant needs to be conserved so that the future generation may benefit by this plant.

Mangrove ecosystem is ideally situated at the inter phase between terrestrial and marine environment and protect against coastal disasters (Das *et al*., 2009). The *in vitro* propagation of *Bruguiera cylindrica* will compliment and strengthen the large-scale plantation activities towards the conservation of mangrove forest. The effect of pH, temperature, incubation time, salinity, source of carbon and nitrogen were tested in submerged fermentation processes in production of alpha –amyl by *Penicillium fellutanum* isolated from coastal mangrove soil (Kathiresan *et al*., 2006).

The main aim of the study is the mass production of the important rare mangrove plant, *Bruguiera cylindrica* through micropropagation methods because the survival rate of seedlings is naturally low. Clonal propagation is the multiplication of genetically identical individuals by asexual reproduction. Clonal propagation *in vitro* is called Micropropagation. Propagation of heterozygous genotypes is of necessity by vegetative propagation as it involves only mitotic cell division. This is the process of plant biotechnology, which is being utilized by industries in India for commercial production of many ornamental plants and fruit trees. In the medicinal and environmental importance of the plant the present study was undertaken to standardize protocols for vegetative propagation through shoot multiplication via shoot tip culture and clonal multiplication through seed culture.

2. Materials and Methods

The explants were selected from young, physiologically active disease-free mother plants. Shoot tips, nodal segments and seeds were used as the explants. The explants were surface sterilized using 0.1% mercuric chloride solution. Shoot tips and nodal segments and other explants of *Bruguiera cylindrica* were cultured on MS (Murashige and Skoog) medium fortified with different concentrations of benzyladenine (BA) and combination of BA and α-Naphthalene acetic acid (NAA), Kinetin, Activated charcoal and $NaCl_2$. Different concentrations of NAA and BA or kinetin were used for media preparation. The explants were inoculated on to the culture tubes. After inoculation the cultures were maintained at 25 ± 2°C on a 12 / 12-hour photoperiod at an intensity of 3000 lux from cool white fluorescent lamps. The relative humidity of the culture room was maintained between 70 - 80%. Indirect organogenesis was initiated through callus induction and subsequent regeneration. Sub- culturing was practiced at a regular interval of 40 days. The shoots without rhizogenesis were separated and transferred into

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MS medium containing 2.5 mg/1 NAA. The rooted plantlets were transferred into a sugar free MS liquid medium on filter paper bridges for hardening and then transferred into an autoclaved vermiculate in plastic cups. The plantlets were nourished with Hoagland's solution (Epstein, 1972). The plantlets were covered with moistened plastic bags and were kept at 25 ±1°C with a light intensity of 3000 lux at 12 /12 hours photoperiod. Plant with newly sprouted leaves were transplanted to pots containing garden soil and sand (3:1) under greenhouse conditions and then to field.

Isolated *in vitro* buds were used for immobilization. After separating each bud, they were grown in separate culture flask containing MS medium. The *in vitro* buds initiated from the cultures were encapsulated in the form of synthetic seeds. The buds were dipped in a gel of o.5% (w/v) Sodium alginate prepared in basal MS medium.Fresh razor cut sections of sample at different stages of *in vitro* development were selected for study.

3. Results and Discussion

Standardization of *in vitro* protocols for micropropagation is a pre requisite for any biotechnological application for crop improvement. The present work suggests protocols for the large-scale multiplication through shoot tip/nodal segment culture and seed culture. *Bruguiera cylindrica* W&A is an important mangrove plant belongs to the family Rhizophoraceae.

In recent years there is an increasing trend of loss of mangrove forest cover primarily due to unplanned developmental activities, habit destruction extreme population pressure. As a result, several economically important plant species are getting lost from their natural habitats. Due to removal of mangrove forest, several plant species either become extinct or under severe threat before their importance is being exploited. Therefore, there is an urgent need to conserve the mangrove forest in general, and rare threatened species in particular. Standardization of rapid protocol for clonal multiplication help in future for large scale cultivation and conservation of specie more over genome manipulation and regeneration of the species. The shoot tip, nodal segment and seeds were used as explants for micropropagation. The explants were cultured on MS basal medium and medium supplemented with different concentrations of hormones for shoot proliferation. They showed varying growth responses.

Healthy shoot tips and nodal segments of field grown plants were used for inoculation. Shoot tips and seeds were cultured on MS basal medium. The seeds, on germination, produced one shoot and three roots after 15 days of inoculation. The response of the explants varied with the growth regulators used in culture. Explants were cultured on MS medium containing 0.5-4mg/l BA higher concentration of BA produced one shoot. Seeds and shoot tips cultured on medium containing combinations of 1-4 mg/l BA and 0.5mg/l NAA from that 3mg/l BAwith 0.5mg/l NAA produced 1shoot and 1 root (Fig. 1a). Seeds inoculated on MS medium containing 2mg/l BA along with 0.5mg/l KIN produced 2 shoots. MS medium with 500 mg/l activated charcoal along with 500mg/l NaCl in the shoot tip it produces one shoot and seeds produced 3 shoots and 6 roots. Rhizogenesis was observed in presence of auxin (NAA). Auxins are routinely used for the induction of roots from the explant. Rooting is an inevitable step in clonal propagation procedure.

