



UNIVERSITI PUTRA MALAYSIA

**SOMATIC EMBRYOGENESIS AND ORGANOGENESIS IN TEAK
(*TECTONA GRANDIS* L.)**

LIM SOOI PING

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**SOMATIC EMBRYOGENESIS AND ORGANOGENESIS IN TEAK
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By

LIM SOOI PING

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Fulfilment of the Requirements for the Degree of Master of Science**

January 2008



***“Happiness comes of the capacity to feel deeply,
to enjoy simply, to think freely, to risk life, to be needed...”***

- Storm Jameson

Specially dedicated to Baby Horatio and my Darling Whye Yu

Abstract of thesis to the Senate of Universiti Putra Malaysia in fulfilment of
the requirement for the degree of Master of Science

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Chairman : Associate Professor Maheran Abdul Aziz, PhD

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Teak is one of the most valuable timber trees of the tropics. Commercially, teak wood is prized for its strength, durability and appearance. The ever-increasing demand for teak timber has resulted in large-scale plantations, both within and outside its range of natural distribution. Nevertheless teak growers often faced difficulties in obtaining sufficient planting materials, particularly those with improved genetic qualities. During the past two decades, the shift towards teak biotechnology in teak improvement has taken place, away from traditional breeding programmes. Use of *in vitro* regeneration as an integral component of tree improvement programmes has been initiated for teak and may indeed be useful to overcome the handicaps associated with teak production. Therefore this project attempts (1) to study the production of callus and development of somatic embryos from embryogenic callus for genetic manipulation purposes, and (2) to develop an improved *in vitro* shoot multiplication and plant regeneration protocol. In the first study, teak callus was successfully induced and categorised into embryogenic and non-embryogenic callus. Based on morphological and anatomical observations, callus production from cotyledon and embryo explants showed similar

morphological responses irrespective of plant growth regulators, media and incubation conditions. Among the six types of callus observed, only type A callus could be classified as embryogenic cell mass based on several criteria i.e. having relatively small (25-35 μ m) and isodiametric cells with distinct nucleus, contained dense cytoplasm rich in starch reserves, and were actively dividing. Type A callus was obtained from embryo explants that were cultured on either half or full strength MS medium with 1.0mg l⁻¹ 2,4-D and 1.0mg l⁻¹ BAP, full strength MS medium with 1.0mg l⁻¹ 2,4-D and 2.0mg l⁻¹ BAP and half strength MS medium with 2.0mg l⁻¹ 2,4-D and 1.0mg l⁻¹ BAP. Only cultures that were incubated in the dark for 24 hours gave rise to embryogenic response. When the embryogenic callus was subcultured in liquid medium in several stages, early stage somatic embryos (globular and early heart stage) were obtained, before further development was arrested. The failure of the somatic embryos to mature beyond this stage was likely due to the requirements for specific maturation conditions. In the second study, proliferation of shoots was achieved with four types of explants namely shoot tip, node, cotyledon and zygotic embryo. The embryo explant was found to produce the highest number of shoots with an average of 9.3 shoots (76% response) per explant on MS medium with 2.0mg l⁻¹ BAP and 0.01mg l⁻¹ NAA. The microshoots from embryo explants developed well on elongation medium with 0.1mg l⁻¹ BAP and subsequently developed stout roots on rooting medium with 0.01mg l⁻¹ NAA. The successful initiation of early stage somatic embryos should be further explored by narrowing the concentration of growth regulators used in this study in order to increase the production of embryogenic callus as well to enhance the maturation of somatic embryos into whole plantlets. Concurrently the shoot proliferation medium that has been established in this study may well be utilised to regenerate the embryos.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

**EMBRIOGENESIS SOMA DAN ORGANOGENESIS DALAM JATI
(*TECTONA GRANDIS* L.)**

Oleh

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Pokok jati merupakan salah satu jenis pokok balak yang paling berharga di kawasan tropika. Dari segi komersial, kayu jati bernilai tinggi disebabkan sifat keteguhan, kelasakan dan kecantikannya. Permintaan untuk kayu jati yang kian meningkat telah mengakibatkan penanaman pokok jati secara besar-besaran di dalam dan di luar kawasan taburan semulajadinya. Walaubagaimanapun, para usahawan ladang jati masih menghadapi masalah mendapatkan tanaman yang mencukupi terutamanya bahan yang mempunyai kualiti genetik yang tinggi. Semenjak dua dekad yang lalu, pembaikan jati telah beralih daripada pembiakbaikan tradisional kepada bioteknologi. Kaedah regenerasi *in vitro* telah diamalkan dalam program pembiakbaikan jati dengan tujuan dapat mengatasi masalah-masalah yang sering dikaitkan dengan jati. Oleh itu, projek ini dilaksanakan untuk (1) mengkaji pembentukan kalus dan penghasilan embrio soma daripada kalus embriogenik bagi tujuan manipulasi genetic, dan (2) membina satu protokol penggandaan pucuk dan regenerasi tumbuhan secara *in vitro* yang lebih berkesan. Dalam kajian yang pertama, kalus jati telah berjaya diperolehi dan diklasifikasikan kepada kalus embriogenik dan kalus bukan embriogenik. Berdasarkan pemerhatian morfologi dan

anatomi, kotiledon dan eksplan embrio dapat menghasilkan kalus yang mempunyai respon morfologi yang hampir serupa walaupun pelbagai rawatan iaitu hormon tumbuhan, media dan keadaan inkubasi yang berlainan digunakan. Hanya kalus jenis A daripada enam kalus yang diperhatikan boleh diklasifikasikan sebagai kalus embriogenik berdasarkan beberapa kriteria iaitu bersaiz kecil ($25-35\mu\text{m}$), isodiametrik dan berupa bulat dengan nukleus yang nyata, mengandungi sitoplasma yang padat dengan simpanan kanji dan mengalami pembahagian sel yang aktif. Kalus jenis A diperolehi apabila eksplan embrio dikultur di atas medium MS dengan kepekatan separa atau penuh dan mengandungi 1.0mg^{-1} 2,4-D dan 1.0mg^{-1} BAP, medium MS berkepekatan penuh yang mengandungi 1.0mg^{-1} 2,4-D dan 2.0mg^{-1} BAP dan medium MS berkepekatan separa yang mengandungi 2.0mg^{-1} 2,4-D dan 1.0mg^{-1} BAP. Hanya kultur dengan rawatan inkubasi gelap sepanjang 24 jam menghasilkan kalus yang embriogenik. Kalus embriogenik yang disubkultur dalam medium cecair dalam beberapa peringkat dapat menghasilkan embrio soma peringkat awalan (jenis globular dan bentuk hati) sebelum perkembangan selanjutnya terjejas. Embrio soma gagal berkembang selepas peringkat ini berkemungkinan disebabkan keperluan syarat-syarat kultur untuk membolehkannya menjadi matang. Dalam kajian yang kedua, penggandaan pucuk telah diperolehi dengan menggunakan empat eksplan iaitu tunas pucuk, nod, kotiledon dan embrio zigot. Dalam medium MS yang mengandungi 2.0mg^{-1} BAP sahaja, min pucuk sebanyak 4.3 dan 4.1 telah diperolehi daripada tunas pucuk dan eksplan nod masing-masing dengan respon sebanyak 80-100%. Eksplan yang berasal daripada benih jati (kotiledon dan embrio zigot) sebaliknya menghasilkan lebih banyak pucuk dengan kehadiran NAA. Enam puluh peratus daripada eksplan kotiledon dapat menghasilkan purata 4.0 pucuk dalam medium MS yang mengandungi 1.0mg^{-1} BAP

dan 0.1mg^{-1} NAA. Walaubagaimanapun, ekplan embrio menghasilkan bilangan pucuk yang paling banyak dengan min pucuk sebanyak 9.3 (respon sebanyak 76%) dalam medium MS yang mengandungi 2.0mg^{-1} BAP dan 0.01mg^{-1} NAA. Pucuk mikro yang diperolehi daripada eksplan embrio bertumbuh baik dalam medium elongasi yang mengandungi 0.1mg^{-1} BAP dan seterusnya menghasilkan akar yang teguh dalam medium pengakaran yang mengandungi 0.01mg^{-1} NAA. Kajian inisiasi embrio soma peringkat awalan perlu dilanjutkan dengan meneliti jenis hormon tumbuhan dan kepekatan yang digunakan dalam kajian ini untuk meningkatkan penghasilan kalus embriogenik dan menggalakkan kematangan embrio soma kepada tumbuhan yang lengkap. Pada masa yang sama, medium pembentukan pucuk yang telah diperolehi dalam kajian ini bolehlah digunakan untuk regenerasi embrio.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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Date:

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ABA	Absciscic acid
BAP	Benzylaminopurine
CPA	p-chlorophenoxyacetic acid
DMRT	Duncan's Multiple Range Test
et al	et alia
etc	et cetera
e.g.	example gracia (for example)
EM	Basic MS medium supplemented with 30gl ⁻¹ of sucrose, 7gl ⁻¹ agar and growth regulators (where required)
FAA	Formaldehyde acetic acid
FRIM	Forest Research Institute Malaysia
gl ⁻¹	Gram per litre
GA ₃	Gibberelic acid
GM	Basic MS medium supplemented with 30gl ⁻¹ of sucrose and 7gl ⁻¹ agar
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
<i>i.e.</i>	<i>id est (that is)</i>
KIN	6-furfuryl-aminopurine (Kinetin)
kPa	Kilo pascal
M	Molar
MS	Murashige and Skoog (1962) inorganic salt
Mgl ⁻¹	Milligram per litre
mM	Millimolar
NAA	α-napthalene acetic acid
NaOH	Sodium hydroxide
RCBD	Randomised Complete Block Design

RM	Basic MS medium at half strength supplemented with 20gl ⁻¹ of sucrose, 7gl ⁻¹ agar, growth regulators (where required) and activated charcoal (where required)
SAS	Statistical Analysis System
SM	Basic MS medium supplemented with 30gl ⁻¹ of sucrose, 7gl ⁻¹ agar and growth regulators (where required)
p=0.05	Probability at 95%
μM	Micro molar
μmolm ⁻² s ⁻¹	Micromole per square per second
2,4-D	2,4-dichlorophenoxy acetic acid
2ip	2- isopentenyl
2,4,5-T	2,4,5-trichlorophenoxy acetic acid
%	Percent
°C	Degree centigrade

CHAPTER 1

INTRODUCTION

Tectona grandis or commonly known as teak is highly sought after for its quality and durable timber which is also resistant to termites and fungi. Owing to declining natural teak forests, this species has been a favourite choice for agro-forestry in the tropics as well as in areas much beyond its natural limits. Of the estimated 187.1 million hectares of global forest plantations in 2000, about 5.7 million hectares were teak, which roughly covers 75% of the world's high-quality tropical hardwood plantations (FAO, 2001).

In Malaysia, teak has been recognised as a potential crop for large-scale plantation programmes to produce short-term rotation timbers (Krishnapillay and Abdul Razak, 1998). In many countries veneer and sawnlog production with shorter rotations of 20-30 years are now being employed for relatively quick returns (Ball *et al.*, 1999). Currently 3,990 hectares of teak plantations have been established in Malaysia (Bhat and Ma, 2004) of which teak plantations in Sabah cover approximately 2,214 hectares (Aminuddin, 2003).

The success of timber plantation such as teak depends on the availability of large quantities of good planting stock. Teak is generally reproduced from fruits which contain one to four seeds per fruit; nevertheless seed germination is often difficult due to various physiological, physical and morphological barriers. Alternatively teak has been propagated *via* cuttings as well as budding and grafting but these methods

have severe limitations, including rooting and incompatibility problems, and only provide a few propagules from selected individuals.

In vitro regeneration system via tissue and organ culture offers an alternative and novel possibility to increase the production and germination rates of viable seeds from elite trees as well as to produce clones of genetically superior planting materials for large-scale plantations. New development in genetic engineering procedures, including somatic hybridization and gene manipulation technologies, offer opportunities to increase teak forest productivity, wood quality, and other desirable properties such as resistance to pest and pathogens, stress and herbicides. These biotechnological approaches, when combined with methods of *in vitro* propagation, could greatly reduce the rotation period for well-established teak plantations, which require between 30 to 80 years under conventional breeding program.

In vitro propagation of *T. grandis* has been well reported in India, Thailand and Indonesia; however, with varying degree of success. In Malaysia, Forest Research Institute of Malaysia (FRIM) has been actively pursuing the research on teak tissue culture. Most of the protocols were developed to multiply shoots by means of shoot tip and axillary bud cultures. Common problems including exudation of phenolic substances into culture medium and risk of contaminations from field-sourced materials are often encountered. Also many papers has shown that the process of repeated cycles of subculturing increases the risk of *in vitro* shoots exhibiting vitrification phenomena, genetic abnormalities and reduced regeneration capacity. Thus selected juvenile materials that are able to undergo rapid shoot multiplication