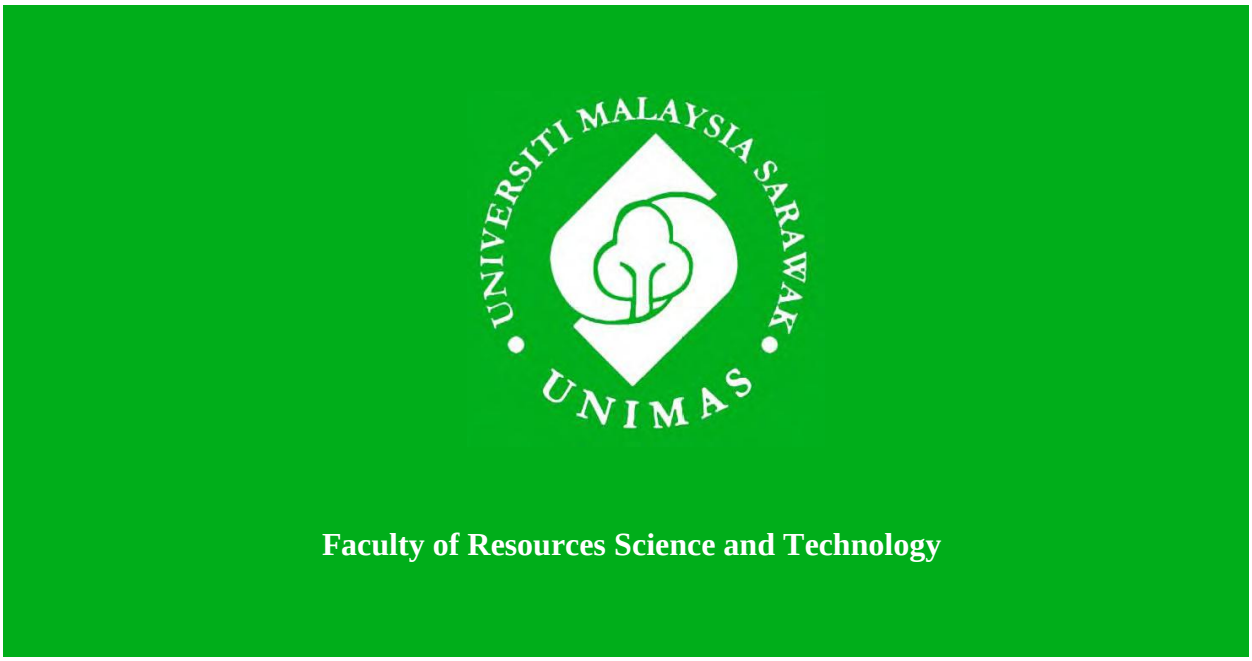

SUSPENSION CULTURE OF *AQUILARIA BECCARIANA* VAN TEIGH.

Khairunisa Binti Ab Aziz

Bachelor of Science with Honours
(Plant Resource Science and Management)
2013

SUSPENSION CULTURE OF AQUILARIA BECCARIANA VAN TEIGH

KHAIRUNISA BINTI AB AZIZ

26604

This project is submitted in fulfilment of the requirements for the Degree of Bachelor of Science with Honours

(Plant Science Resource and Management)

Faculty of Resource Science & Technology

UNIVERSITI MALAYSIA SARAWAK

2013

APPROVAL SHEET

Name of candidate : Khairunisa Binti Ab Aziz

Title of dissertation : Suspension Culture of *Aquilaria beccariana* Van Teigh

_____

(Prof. Dr. Hamsawi Sani)

Supervisor

_____

(Dr Rebicca Edward)

Coordinator of Plant Resource Science and Management Programme

Department of Plant Science and Environmental Ecology

Faculty of Resource Science amd Technology

Universiti Malaysia Sarawak (UNIMAS)

DECLARATION

I hereby declare that this Final Year Project 2013 is based on my original work except for quotations and citations, which have been duly declared that it has not been or concurrently submitted for any degrees at UNIMAS or other institutions of high education.