Seeds cultured on MS medium containing 1-4 mg/l BA, 500mg/l NaCl and 500mg/l activated charcoal. Shoot development was observed in all the concentrations of the hormone. MS medium supplemented with 3mg/l BA, 500mg/l NaCl along with 500mg/l activated charcoal produced maximum shoot and four roots after 15 days of inoculation.

Shoot culture is the most commonly used *in vitro* method for micropropagation (George and Sherrington, 1984). In the present study seed culture in *Bruguiera cylindrica* on MS medium with 500mg/l activated charcoal and 500mg/l $NaCl_2$ produced best result (Table 1). In direct organogenesis, adventitious shoots develop directly from the explant. According to George and Sherrington (1984) balanced auxins/ cytokinin interaction is effective for organogenesis. Tissue cultured plants were raised from stem explants on MS medium supplemented with various concentration of growth hormones (Kumar and Bhavanandan, 1989).

In the case of *Bruguiera cylindrica* the basal MS medium produced maximum shoot growth and number of shoot buds produced were greatly enhanced when charcoal and $NaCl_2$ were used in the medium. The applications of cytokinins promotes the growth of lateral buds by reducing the dominance of apical bud. The synthetic cytokinin BA is routinely added to tissue culture medium to stimulate shoot proliferation (Thomas and Blackesley, 1987)

The medium supplemented with 2mg/l BA and 500mg/l NaCl, rhizogenesis was also observed together with shoot bud production (Fig. 1b). MS medium amended with 2mg/l BA showed gradual initiation of shoot bud in *Bruguiera cylindrica*. Similar results were observed for shoot multiplication in some horticultural crops (Torres, 1988).

Leaf segments of *Bruguiera cylindrica* was cultured on MS medium supplemented with different concentrations of 2, 4-D (0.5mg/l,1mg/l,1.5mg/l and 2mg/l) with constant concentration of BA(1mg/l). Callus induction was highest in 1mg/l 2,4-D with 1mg/l BA (Fig. 1d) and lowest in 2mg/l 2, 4-D with 1mg/l BA.

Production of artificial seed consisting of somatic embryos or shoot buds enclosed in a bio-degradable protective coating is low cost, high volume propagation system (Sajina *et al*., 1997). *In vitro* buds initiated from culture containing 1 mg/l BA and NAA encapsulated in calcium alginate and germinal synthetic seed were developed (Fig. 1e). The buds were washed and in sterile distilled water for removing agar from the basal medium they were blot dried before immobilization. 0.5 to 5.0% (w/v) sodium alginate dissolved in MS basal medium and 2.5 % (w/v) $CaCl_2.2H_2O$ solution were used for encapsulation. Spherical and durable beads were obtained in 4% (w/v) sodium alginate and the bead become fragile when lower concentration (below 3% w/v) was used. Higher concentration of sodium alginate showed reduced viability of the synthetic seeds and above 5% w/v none of the seed

Fig. 1a. 1a. Shoot bud development from seed, **1b.** Root formation within culture medium, **1c.** Multiple shoot initiation, **1d.** Callus induction from leaf segment, **1e.** Encapsulated *in vitro* buds

Table 1. Comparative study of BA, NaCl and charcoal on root development

Explant	Medium	Hormone BA(Mg/l)	Activated charcoal (Mg/l)	NaCl (Mg/l)	No. of shoots and roots (Mean±SE)	
					Shoots	Roots
Seeds	MS	1	500	0	1.0±0.03	1.20±0.02
		2	500	0	1.0±0.03	1.50±0.02
		3	500	0	1.3±0.03	1.00±0.02
		4	500	0	1.4±0.03	1.12±0.02
		1	500	500	1.01±0.03	1.40±0.02
		2	500	500	1.31±0.03	1.05±0.02
		3	500	500	1.60±0.03	4.08±0.02
		4	500	500	1.30±0.03	2.00±0.02
		1	0	500	1.00±0.03	1.04±0.02
		2	0	500	1.01±0.03	2.01±0.02
		3	0	500	1.20±0.03	2.30±0.02
		4	0	500	1.50±0.03	1.40±0.02

germinated. Synthetic seed stored at 4±1° showed maximum frequency germination. Viability of such seeds was retained for more than three months, and 95-100% of germination was observed after 30 days. Those seeds kept at 24±1° showed 100% germination within 30 days but a gradual decrease was noticed after 30 days. Germination rate was less in the case of encapsulated buds kept in room temperature after 30 days. None of the seeds germinated after 60 days, when they were kept in room temperature. Explants takes time to recover from cold storage stresses after return to proliferation culture conditions (Ballester *et al*., 1997; West *et al*., 2006).

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