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Developing a Protocol for *in vitro* Propagation of the Grey Mangrove - *Avicennia marina*

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Abstract

Very little work has been done to date on the micropropagation of mangrove species, yet they have a high economic, ecological and social significance in many countries. As mangroves have few, short-lived propagules, a micropropagation protocol is essential to ensure an ongoing supply of seedlings. However, developing a method to clean explants from both field and glasshouse derived *Avicennia marina* plants proved very difficult (< 10% success rate). Using actively growing tips and the first binodal stem sections, six different chemical sterilants were tried with a 0.1% solution of mercuric chloride being the only successful one. Spraying the foliage with Benlate several days before collection improved the results.

The apical tips were the easiest to clean but did not respond well to the plant growth regulators (PGRs) whereas the binodal explants that survived the cleaning regime often gave rise to two growing buds.

To provide enough plant material for media trials, embryos were excised from seeds, established in culture, and the subsequent seedling tips used. Field derived explants were then grown on the most successful formulæ.

Mineral salts, at various concentrations, were tested. These included Murashige and Skoog (MS), Woody Plant medium (WPM), Rao Medium ‘X’ (X) and Gamborg B5 (B5) of which 1/2 MS and B5 were found to be the most suitable. For multiplication, binodal explants were the most responsive in a medium containing 1/2 MS macro salts + MS micro salts + 2 mgL\(^{-1}\) Glycine + 1 mgL\(^{-1}\) Thiamine + 400 mgL\(^{-1}\) Inositol + 0.5 mgL\(^{-1}\) Nicotinic acid + 0.5 mgL\(^{-1}\) Pyridoxine + a combination of 3 mgL\(^{-1}\) BAP, 1 mgL\(^{-1}\) Zeatin, 0.25 mgL\(^{-1}\) IBA and 1 mgL\(^{-1}\) GA\(_3\). Gelcarin replaced phyto gel as the gelling agent to reduce hyperhydricity. A soft gel also seemed to cause softening and necrosis of the stem. Successful root development has been produced in the above medium using both 1 mgL\(^{-1}\) of IBA or 1 mgL\(^{-1}\) NAA. Plant-out trials are currently being undertaken.

Keywords

Tissue Culture, re-afforestation, Vietnam, ACIAR, micropropagation

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Introduction

Mangrove forests are a feature of the intertidal zone of tropical and subtropical coastlines. They have developed several unique morphological and physiological characteristics to adapt to their shifting, saline and muddy environment as they play an important role in coastal protection and maintenance of both water quality and biodiversity. Mangroves have high economic, ecological and social significance but are continually under threat from coastal development, excessive wood gathering, fishpond operations, diversion of freshwater and mining or extraction of minerals.

Although there is greater interest in mangrove afforestation programs, difficulties occur in ensuring a reliable supply of selected propagules for increasing the number and scale of plantings. Mangroves present particular problems of seed collection due to the nature of the habitat, of seed viability, which is only for short periods each year, and of seed storage since storage life is short and most seeds are large. The survival of mangrove seedlings in the field is also generally poor because of the dynamic nature of their habitat, thus replacement plantings often need to be undertaken for up to three years (Saenger and Siddiqi, 1993).

