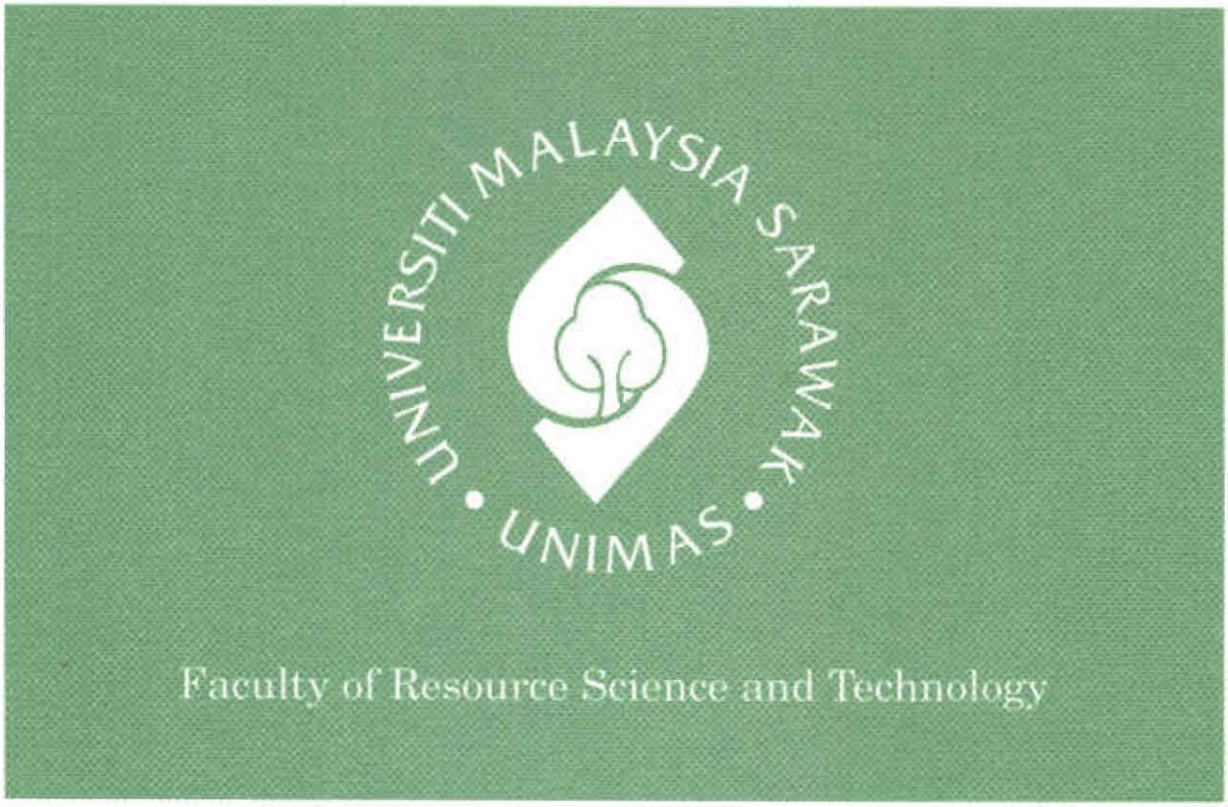

ESTABLISHMENT OF CONTAMINATION-FREE CULTURE AND *IN VITRO REGENERATION* OF *SHOREA LEPROSULA* MIQ.

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Bachelor of Science with Honours
(Resource Biotechnology)
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Establishment of Contamination-free Culture and *In Vitro* Regeneration of *Shorea leprosula* Miq.

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This Final Year Project Report is submitted in partial fulfillment of requirements for the degree of Bachelor of Science with Honours in Programme Resource Biotechnology

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DECLARATION

I hereby declare that Final Year Project Report 2009 is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been or concurrently submitted for any other degree at UNIMAS or other institutions of higher learning.

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TABLE OF CONTENTS

APPENDICES

LIST OF ABBREVIATIONS

BAP	6-Benzylaldenine Purine
CRD	Completely Randomized Design
GA_3	Gibberellic Acid
IBA	Indole-3-butyric Acid
MS	Murashige and Skoog Medium
NAA	Naphthalene acetic acid
PPM	Plant Preservative Mixture
RO	Reverse Osmosis Water
TDZ	Thiadiazuron
WPM	McCown Woody Plant Medium
2iP	N6-[2-isopentyl]adenine

LIST OF TABLES

LIST OF FIGURES

Establishment of Contamination-free Culture and *In Vitro* Regeneration of *Shorea leprosula* Miq.