_____

Khairunisa Binti Ab Aziz

Plant Resource Science and Management

Department of Plant Science and Environmental Ecology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak (UNIMAS)

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest appreciation to my supervisor, Prof. Dr. Hamsawi Sani, for his guidance, support, advice, and help in complete my Final Year Project (FYP) on suspension culture.

I also would like to thank Mr Zul Helmey Mohamad Shabdin and Ms.Cherrie Lim Han Rou, the postgraduate students in our laboratory for the guidance, suggestion of essential references and giving me plenty useful information and technique about tissue culture. A special thanks to my laboratory mate, Ms Noor Izzieana Ibrahim for her full support and assistance in finishing my project. Thus, I also would like to express my gratitude to Ms Nor Emeylia Md Zain for her help and encouragement. A special thanks to my laboratory mates Ms.Siti Zalikha Mohd Ridzuan Jamil and Ms.NurAmalia Kamal Abidin Shah.

Last but not least, deepest thank to my parents and other family members for their care, love, support and understanding me in completion this study.

Suspension Culture of *Aquilaria beccariana* Van Teigh

Khairunisa Binti Ab.Aziz

Plant Resources and Management Programme
Faculty of Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Aquilaria beccariana van Teigh (Thymelaceae), is a non timber forest product. The tree is valuable for its fragrant wood. Agarwood which is important for incense, traditional medicine, perfumery and others products. The population of Aquilaria has been reduced drastically due to the indiscriminate collection, over exploitation and lacking of rapid natural regeneration. The population of this tree became declined and the production of gaharu is less compared to highly demand of the user. Thus, *in vitro* technique was selected because it offer rapid regeneration and conservation of these endangered species and this study, attempts were done to produce callus from *Aquilaria* sp. Works were done to induce callus from lamina explants on MS solid and MS liquid medium supplemented with different combination of hormone (2,4-D, picloram and NAA) and varied concentration of sucrose (20-50 g/L). The callus culture was successfully established in MS solid media supplemented with combination of 2.0 mg/L 2,4-D plus 0.5 mg/L BAP. While in MS liquid medium, the suspension culture successfully established in combination of 3 mg/L NAA plus 0.5 mg/L BAP by obtaining highest frequency of fresh weight (2.09g). Thus, 40 g /L found to be the best sucrose concentration with (1.95 g) of fresh weight. While, combination of 0.25 mg/L picloram with 2.0 mg/L 2,4-D and 0.5 mg/L BAP gave high frequency of fresh weight (1.06 g) in Picloram effect. Suspension culture of callus gave friable and compact in texture and globular and irregular in shape.

Keywords: *Aquilaria beccariana* van Teigh, *in vitro* technique, suspension culture, callus induction, fresh weight

ABSTRAK

Aquilaria beccariana van Teigh (Thymelaceae), bukan produk hutan kayu. Pokok ini bernilai untuk kayu wangi itu. Gaharu penting untuk kemenyan, ubat tradisional, produk minyak wangi dan lain-lain produk. Populasi Aquilaria telah berkurang secara drastik kerana koleksi sembarangan, lebih eksploitasi dan kekurangan pertumbuhan semula yang pesat. Populasi pokok ini menjadi berkurangan dan pengeluaran gaharu adalah kurang berbanding dengan permintaan pengguna. Oleh itu, teknik vitro menawarkan kaedah cepat dan pemuliharaan spesis terancam dan kajian ini, usaha telah dilakukan untuk menghasilkan kalus daripada pokok karas. Kerja-kerja telah dilakukan untuk kalus daripada eksplan lamina pada MS pepejal dan MS medium cecair ditambah dengan kombinasi yang hormo yang berbeza (2,4-D, picloram dan NAA) dan kepekatan pelbagai sukrosa (20-50 g / L). Pertumbuhan kalus telah berjaya dalam MS media pepejal ditambah dengan gabungan 2.0 mg / L 2,4-D serta 0.5 mg / L BAP. Dalam MS medium cecair, pertubuhan kalus berjaya dalam gabungan 3 mg / L NAA serta 0.5 BAP dengan mendapatkan kekerapan tertinggi berat basah (2.09g.)Dalam, 40 g / L mendapati kepekatan sukrosa terbaik diperolehi (1.95 g) berat basah. .Gabungan 0.25 mg / L picloram dengan 2.0 2,4-D mg/L dan 0.5 mg / L BAP memberikan frekuensi tinggi berat badan segar (1.06 g) berkuat kuasa Picloram. Budaya penggantungan kalus memberikan rapuh dan padat dalam tekstur dan bulat dan tidak teratur dalam bentuk.