To date, very little tissue culture work has been done on mangroves. Limited work on Bruguiera gymnorrhiza (Satwong et al., 1995) was conducted using hypocotyls as explants on MS and WPM supplemented with 2,4-D and Kinetin, which produced callus on about 6% of cultures. Multiple shoot and some root initiation occurred on sugar-free media with IBA and phloroglucinol when cultures were kept in the dark. However, no further development could be induced. Baba and Onizuka (1997) attempted to refine the techniques of re-differentiation from callus cultures of Bruguiera gymnorrhiza, Kandelia candel, Pemphis acidula and Rhizophora stylosa but without success. Akatsu et al. (1996) were able to maintain callus of Sonneratia alba on MS supplemented with a range of hormones and 3% sucrose but no further work was reported. At the ‘International Symposium on Mangrove Ecology & Biology’, Kuwait 1998, Sudhersan et al. (1998) used embryo explants of Avicennia marina to mature into seedlings on modified MS medium or into callus using growth hormones producing numerous meristematic nodules after 3 months but no details were provided. At the same event, Rao (et al., 1998a) reported the development of micropropagation techniques for Exoecaria agallocha, Avicennia marina and Acanthus ilicifolius using a new macronutrient combination with MS micronutrients and vitamins. Specific details for Exoecaria agallocha were soon published (Rao et al., 1998b). Using nodal explants, axillary shoots were produced on medium ‘X’ supplemented with BAP (13.3 μM), Zearin (4.65 μM) and IBA (1.23 μM). Rooting was initiated in medium ‘X’ containing IBA (0.23 μM) and regenerated plants planted out successfully. Eganathan and Rao (2001) published protocols for three mangrove species including Avicennia officinalis which also multiplied in medium ‘X’ supplemented with 1 mgL⁻¹ BAP and 0.5 mgL⁻¹ IBA.

At Central Queensland University (Mills et al., 1999) various techniques of disinfesting eight mangrove species were conducted with limited success. Clean explants of Avicennia marina were not produced until mercury chloride was used as the chemical sterilant (P. Bhatia pers. comm. 2000).

Micropropagation is the most useful and widely used technology in tree improvement programs. Sharp (1980) concluded that micropropagation may be useful for forest trees characterised by poor seed set, absence of uniform seed production, and seed prone to genetic damage or loss of viability during storage – features common in mangroves (Saenger, 1982). Thus, successful in vitro propagation of mangroves would not only provide ample planting material for restoration projects but a source of material for nutrient trials, a method of germplasm storage, germplasm exchange and an essential technology for long term objectives of genome modification such as salinity and frost tolerance or insect resistance (Karnosky, 1981; Maity, 1994).
This work is being carried out in conjunction with a broader project on the “Genetic Diversity and Propagation of Mangroves” funded by ACIAR and in collaboration with Silvicultural Research Division, Royal Forest Department, Bangkok, Thailand and Mangrove Ecosystem Research Division, Vietnam National University, Hanoi, Vietnam.

Initial work is on the common grey mangrove Avicennia marina. Objectives include an assessment of the extent of genetic variation within and between populations of mangroves, the extent to which desirable traits are genetically determined and the development of screening and selection procedures for identifying desirable genotypes as well as a micropropagation technique.

Materials and Methods

Plant material

Explants chosen were actively growing apical and axillary buds from 2 to 3 year old seedlings and adult trees of Avicennia marina. Clones from adult trees are necessary if certain phenotypic characteristics observed in the field are sought after. This material, however, is the most difficult to establish in culture due to the high level of contamination in the mangrove environment, so seedlings grown under glasshouse conditions were also used as a source of explant material.

Seeds, which are more easily sterilised, were dissected and established in culture to provide a source of sterile growing tips for media trials.

Sterilisation and culture initiation of Avicennia marina seeds

Fresh seeds (>2cm) with peduncle still attached were collected from mature trees, discarding those with insect attack from the fruit fly Euphranta marina (P. Mackay pers comm. 2000). Seeds were sterilised for 10 minutes in 10% sodium hypochlorite (NaOCl) with Tween 20. Immature propagules with a single cotyledon were then dissected and established on a simple medium of 1/2 MS (Murashige and Skoog, 1962) macro salts, MS micro salts, MS vitamins, 1 mLL⁻¹ Plant Preservative Mixture (PPM), 30 gL⁻¹ sucrose and 2 gL⁻¹ phytogel.