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ABSTRACT

Shorea leprosula Miq., a fast growing dipterocarps, has potential for reforestation of degraded forest. However, planting stock of this species is scarce because fruiting is irregular and the seeds are recalcitrant. Micropropagation has been considered as an alternative to mass produce the planting stock. The objective of this study was to develop an effective surface sterilization protocol to establish contamination-free culture as the first step towards development of micropropagation of *S. leprosula*. The surface sterilization protocol developed so far started with soaking the field-derived shoot-tip and nodal explants in a fungicide solution for one and three hours respectively. The explants were then dipped in 75% ethanol for one minute and surface sterilized in 5% Clorox added with Tween 20 for 10 minutes and Chlorine Dioxide at 50 ppm for 10 minutes. The explants were then rinsed with autoclaved-RO water for at least three times. Nodal explants were pulse-treated with 10 ml/L PPM for overnight whereas shoot-tip explants were pulsed-treated with PPM at 5 ml/L for an hour. Surface-sterilized explants were trimmed and cultured on MS basal medium supplemented with 20 g/L sucrose. BAP and TDZ were found to be effective in inducing new buds growth on nodal explants. Nodal explants inoculated on MS basal medium supplemented with 2 mg/L BAP and 0.1 mg/L NAA showed new buds growth.

Key words: *Shorea leprosula* Miq., *in vitro* regeneration, surface-sterilized, inducing new buds growth

ABSTRAK

Shorea leprosula Mique mempunyai kadar tumbesaran yang cepat dan berpotensi digunakan untuk tujuan penanaman semula hutan. Walau bagaimanapun, stok spesis ini tidak mencukupi kerana perbungaan spesis ini tidak konsistent dan masa perbuahan yang lama. Mikropropagasi merupakan satu alternatif untuk menghasilkan stok tumbuhan ini secara besar-besaran. Objektif kajian ini adalah untuk menghasilkan satu protokol pensterilan pernukaan yang berkesan untuk menghasilkan kultur yang bebas dari pencemaran sebagai langkah yang pertama dalam S. leprosula mikropropagasi. Protokol pensterilan permukaan bermula dengan menrendamkan eksplan nodus dan hujung pucuk yang berasal daripada luar dalam racun kulat selama satu dan tiga jam masing-masing. Eksplan direndamkan dalam 75% ethanol selama 60 saat dan disteril dalam kombinasi 5% (v/v) Clorox and Tween 20 selama 10 minutes dan Clorine dioxide (50ppm) selama 10 minute masing-masing dengan goncangan yang perlahan. Selepas itu, eksplan hujung pucuk disteril dengan 5 ml/L PPM selama satu jam manakala eksplan nodus disteril dengan 10 ml/L PPM selama dua puluh empat jam. Eksplan yang telah disteril ini dikultur dalam MS media yang ditambahkan dengan 20 g/L sucrose. Dalam kajian ini, BAP and TDZ didapati memberi kesan yang baik dalam induksi pertumbuhan pucuk bagi eksplan nodus. Eksplan nodus yang dikultur dalam MS media yang ditambahkan dengan 2 mg/L BAP dan 0.1 mg/L NAA menghasilkan pucuk baru.

Kata Kunci: Shorea leprosula Mique, in vitro regenerasi, pensterilan permukaan, induksi pertumbuhan banyak pucuk

1 INTRODUCTION

Dipterocarpaceae hold the distinction of being the most well known trees in the tropics. This family, consisting 580 species in 15 genera, is of palaeotropical distribution and found chiefly in Indomalaysia. Dipterocarp species have long been recognized in Southeast Asian and other regions for both their hardwood timber and non-timber products. Timber of these trees makes up about 25 percent of the total global tropical hardwood timber trade (Smits, 1987). In addition, several *Shorea* species (e.g. *Shorea javanica)* also yield other products, such as resin for the production of varnishes and turpentine. Fruits of several dipterocarps contain fat which can be used in chocolate and cosmetic industries (Sidiyasa, 1995). However, growth rates of Dipterocarp species are too slow. The exploitation of the dipterocarp forests by logging operations also caused a sharp decrease in this resource.