Kata kunci: *Aquilaria beccariana van Teigh, in vitro teknik, cecair kultur,induksi kalus,berat basah*

TABLE OF CONTENTS

LIST OF APPENDICES

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlropenoxacetic acid
ANOVA	Analysis of variance
BAP	6-benzylaminopurine
IBA	Indole 3-butyric acid
MS	Murashige and Skoog medium
NAA	1-Napthaleneacetic acid
Picloram	4-amino-3,5,6-trichloropicolinic acid
FW	Fresh weight
DW	Dry weight

1.0 INTRODUCTION

1.1 Introduction

Agarwood, locally known as gaharu is belongs to the Genus *Aquilaria* under family Thymeleaceae. Agarwood is a natural forest product that obtained from *Aquilaria* spp. There are 20 species of *Aquilaria* distributed in tropical and subtropical Asia. According to Lau & Chua (2011), they are five known species in Malaysia which are *A. beccariana*, *A. malaccensis*, *A. macrocarpa*, *A. hirta* and *A. rostrata*. The tree is valuable for its fragrant wood which is used for perfumery, incense (Soehartano & Newton, 2000) and medicinal purpose. It's produced resin with aromatic smell.

Previous study by Wetwitayaklung *et al.*, (2009), state that gaharu is naturally produced by infection of fungi, bacterial, insects, physical cut and chemical stimulation. However, the resin production could be achieved by applying tissue culture technique. The callus from *in vitro* technique will infect by some fungus for resin production. This method tends to be popular in order to avoid long period in resin production.

In recent years, the demand for agarwood has been increased, so that this species are critically endangered because of indiscriminately cut down in natural forest. As a result, the population of natural gaharu tress has been drastically decreased (CITES, 2004). There are effort to plant gaharu in large scale; however most of the project has been hampered by the storage of planting stock. With the shortage of seedling for large scale planting, the next alternative is to produce gaharu by vegetative propagation such as by cutting and *in vitro*.

In vitro suspension could be an alternative method to produce gaharu. This can be done through formation of callus by suspension culture. Study by Okudera and Ito (2009), showed the callus and suspension cell culture successfully in production of fragrant compound contain in *Aquilaria* sp. The major compound in agarwood like sesquiterpenoid was successfully found in their studies.

Thus, suspension cultures have ability to produce friable callus would determine the success of gaharu production *in vitro*. Proper combination of auxin and cytikinin will produce the friable callus in large scale production and being used for fungus inoculation in gaharu production.

1.2 The objective of this study were

i. To establish a working protocol for callus induction by using MS (Murashige & Skoog, 1962)
ii. To determine the effect of different levels of 1-Naphtaleneacetic acid on cell proliferation
iii. To examine the effect of sucrose concentration on cell proliferation and
iv. To identify the different of level of picloram on cell proliferation in MS liquid media

2.0 LITERATURE REVIEW

2.1 *Aquilaria* sp

Aquilaria is one of fifteen genera of trees in Thymelaeaceae family, which is native to Southeast Asia, Northern India and Vietnam. The tree can grow up to 40 meters in height and reach a diameter of 60 cm. The tree usually grows straight, but sometimes fluted or with thick (10cm) buttress up to two meters high and commonly found in mixed hill forests across Southeast Asia (Whitmore, 1972).

The leave of this tree is alternate, pinnately vein, without translucent glandular dots and fibrous. The flowers are 5-merous, the fruits loculicidal capsules and have smooth, pale whitish bark (Tawan, 2004). Besides that, others species in *Aquilaria* like *A.crassana* is medium size tree of 15-20 cm height and 40-50 cm diameter (dbh) at maturity (Hoang and Nguyen, 2002). While the others species such as *A.malacensis* show differ by achieved 20-40 cm in height and 60 cm (dbh) (Lata, 2007). According to Lata (2007), both of the species white in colour, light in weight and soft in density.

2.1.1 Taxonomic classification and nomenclature of Gaharu

According to Lau and Chua (2011), *Aquilaria* sp is also known as Malayan eaglewood, agarwood, aloeswood, gaharu, engkaras (Iban), Karas (Malay). Agarwood is classified as non wood forest product (Lata, 2007). The taxonomic classification and nomenclature of *A. beccariana* is shown in Table 1.

Table 1: Taxonomic classification of *A. beccariana*

Kingdom	**Plantae**
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Myrtales
Family	Thymeleaceae
Genus	*Aquilaria*
Species	*Aquilaria beccariana* van Teigh

Source: (Yusof, 2008)

Basically *A. beccariana* is widely distributed at Sumatra and Peninsular Malaysia. In Malaysia, *A. beccariana* locally known as Karas where the species that producing gaharu. According to Tawan (2004), this evergreen species can grow up to 20 m tall and 36 cm diameter. It can be found in mixed dipterocarp forest which altitude to 1000 m above the sea level. The leave is 7-27 × 3-8 cm with cuneate to attenuate base. The flower produced in umbel; the fruit is producing from top of calyx tube. Figure 1 showed the morphology and vegetative part of *A. beccariana* sp.

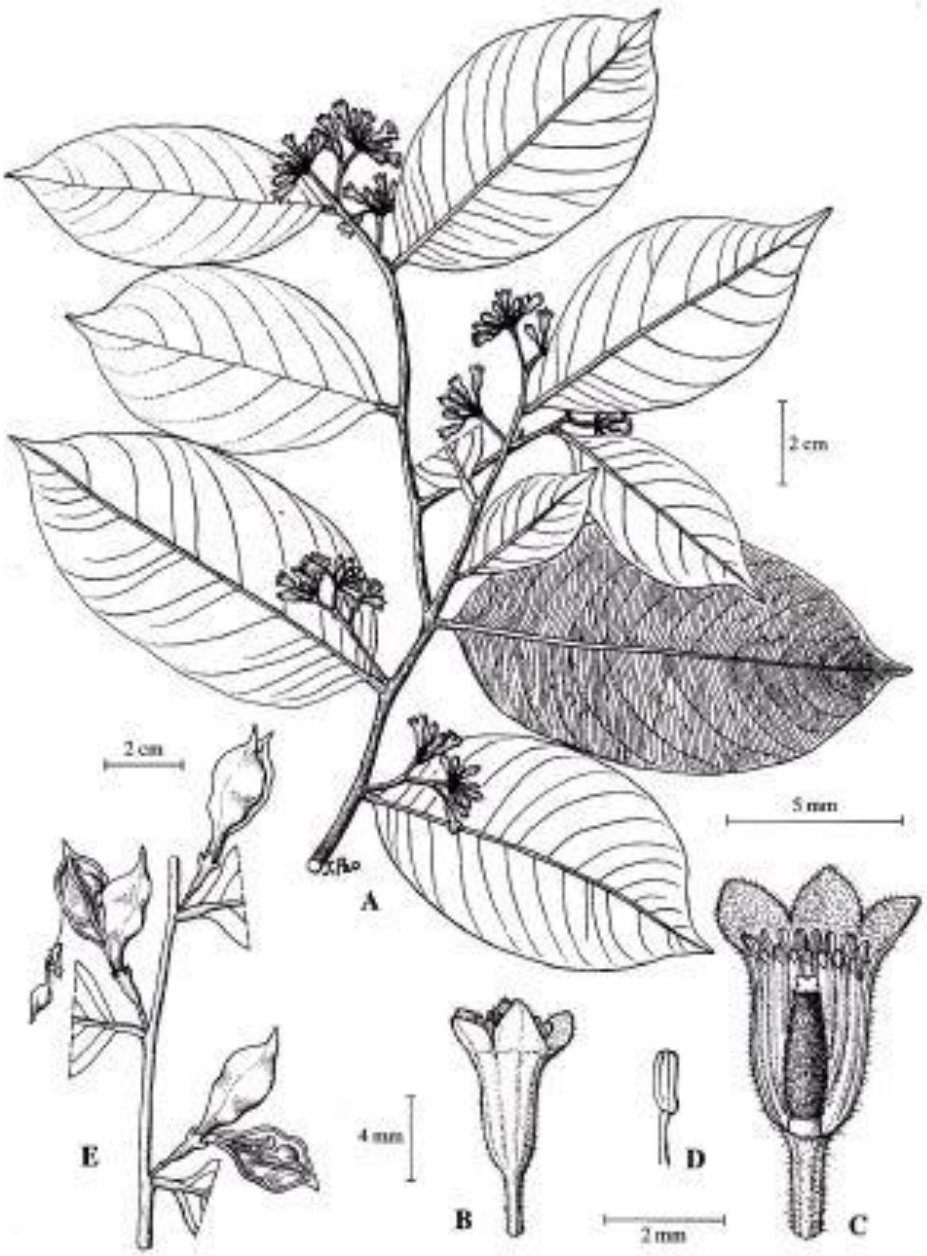

Figure 1: morphology and vegetative part of *A. beccariana* sp.
Source: Tawan (2004).

(a). Flowering leafy twig
(b). Open flower
(c). Longitudinal section of open flower
(d). Stamen
(e). Fruiting leafy twig

2.1.2 Distribution and ecology

Aquilaria sp is widely distributed all over the world. This species could be found in Malaysia, India, Burma and Thailand. According to (Ding, 1960), these species could be found in southern Asia from India to China and much of Indonesia. Others report also stated that, agarwood producing species found from India eastward to Island of New Guinea which includes all Southeast Asian country and north to Hainan island where located at Southeast China. In Thailand, there are four indigenous species reported which are *A. crassana, A. malaccensis, A. subintegra* and *A. baillonii* (Wetwitayaklung *et al.,* 2009).

According to Nor Hasnida *et al*., (2011), there are total of 19 spp of agarwood native to Malaysia (Peninsular: 13 spp, Sabah; 11 spp, Sarawak; 15 spp). Other report stated that, there are fifteen species in *Aquilaria* spp and eight of them are known to produce gaharu (Ahmad Junaidy, 2008). Mean while, there are only 5 species producing gaharu reported in Malaysia. They are *A. macrocarpa, A. malaccensis, A. beccariana, A. hirta and A. rostrata* (Whitmore, 1972; Huey, 2008).

Thus, *Aquilaria* consists of species that are adapted to rocky, sandy or calcareous, well drained slopes and ridges and near swamps (Barden *et al,* 2000). According to Chakrabarty *et al.*, (1994), *A. malaccensis* generally found growing up to an elevation of 1000 m and locally in the foothills and undulating slopes of evergreen and semi-evergreen area.

2.1.3 Production of gaharu

Not all *Aquilaria* sp produced gaharu. Agarwood is an accumulation of resinaceous substances produced by the tree and deposited in heartwood (Rahayu, 2010). Naturally,

gaharu obtained from natural forest. According to Soehartano and Newton (2002), gaharu derived from tropical tree of *Aquilaria* Lam. According to Chakrabarty *et al.,* (1994), agarwood frequently found in young tree about 20 years old but the infestation take time to mature. Trees about 50 years old have shown the highest concentration yield approximately 2 to 3 kg per tree.

