[BIO29] *In vitro* regeneration system of teak (*Tectona grandis* L.)

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Introduction

Tectona grandis, commonly known as teak, is a member of the Verbanaceae family. It is one of the world's premier appearance grade timbers and is highly sought after for its attractiveness, durability and resistance to termite and fungal infection (Appanah and Weinland, 1993). The species is indigenous to India and the Southeast Asian region (Mynmar-Thailand-Laos) where it occurs in tropical deciduous forests with pronounce dry season (Tewari, 1992). Teak is amenable to plantation cultivation and is planted on four continents.

The concept of plantation timber is relatively new in Malaysia. Considering significant factors of depleting forest resources and ever-growing consumer demand for timber and other woody products, mass production of high value timbers with short gestation period, if feasible, is inevitable. Teak wood, which commands a price of US$ 1500 to 2000 per ton in the world market, has been identified to be well suited for this purpose (Baskaran *et al.*, 1998). Currently, the total area planted in Peninsular Malaysia and Sabah is 2279 and 2362 hectares respectively (Trockenbrodt and Josue, 1999). The establishment of teak plantations in Malaysia is being actively promoted by the Department of Forestry, the Forest Research Institute of Malaysia (FRIM), other government agencies and the private sector.

The success of teak plantations depends on the availability of large quantities of good quality planting stock. The conventional method of propagating teak *via* fruits (seeds) and cuttings have severe limitations including time consuming, low germination rate and rooting difficulty. *In vitro* propagation via tissue and organ culture offers an alternative, novel possibility to increase production and germination rates of viable seeds from elite trees as well as to produce clones of genetically superior seedlings. In forest biotechnology, the system has been applied to multiply stocks for large-scale planting purposes. Ever since Gupta and friends (1980) reported teak culture from seedling explants and buds of 100 year-old teak tree, teak tissue culture has been widely researched in Thailand, Indonesia and India. This paper reports on *in vitro* regeneration system of teak that is sourced from Mata Ayer Forest Reserve Plantation in Perlis, one of the pioneer projects of teak plantation in Peninsular Malaysia.

Materials and Methods

Plant material

Mature teak fruits were collected from the clone bank established in FRIM sub-station in Mata Ayer, Perlis. Teak fruit is enclosed within a thin papery outer layer that is easily removed. To extract the seeds (usually 1-3 seeds per fruit), the stony endocarp was spilt open with a seed cutter. Then the seeds were sterilized in 10% commercial bleach (Clorox) for 15 minutes and rinsed three times in sterile distilled water in the laminar air flow cabinet. The seed coat was soften and removed by soaking the seeds in sterile distilled water for approximately 1 hour.

Shoot induction

To induce axillary shoot formation, 4-6 week old *in vitro* germinated seedling was separated into shoot tip and nodal explants. Cotyledons and embryonic axis that were excised from the seeds were also used to induce formation of adventitious shoots. All four types of explants as mentioned were subjected to 21 treatments of basal medium supplemented with combinations of an auxin, α-naphtalene-acetic acid (NAA) at 0, 0.01 and 0.1mgl^{-1} and a cytokinin, 6-benzylaminopurine (BAP) at 0, 0.1, 0.5, 1.0, 2.0, 3.0 and 5.0mgl^{-1}. Each treatment was represented by 5 explants and was replicated 5 times. The basal medium consisted of MS

inorganic salts (Murashige and Skoog, 1962), Gamborg B5 vitamins (Gamborg *et al.*, 1968), 0.1gl^{-1} myo-inositol, 30gl^{-1} sucrose and solidified with 7gl^{-1} agar (Type 900). The pH was adjusted to 5.8 before autoclaving at 121ºC for 15 minutes. All cultures were maintained at 25±2ºC with 16-hour photoperiod provided by cool white fluorescent lamps (13-25µmol^{-2}s^{-1}). All data were subjected to analysis of variance and the treatment means were compared by Duncan's Multiple Range Test (DMRT).