Shorea appears to be the largest and economically most important genus in the family of Dipterocarpaceae (Symington, 1943). The genus *Shorea* consists of 188 species and is widely distributed from Southern Thailand (Pattani), throughout Peninsular Malaysia, Sumatra to Borneo (Lee, 2000). Among *Shorea* species, *Shorea leprosula* Miq. has been identified as one of the most important timber species in tropical Asia (Joker, 2002). It is commonly known as Meranti Tembaga in Malaysia. This species is classified as light hardwood with the density of 0.3–0.55 g/cm^3. It is excellent for joinery, furniture, panelling, flooring, ceiling and plywood manufacturing. Besides that, it also produces resin called ‘Damar Daging’ with medicinal property. The bark can be used for tannin production (Joker, 2002).

S. leprosula is a fast growing dipterocarps (Masano *et al.*, 1987) and have potential for reforestation of degraded forest (Sakai *et al.*, 2001). A stand of this species achieved an average height and diameter of 44.6 m and 77 cm respectively in 35 years (Appanah & Turnbull, 1998). Flowering normally occurs every three to five years. The fruits are ripe some 14 weeks after flowering. If a dry spell occurs during this period, fruit fall will be delayed, thus the fruits will not be well developed (Joker, 2002). Moreover, the early falling seeds are often immature and attacked by insects or animals.

This dipterocarp species has been propagated mostly through seeds and cuttings. Seed production of this species is erratic and infrequent. Furthermore, the recalcitrant seeds deteriorate rapidly in storage. Recalcitrant seeds undergo little, or no, maturation drying and remain desiccation sensitive both during development and after they are shed (Berjak & Pammenter, 2003). The seeds do not tolerate temporary storage as a result of high moisture content (almost 50%) at the time of collection. Study indicated that the seeds tolerate drying to 25-30% moisture content with little loss in viability (Joker, 2002). Thus, the seeds cannot be preserved or stored for long periods as they lack of dormancy. In addition, production of cuttings from mature trees is difficult and plagiotropic growth of shoots from cuttings is common. In order to address these common problems of timber species, researches have been using plant tissue culture techniques to mass clonally propagate this species. Nakamura *et al.* (1999) had reported the efficient mass propagation of *Shorea roxburghii* and *Gmelina arborea* by shoot-apex culture. Nevertheless, there is an increasing interest in clonal propagation of dipterocarp species by tissue culture due to the rapid disappearance of the Dipterocarpaceae forest.

Furthermore, tissue culture is the best and reliable approach to produce a large quantity of genetically true-to-type planting stock. A basic premise in commercial micropropagation, as in all mass production, is the guarantee of a consistently high degree of likeness between the template, here the source plant, and the product. Seed-raised plants show wide variations in the field due to heterozygous of the parents naturally. The techniques of plant tissue culture give an exponential increase of the propagation coefficient. Depending on the multiplication rate, thousands and millions of plants can be rapidly produced *in vitro* from relatively few selected source plants (Altman & Rita, 1997). Hence, plant tissue culture techniques can rapidly increase the number of individuals of endangered species with reproductive problems and/or extremely reduced populations due to deforestation (Iriondo & Pérez, 1990). These techniques could be useful for *in vitro* propagation of *S. leprosula*. Thus, the objective of this study is to establish an effective protocol for *in vitro* regeneration of *S. leprosula* for use in large-scale propagation.

2 LITERATURE REVIEW

2.1 The Plant-*Shorea leprosula* Miq.

Shorea leprosula is also known as *Hopea maranti* Miq., *Shorea maranti* Burck and *Shorea astrostricta* Scort. Ex. Foxw. This species is widely known as Meranti Tembaga in Malaysia and Indonesia. It also has other vernacular names such as Meranti Pusuh (Sarawak), Seraya Tembaga (Sabah), Meranti Merah, Kontoi Bayor (West Kalimantan) and Lempong Kumbang (East Kalimantan). In Thailand, it is known as Saya-Daeng, Kalo Khao, Pha Yom Daeng and Ta Yom (Peninsular Thailand).