Sterilisation and culture initiation of Avicennia marina vegetative material

Sterilising explants sourced from the field or glasshouse was much more difficult. Growing tips with 2 binodal points were collected and subjected to various combinations of both physical and chemical methods. Physical methods included: trimming, gentle scrubbing with antibacterial soap, agitating in a 2% solution of Tween 20, immersion in 70% Ethanol (EtOH) and a wash under running tap water. Chemical sterilants included various concentrations and exposure times to NaOCl, calcium hypochlorite (CaOCl), mercury chloride (HgCl₂), silver nitrate (AgNO₃), benzalkonium chloride and tea tree oil. After repeated rinses in sterile deionised water and final dissection of nodal pieces, all explants were initially cultured on a simple medium of 1/2 MS macro salts, MS micro salts, MS vitamins, 2 mgL⁻¹ glycine, 30 gL⁻¹ sucrose and 2 gL⁻¹ phytogel.

Media determination

To determine a suitable multiplication media for Avicennia marina, the basal salts of a series of well known published media were first tested using growing tips of equal length from seedlings growing in culture. Results from this plant material source may not be directly applicable to nodes from adult material but does provide a starting point (de Fossard, 1993). Since the prime motivation for this micropropagation work is commercial production, the response of cultures to treatments were scored rather than accurately measured to aid a more rapid screening of options and to use the findings as stepping-stones to the next experiment (de Fossard, 2000).
All media trials consisted of 10 experimental units each containing a single explant. Media was adjusted to pH 5.7 before dispensing 10 mL into 30 mL polycarbonate tubes and autoclaved at 121°C and 1.05 kg cm⁻² for 20 minutes. Cultures were placed in a growth room maintained at a temperature of 25 °C ± 2 °C on mesh shelves and exposed to 16 hours light / 8 hours dark from fluorescent lighting suspended from the shelf above (NEC Standard Daylight High-Grade and NEC Biolux) with a light intensity of 35 μmol m⁻² s⁻¹.

For all experiments, ten shoot tips were established in each medium and growth scored after 30 days on a 0 to 5 scale without removing the plant material from its culture vessel where 0 = no change, 1 = < 1 mm stem growth, 2 = 1–10 mm stem growth, 3 = 10–20 mm stem growth, 4 = 20–30 mm stem growth, and 5 = 30–40 mm stem growth. Losses, which may have been caused by microbial contamination or an imbalance in the culture condition, were removed from the data. Where possible, a single subculture onto the same medium was carried out and scored again after 30 days. A repeat trial was also established if sufficient plant material was available.

**Experiment 1**: The initial experiment compared four concentrations (1/4, 1/5, 3/4 and full strength) of MS + 20 g L⁻¹ sucrose, 2 g L⁻¹ phytagel and adjusted to pH of 5.7. The 1/2 MS medium was the most successful one which was then trialed against three other published media namely McCowan et al. (1981) woody plant medium (WPM), medium 'X' (Rao et al., 1998b) and B5 (Gamberg, et.al. 1968). See Table 1 for media composition.

**Experiment 2**: 1/2 MS was prepared with the addition of varying concentrations (0.1, 1.0, 5.0, 10.0 mg L⁻¹) of 6-benzylaminopurine (BAP) and Kinetin, two commonly used cytokinins for woody plants were trialed individually and in combination.

**Experiment 3**: BAP and Kinetin were each trialed with the addition of Naphthaleneacetic acid (NAA) using 1/2 MS basal medium.

**Experiment 4**: A combination of three PGRs (3 mg L⁻¹ BAP + 1 mg L⁻¹ Zeatin + 0.25 mg L⁻¹ Indolebutyric acid (IBA)) used successful in *E. agallocha* (Rao et al., 1998b) were trialed in combinations with three basal salts (1/2 MS, B5 and modified 'X'). A modified trial with the elimination of Zeatin was also conducted due to the cost of this cytokinin (an important consideration in commercial production).

**Experiment 5**: Since explants of *A. marina* are slow to respond in culture, two pulse treatments were trialled. Explants were established in 1/2 MS supplemented with 6.675 μM thidiazole (TDZ, contained in 3 mg L⁻¹ of Dropp, a commercial cotton defoliant) or 1 mg L⁻¹ GA₃ for 10 days before subculture.

**Experiment 6**: A dose response trial of coconut water (50, 150, 400 mLL⁻¹) added to 1/2 MS + the 3 PGRs + 20 g L⁻¹ sucrose. This experiment was replicated in Hanoi but the freshness and quality of the coconut water was considerably different due to local availability of coconuts at that time of year.

**Experiment 7**: Sucrose was increased from 20 g L⁻¹ to 30 g L⁻¹ in the medium, 1/2 MS + the 3 PGRs. A greater range of sucrose concentrations could not be established at this time due to lack of availability of sterile plant material.

**Experiment 8**: A dose response trial of Inositol (100, 200, 400 mgL⁻¹) on 1/2 MS and B5 basal salts + the 3 PGRs (S. Rao pers. comm., 2001).

**Experiment 9**: An omission and dose response trial for GA₃ (0, 0.5, 1.0, 2.0 mgL⁻¹) on 1/2 MS + the 3 PGRs.

**Experiment 10**: Stage III trial of two auxins, NAA (0.5, 1.0 mgL⁻¹) and IAA (0.5, 1.0 mgL⁻¹) on both 0.5 MS and B5 basal media.
Results and Discussion

Stage I

The successful cleaning regime for *Avicennia marina* was dependant on the time of year, the source of the vegetative material and the type of explant (apical tip, first or second binodal segment). Field explants taken from 1 to 2 year old seedlings were more successful than from mature trees possibly due to the reduction in phenolic exudation. This dark discolouration of the medium surrounding the explant, which causes eventual necrosis of the tissue, can be overcome by frequent subculturing or by addition to the medium of polyvinylpyrrolidone (PVP) or PPM (Guri, 1997). In this case, PPM proved to be the most effective. Explants sourced from glasshouse plants were more successful than field collected material and fungal contamination was further reduced if Benlate (0.5 g L\(^{-1}\)) was sprayed on the plant several days prior to collection. First binodal segments were the most responsive, often producing two or three shoots, but were also the most difficult to clean.

The final cleaning regime included trimming of excess plant material from stem sections before gently scrubbing with antibacterial soap or agitating in 2% Tween 20 for 5 minutes. The plant material were then washed under running tap water for 1 hour then trimmed further before agitating in 0.1 % HgCl\(_2\) for 5 to 20 minutes. Plant material was then rinsed up to five times with sterile distilled water before explants were dissected into apical or binodal buds. The addition of PPM to the medium reduced the contamination rate but when no longer added, a bacterial contaminant often occurred. Further trials are necessary to determine the origin of these prevalent bacteria as they may be endogenous.

Stage II

Deciding on the 'type' of multiplication desirable for *A. marina* took into consideration the difficulties often faced with reversal of habit in culture. Thus the development of cultured buds into a shoot with normal or greater elongation of internodes, so that each node could be excised and cultured further, was chosen instead of overcoming apical dominance by chemical means to develop multiple shoots. Each experiment was based on the results of the previous one, however extensive trials were limited by the availability of explant material. Growth rates were slow but this could be attributed to the use of apical rather than nodal buds from cultured seedlings – the only material available in the required quantity.

Experiment 1

Mean frequencies for each stem growth category, representing the surviving plantlets from each trial of 10, are shown in Figure 1. The trials were repeated as different basal salt media were added at a later date. Initially, the most suitable medium was 1/2 MS macro salts + MS micro salts + MS vitamins + 20 g L\(^{-1}\) sucrose. This medium was used for successive trials with various plant growth regulators. Later, B5 medium incorporating MS (iron + EDTA) + B5 vitamins was also found to be suitable. Medium 'X' (Rao, pers. Comm., 2000) was not successful for *A. marina* at any stage.
Figure 1: Growth response of *A. marina* on various concentrations of Murashige and Skoog (MS), Woody plant medium (WPM), medium 'X' and B5 medium.

Experiment 2

Plant material responded best to a combination of BAP and Kinetin (Figure 2).

Figure 2: Growth response of *A. marina* on 1/2 MS + combinations of BAP (B) 1 and 5 mgL\(^{-1}\) + Kinetin (K) 1 and 5 mgL\(^{-1}\)

Experiment 3

Survival rate and growth were higher in the medium supplemented with BAP and NAA (57%) than in the medium supplemented with Kinetin and NAA (19%). Where the concentration of auxin was equal to or greater than the concentration of cytokinin, strong roots developed, indicating that root initiation may not be too difficult for *A. marina*. These explants, however, had not been exposed to multiplication medium which may affect the response to root development.
Experiment 4

This combination of PGRs was chosen in response to the published work of Rao (1998b). Zeatin is not usually found to be effective in woody species was replaced by Kinetin and IBA was replaced by NAA. Shoot tips responded favourably on both 1/2 MS and B5 but the medium 'X' resulted in necrosis (Figure 3).

Curling and twisting resulting from high cytokinin concentrations in a significant number of new leaves led to several trials with reduced cytokinin levels since high cytokinin levels have been known to cause small shoots incapable of elongating, aberrant leaf morphology and difficulties in rooting (George 1996). Results at this stage were inconclusive.

![Figure 3: Growth response of A. marina on 3 different basal salts ('X', B5 and 1/2MS) supplemented with 3 PGRs (3 mgL⁻¹ BAP + 1 mgL⁻¹ Zeatin + 0.5 mgL⁻¹ IBA)](image)

Experiment 5

Explants exposed to Dropp for 10 days yellowed and died, however exposure to GA₃ showed some growth. The slow response of explants may be due to a lag or stabilisation phase but with limited amount of plant material available, this avenue was discontinued and a follow up dose response experiment of GA₃ designed.

Experiment 6

'Undefined' substances such as coconut water are often considered undesirable, because their chemical nature are not precisely known. However coconut water is a source of many nutrients including Zeatin, GA₃, inositol and sugar. Since this work is also being conducted in Vietnam with the desired outcome being mass production in that country, coconut water may provide a readily available and cheap source of these growth factors.

A. marina responded well to the addition of coconut water with both axillary buds developing within 30 days on 16% of plants cultured on 1/2 MS with no additional PGRs (Figure 4). The increase in leaf abnormalities (curling) on medium supplemented with 400 mL of coconut water and the 3 PGRs (Figure 5) may indicate that the total cytokinin level is too high. This experiment was replicated in Vietnam but data has not been compared due to the variability in the coconut water and the explant material.

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Figure 4: Growth response of *A. marina* to concentrations of 1/2 MS medium supplemented with coconut water (150 or 400 mLL⁻¹)

Figure 5: Growth response of *A. marina* to the addition of various concentrations of coconut water to 1/2 MS + the 3 PGRs (3 mgL⁻¹ BAP +1 mgL⁻¹ Zeatin + 0.5 mgL⁻¹ IBA)

Experiment 7

Although not an extensive trial, and the growth rates not markedly different between the two sucrose concentrations, plants looked greener and healthier after 30 days in the medium containing 30% sucrose.

Experiment 8

Although inositol is classed as a member of the Vitamin B complex, many believe that in micropropagation it should also be regarded as a supplementary carbohydrate and that it may favour bud formation and retard necrosis (George, 1993). It is also considered part of the growth promoting property of coconut water (Pollard *et al*., 1961). Inositol has rarely been considered as an important variable in tissue culture but was trialed here in response to personal communication with S. Rao (2000). Results indicated that increased inositol from 100 mgL⁻¹ to 400 mgL⁻¹ improved the growth response of axillary buds, thus producing 2 or 3 shoots instead of 1 from each nodal stem section. This response was greater on B5 salts (33%) than on 1/2MS salts (18%).
Experiment 9

Addition of GA$_3$ to the medium 1/2 MS + the 3 PGRs improved the growth rate and stimulated greater axillary bud growth (18%). However, leaf abnormalities (curling) still occurred. Subculturing was successful using nodal stem sections from the main shoot and axillary branches on 1/2 MS + 1 mgL$^{-1}$ GA$_3$ + the 3 PGRs + 30 gL$^{-1}$ sucrose.

![Graph showing growth response to GA$_3$ concentration](image)

**Figure 6:** Growth response of *A. marina* to various concentrations of GA$_3$ with 1/2 MS + 3PGRs (3 mgL$^{-1}$ BAP + 1 mgL$^{-1}$ Zeatin + 0.5 mgL$^{-1}$ IBA)

Experiment 10

Rooting was achieved easily with NAA in Experiment 3 but proved more difficult after the plants had been exposed to multiplication media. Roots did occur in 50% of field-derived plantlets cultured on both 1/2 MS and B5 media supplemented with either IBA or NAA (0.1 and 1.0 mgL$^{-1}$) after 30 days. Subculturing did not improve this result. Further testing on hormone-free media or with the addition of activated charcoal to remove traces of GA$_3$ (which may be inhibitory to root formation) is required but plant material derived from Stage II is limited.

Work here demonstrates that explants from *Avicennia marina* have been successfully established *in vitro* (Stage I), multiplied (Stage II), roots developed (Stage III) and planted out in pots (Stage IV) but all with varying degrees of success. At this stage the most successful medium is Gamborgs B5 basal salts or 1/2 MS macro salts + MS micro salts with the addition of 2 mgL$^{-1}$ Glycine + 1 mgL$^{-1}$ Thiamine + 400 mgL$^{-1}$ Inositol + 0.5 mgL$^{-1}$ Nicotinic acid + 0.5 mgL$^{-1}$ Pyridoxine + 3 mgL$^{-1}$ BAP, 1 mgL$^{-1}$ Zeatin, 0.25 mgL$^{-1}$ IBA and 1 mgL$^{-1}$ GA$_3$ + 30 gL$^{-1}$ sucrose + 7 gL$^{-1}$ Gelcarin. The growth rate is still slow but this may improve with subsequent subcultures. Where possible, field-derived material has been subcultured on this medium, stimulating further growth. At this stage only a limited number of specimens have progressed to producing roots in culture and plant out trials.

Continued research on tissue culture of *Avicennia marina* is needed to further optimise physical and chemical factors. The addition of sodium chloride (NaCl) to the media has been excluded to date perhaps because it is commonly believed that the importance of salt lies in the fact that mangroves are slow growing and cannot compete with faster growing species unless salt is eliminated from media (Hutchings and Saenger 1987). However, in *in vivo* trials, Tuan et al. (1996) reported that salinity is an important factor affecting the establishment and growth of *Avicennia marina* seedlings. The germination and growth rate increased with lower salinity, the optimum
concentration being 25% seawater, but it was also noted that seedlings looked healthy at all salinities. Thus, current studies include a factorial treatment of sodium chloride (NaCl). Future work includes further trials with coconut water, as this has been the most successful medium in Hanoi, and decreasing the concentration and/or combinations of cytokinins in Stage II may ultimately reduce leaf abnormalities and increase the multiplication rate.

Acknowledgements
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References


**Table 1:** Compositions of media used in experiments.

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<th>B5 MEDIUM mmolL⁻¹</th>
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MS Murashige and Skoog Medium (1962)
WPM Woody Plant Medium (McCowan & Lloyd - 1981)
B5 Gamborg's Medium (1968)
RAO 'X' Medium for Excoecaria agallocha (1998b)
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