The resinous substance derived from gaharu produced by tree that response to attack by pathogenic fungi (Ng, Chang & Kadir, 1997). The infection of the fungus spread slowly to all side with increasing and ageing of the initial infection. Generally, *Aquilaria* sp infected by imperfecti fungus such as *Aspergillus* sp, *Penicillium* sp and *Fusarium* sp (Chakrabarty *et al.,* 1994). Other study reported that, the resin created in response to an attack from *Phialophora* parasitica which is parasite fungus (Nurdiyana, 2008). Besides that, Rahayu (2010) stated that the resin produced also part of defense reaction toward mechanical damage apart from attacking by fungus.

While, there are some features in identifying deposited agarwood in the tree. The features are classified below by Chakrabarty *et al.,* (1994):

(a) A poor crown, decayed branches, an even bole

(b) Swelling or depression and cankers on the bole

(c) The appearance of hordes of ant in the fissures

(d) A distinctly yellowish tinge in the wood under outer bark

2.1.4 Chemistry of gaharu

Generally, gaharu oil greatly valued as perfumery ingredient and incense. Thus, agarwood oil is classified to five types which Super A, A, B, C and D. The study done by Nor Azah *et al*., (2008) stated that majority of essential oil made up from sesquirterpenoids and other oxygenated derivatives. The chemical compound like 3-phenyl-butanone, α-guaiene, β-agarofuran, α-agarofuran, agarospirol and jinkoh-eremol were detected in Malaysian gaharu.

Besides that, other study on chemical composition in gaharu showed that, selinene selina-4,11-dien-14-al and selina-4,11-dien-14-al were present in *A. crassana* by applied water distillation and supercritical fluid carbon dioxide extraction (Wetwitayaklung *et al.,* 2009).

In addition, other study claimed that, the chemical compositions founded in *A. agallocha*. Some of them are cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-, caryophyllene oxide and 7-isopropenyl-4a-methly-1-methylenedecahydronaphthalene (Md. Nazrul *et al.,* 2009). These types of chemical obtained from infected plant agar (super agar) by Gas chromatography mass spectrometry (GCMS) method.

Other study done by Zul Helmey *et al*., (2011a) showed that, the chemical components can be determined by GCMS method. In this study, they found some chemical composition in two species of *Aquilaria* which is *A. malaccensis* and *A. microcarpa*. There are varies in number of chemical composition founded. 20 compounds were founded in *A. malaccensis* while 16 compounds were found in *A.microcarpa*. However, they indicate four main chemical compounds such as sesquiterpene hydrocarbon, chromone derivative, oxygenated sesquiterpenenes and fatty acid. Thus, both of the species share same chemical compounds

like α-guaiene, oxo-agarospirol, α-humulene, caryophellene oxide and 2-(2-phenylethyl) chromones (Zul Helmey *et al*., 2011a)

2.1.5 Uses of gaharu

Gaharu is widely used because of its aromatic smell which produce due to the chemical components extracted from agarwood. Generally, the agarwood is used for incense, medical purpose, perfumery and others. According to Chua (2008), agarwood grade is not uniform as it characterized according to various grading system that differ in each country producing agarwood. Thus, in Malaysia the agarwood used as aromatic range, religious, ceremonial and decorative carves.

2.1.5.1 Incense

One of the gaharu usages is incense. The uses of gaharu as incense is widely discovered around the many countries (Yinzeng *et al.,* 2007) as it has strong heavy scent of gaharu which is unique and complex, that is why the scent becomes stronger when it is burnt. Application of incense done by burned a pieces of wood in order to produced pleasant aroma where used as general perfume to element of important religious occasion (Barden *et al*., 2000). The incense also applied in some country like Egypt, Arabia and throughout the Northeast part of Bangladesh (Md. Nazrul *et al.,* 2009).

Besides that, the Arabic and Japanese enjoy the strong fragrant and incorporate the essence in incense product. According to Nurdiyana (2008), the smell produced from burned agarwood used as important religious occasion. In Japanese, the gaharu applied in Japanese appreciation of incense wood which called Koh doh (incense ceremony).