Elongation and rooting of shoots

Miniature shoots that were proliferating from the expanding embryonic axis were subjected to 6 elongation treatments namely MS basal medium and basal medium supplemented with 2.0mgl^{-1} BAP + 0.01mgl^{-1} NAA (control); 0.1mgl^{-1} BAP; 0.1mgl^{-1} BAP + 0.1mgl^{-1} NAA; 0.1mgl^{-1} BAP + 0.1mgl^{-1} gibberellic acid (GA_3); and 0.1mgl^{-1} BAP + 0.05mgl^{-1} kinetin (KIN). After 4 weeks, the elongated shoots were subjected to 11 rooting treatments consisting of various combinations of NAA (0.01-1.0mgl^{-1}), 3-idolebutyric acid (IBA) (0.01-1.0mgl^{-1}) and activated charcoal. Observation for rooting response was carried out after 6 weeks of incubation.

Induction and formation of callus

Seed derived explants (cotyledon and embryonic axis) were used to induce callus for the study of plant regeneration via somatic embryogenesis system. The explants were subjected to various treatments consisting of three types of plant growth regulators namely 2, 4-dichlorophenoxyacetic acid (2, 4-D), NAA and BAP, alone or in combinations; two types of media, half or full strength plant growth regulator supplemented MS basal medium; and incubated under light or dark condition.

Classification of callus

The callus were categorized into embryogenic callus (Type A) and non-embryogenic callus (Type B – F) at macroscopic and microscopic level. For morphological classification, the callus were observed under 3-D light microscope and categorized according to colour, texture (degree of friability), structure and size. At microscopic level, histology study was carried out whereby samples of the callus were fixed in formaldehyde acetic acid, dehydrated in serial alcohol, embedded in paraplast medium, sectioned at 12µm and stained with toludine blue O. The samples were then viewed under light microscope and were categorized according to the size, structure and content of cell or group of cells. The induced callus was subcultured every 2 weeks in semi-solid and liquid regeneration media. Observation was carried out weekly for indication of morphogenesis.

Results and Discussion

Shoot induction from seedling and seed derived explants

Analysis of variance showed that the percentage of explant that formed shoots and the mean number of shoots per responding explant were significantly affected by growth regulator concentrations. Generally, positive results were obtained at 1.0-3.0mgl^{-1} BAP, without or with 0.01mgl^{-1} NAA (Table 1). Less than 50% explants (except nodal explants) responded at concentration of BAP below 1.0mgl^{-1} (data not shown) while at concentration of BAP above 3.0mgl^{-1}, less than 30% explant (except nodal explant) responded (data not shown). Even though shoot tip and nodal explants showed very high response (80-100%), the number of shoots produced per responding explant was low (the highest being 4.3 and 4.1 shoots respectively at 2.0mgl^{-1} BAP) (Table 1). 60% of the cotyledonary explant responded to 1.0mgl^{-1} BAP combined with 0.1mgl^{-1} NAA and produced an average of 4.0 shoots (Table 1). Among the four types of explants used, the embryonic axis produced the highest number of shoots (9.3) at 2.0mgl^{-1} BAP in combination with 0.01mgl^{-1} NAA (Table 1). Explants that did not develop shoots either turned necrotic or only produced callus.

Production of plants from axillary shoots *via* shoot tip and nodal culture has been proven to be the most generally applicable method of *in vitro* propagation. These explants have pre-formed meristems that are destined to become shoots and will proliferate when subjected to appropriate stimuli (growth regulator) (George, 1993). However, the rate of axillary shoots formation from the shoot tip

TABLE 1 Effect of BAP and NAA on shoot induction from shoot tip (ST), nodal (N), cotyledonary (C) and embryonic axis (EA) explant.

Growth regulator (mgl^{-1})		Percentage of explants producing shoots (%)[a]				Mean number of shoots per responding explant[a]			
BAP	**NAA**	**ST**	**N**	**C**	**EA**	**ST**	**N**	**C**	**EA**
1.0	0	68^{b}	100^{a}	0^{d}	60^{bc}	2.7^{cd}	2.7^{b}	0.0^{e}	4.4^{f}
1.0	0.01	48^{d}	64^{d}	0^{d}	76^{ab}	2.6^{de}	2.0^{d}	0.0^{e}	6.6^{c}
1.0	0.1	20^{h}	44^{e}	60^{a}	36^{de}	2.0^{h}	2.0^{d}	4.0^{a}	4.1^{fg}
2.0	0	80^{a}	100^{a}	0^{d}	56^{cd}	4.3^{a}	4.1^{a}	0.0^{e}	6.0^{d}
2.0	0.01	60^{c}	100^{a}	60^{a}	76^{a}	2.5^{ef}	2.1^{cd}	2.6^{b}	9.3^{a}
2.0	0.1	0^{i}	0^{f}	60^{a}	48^{cde}	0.0^{i}	0.0^{e}	2.2^{bc}	5.3^{e}
3.0	0	48^{de}	100^{a}	52^{b}	56^{cd}	3.4^{b}	2.2^{c}	1.5^{d}	5.2^{e}
3.0	0.01	48^{de}	100^{a}	28^{c}	56^{cd}	2.4^{ef}	2.1^{cd}	2.0^{c}	7.3^{b}
3.0	0.1	0^{i}	0^{f}	24^{c}	36^{de}	0.0^{i}	0.0^{e}	1.9^{cd}	4.5^{f}

[a]Means within a column followed by the same letter are not significantly different at 5% level according to Duncan's multiple range test.

and nodal explant of teak was slow, which may be due to apical dominance characteristic of teak tree. Cytokinin is usually incorporate into the medium to remove the dominance of the apical meristem so that axillary shoots are produced. Exposing the cotyledon and embryonic axis to growth regulators has apparently stimulated *de novo* or adventitious buds that developed into shoots (Figure 1a). Transverse section of the hypocotyls segment from the expanding embryonic axis showed that the shoots arised from the differentiated parenchymatous cells. Morphogenesis was apparently direct as the procambium of developing shoots was attached to the procambium of embryonic axis (Figure 1b).

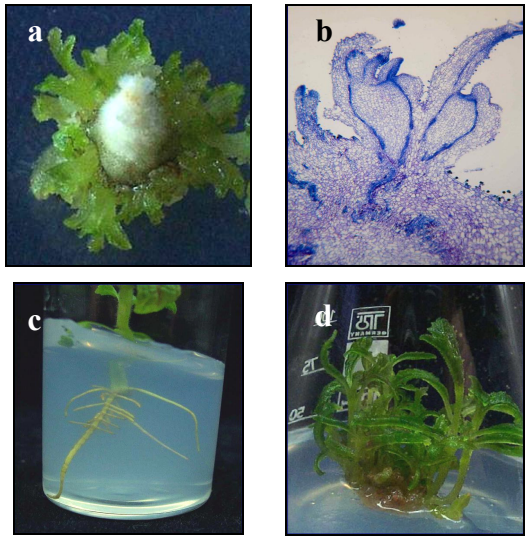

FIGURE 1 (a) Shoots proliferation from embryonic axis, (b) Elongation of shoots on MS basal medium supplemented with $0.1mgl^{-1}$ BAP, (c) Stout roots developing from base of shoot, (d) Attachment between the shoot

Elongation and rooting of shoots

Miniature shoots that developed from the expanding embryonic axis quadrupled their initial length of approximately 0.5cm to 2.1cm during the 4 weeks of culture in $0.1mgl^{-1}$ BAP (data not shown) (Figure 1c). Addition of $0.1mgl^{-1}$ NAA, $0.1mgl^{-1}$ GA_3 or $0.05mgl^{-1}$ KIN to medium supplemented with $0.1mgl^{-1}$ BAP helped to elongate the shoots but also produced poor to moderate callusing response. When the elongated shoots were transferred to rooting medium supplemented with $0.01mgl^{-1}$ NAA, 74% of the shoots produced an average of 3.2 roots per shoot (data not shown). The main roots were stout and secondary root hairs can be seen growing along the axis (Figure 1d). Rooting medium containing $0.1\text{-}2.0mgl^{-1}$ IBA only produced 34-44% response (data not shown). Addition of $2gl^{-1}$ of activated charcoal into MS basal medium produced a high rooting response (60%) (data not shown) but approximately 50% of the roots that developed were thin and weak.

Most of the shoots from the embryonic axis developed in clusters and thus, another step, the elongation step was required. It was necessary to specially elongate shoots to facilitate the separation process. Addition of $0.1mgl^{-1}$ BAP into the basal medium apparently reduced axillary shoot formation but promoted shoot growth as compared to other basal medium. Rooting of shoots is also an important step as the survival of seedling in the nursery and subsequently in the field will depend on the vigour of the growing roots.

Tree species is usually difficult to root. However in this case, stout roots of considerable length were obtained when the shoots were cultured for 6 weeks in low concentration of NAA and no subculture was required.

Morphogenesis of callus from seed derived explants

Irrespective of the initial material (cotyledon and embryonic axis), combinations of growth regulator, media and incubation condition, the callus elicited similar response in terms of morphology. Embryogenic callus (Type A) which was yellowish, friable and has granular texture was only obtained from embryonic axis cultured on half-strength MS media supplemented with 1.0-2.0 mgl^{-1} 2,4-D in combination with 1.0mgl^{-1} BAP or full-strength MS medium supplemented with 1.0mgl^{-1} 2,4-D in combination with 1.0-2.0mgl^{-1} BAP (data not shown) (Figure 2a). Only dark condition produced embryogenic response. Other combinations of 2, 4-D, NAA and BAP produced non-embryogenic callus (Type B-F) that is whitish to greenish, semi-friable to compact and has irregular protuberance or is highly vacuolated. Most of the cotyledons produced compact callus which was not amenable to liquid culture. Type A callus that was placed on semi-solid regeneration medium showed poor morphogenetic response by turning necrotic or compact. However, in liquid MS basal medium, the suspension (from the embryogenic callus) which initially comprised of small (25-35µm), generally rounded, relatively dense cytoplasmic cells with distinct nucleus and starch grain (Figure 2b) demonstrated early stages of somatic embryo development i.e. the cells underwent rapid division and produced compact and discrete group of cells which developed into embryonal structure (Figure 2c). By 6-7th subculture, somatic embryos at globular or early heart-stage were observed in culture (Figure 2d).

Plant cells can be induced to give rise to somatic embryos directly or indirectly (*via* callus). Somatic embryos are *de novo* embryos formed from somatic cells through a series of developmental process i.e. globular, heart, torpedo and cotyledonary stage (George, 1993). The system is amenable to genetic manipulation and can be incorporated in automated mass propagation and artificial seed production. Although somatic embryogenesis has been reported in more than 200 plant species, the whole induction and developmental process requires a delicate balance between the growth regulator stimulus, plant material and physical environment. It was an interesting breakthrough that embryogenic response was obtained in this teak material. With further research, this system will not only be used for mass propagation, but will also facilitate tree improvement programs.

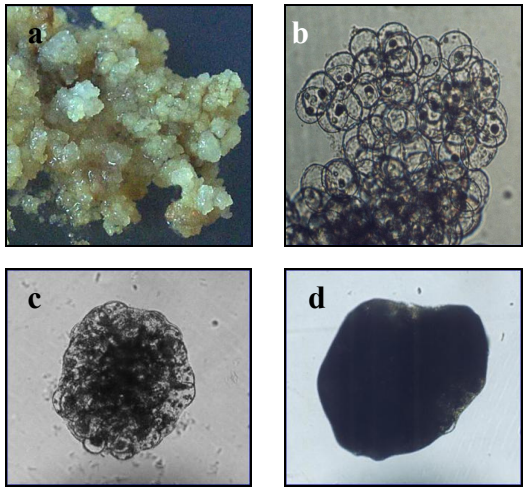

FIGURE 2 (a) Yellowish, friable and granular embryogenic callus induced from embryonic axis, (b) Embryogenic cells that are usually small (25-35µm), generally rounded, and have relatively dense cytoplasm with distinct nucleus and starch grain, (c) Proembryo, (d) Somatic embryo at globular stage.

Acknowledgements

The authors wish to thank the Ministry of Science, Technology and Innovation (MOSTI), Malaysia for the National Science Fellowship awarded to Lim Sooi Ping and for IRPA grant awarded to Maheran Abdul Aziz. The authors are also grateful to Forest Research Institute of Malaysia (FRIM) for providing the teak seeds and all staff at Tissue Culture & Micropropagation Laboratory, FRIM, for their experience and technical help in the research.

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