The taxonomic classification of *Shorea leprosula* is as below:-

Kingdom	:	Plantae
Subkingdom	:	Viridaeplantae
Phylum	:	Tracheophyta
Subphylum	:	Spermatophytina (auct.)
Infraphylum	:	Angiospermae auct.
Class	:	Magnoliopsida
Order	:	Clusiales
Family	:	Dipterocarpaceae
Tribe	:	Shoreae
Genus	:	*Shorea*
Specific epithet:		*leprosula* Miq.
Botanical name:		*Shorea leprosula* Miq.

2.1.1 Distribution and Habitat

Shorea leprosula is distributed from Southern Thailand (Pattani), throughout Peninsular Malaysia, Sumatra to Borneo (Lee, 2000). It is a common species in the lowland dipterocarp forests below 700 m altitude where it colonises gap openings in disturbed forests. It can grow on a variety of soils but does not tolerate waterlogged sites, especially peat soils. Rainfall of 1500-3500 mm per year and short dry periods are conducive for its growth and regeneration. It is seldom found on ridges. Plantation trials have shown that it grows better in the foothills than on ridge tops (Joker, 2002).

At the lowland dipterocarp forest of Pasoh Forest Reserve in Peninsular Malaysia, the density of *Shorea leprosula* with diameter > 30 cm is three trees per ha. However, it is much less common in Borneo where it is apparently replaced by *Shorea smithiana.* In natural habitat, it is dynamic with high seedling mortality and high percentage of recruitment. It requires partial shade for the initial establishment but its later growth responds greatly to light. Spatial distribution studies in lowland and hill dipterocarp forests of Peninsular Malaysia have showed that the species is highly aggregated (Lee, 2000).

2.1.2 General Morphology

The trees are readily recognized from a distance by the coppery crown, due to yellow-brown or grey-brown tomentose on the underside of the leaves. A large tree can reach up to 60 m in height and 175 cm in diameter with clear bole up to 35 m (Lee, 2000). Buttresses which are prominent are usually not very large (Joker, 2002). They can reach to

1.5 m in height which are spreading and concaving (Lee, 2000). The crown is wide, umbrella-shaped and characteristically yellowish brown (Joker, 2002). The bark of this tree is light gray to gray brown, smooth when young and irregularly longitudinally fissured with concave ridges in older specimen. Slash inner bark is reddish brown, sapwood is cream-colored and heartwood is light red-brown with conspicuous radial resin canals (Lee, 2000).

The leaves shape is elliptic to ovate with 8-14 cm long and 3.5-5.5 cm wide. Lower leaf surface is cream scaly. Most of the lengths of midrib are beset with domatia at least in young trees with tertiary veins forming a densely ladder-like structure. The flowers are small with pale yellow corolla with narrow petals which incurved like a clutching hand (Joker, 2002).

2.1.3 Flowering and Fruiting

Flowering behavior of *S. leprosula* is sporadic throughout the year and gregarious at intervals of three to five years. Most of the mature trees in population may flower heavily and synchronously during a general flowering. It is the last species in the flowering sequence of section *Muticae*. The flowers open in the evening. They are strongly scented and are pollinated by flower thrips. The tree which is mainly outcrossing is a diploid (2n=14). The fruits are ripe some 14 weeks after flowering. If a dry spell occurs during this period, fruit fall will be delayed and the fruits will not be well developed. Seed dispersal is mainly gravital and seldom exceeds 50 m from the mother trees. In the area of natural

distribution, collection takes place in March-July, typically a few months after a prolonged dry period (Lee, 2000).

The fruit is a nut enclosed in the enlarged calyx lobes. Calyx is sparsely pubescent with 3 longer lobes up to 10 cm long and 2 cm wide which are spatula-like and 2 shorter lobes up to 5.5 cm long with 0.3 cm wide. The nut is about 2 cm long and 1.3 cm in diameter. It is ovoid and pubescent with a sharp pointed end from the remains of the style. The unit for sowing and testing is the fruit and the lower part of the calyx which is left after the wings have been removed. There are 1300-2100 de-winged fruits per kg (Joker, 2002).

2.1.4 Economic Significance

S. leprosula is one of the most common light red meranti timbers with a density of 425-685 kg/m^3. It is valuable for joinery, furniture, panelling, flooring and also used for plywood manufacture. The resin, called ‘damar daging’ is found between the roots and is used in traditional medicine. The bark is used for tanning (Lee, 2000).

2.2 Plant Tissue Culture

Plant tissue culture can be referred as the science of growth and development of axenic plant cells, tissues or organs isolated from the mother plant on nutrient medium. Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency).