In vitro cultures of *Aquilaria malaccensis* for agarwood production

by

Sara Jane Chiu Soo Hoon

December 2014

A thesis submitted to the University of Nottingham in partial fulfilment of the requirements governing the award of the degree of

Doctor of Philosophy

School of Biosciences

University of Nottingham

Supervised by: Dr Peter Alderson

School of Biosciences

University of Nottingham, Malaysia

Dr Kodiswaran Kandasamy

Malaysian Biotechnology Corporation Sdn Bhd

Dr Winnie Yap

School of Biosciences

University of Nottingham, Malaysia

I dedicate this thesis to

My family for their unconditional love, patience, and support ….

Abstract

This thesis describes the results of a series of plant tissue culture, chemical and molecular based experiments aimed at developing an *in vitro* system to study the fundamental changes in chemical composition or activation of specific chemical pathways which take place during the onset and production of agarwood in *Aquilaria malaccensis*.

Cell suspension cultures were established using callus initiated from shoot and leaf segments excised from *in vitro* grown plantlet of *Aquilaria malaccensis*. Callus was successfully established and maintained by culturing leaf segments on MS medium supplemented with 3% sucrose, 0.3% phytagel, 2.2µM of 2,4-D and 2.3 µM of BAP, and cultured under ambient culture condition i.e. 28 ± 2 ºC under continuous dark conditions. Cell suspensions were initiated from the callus lines using the same medium composition, but without the gelling agent and placed on a rotary shaker at 75 rpm. Leaf-derived callus (CS 11) was identified as the preferred source of callus due to the formation of a more homogenous cell suspension cultures which maintained continuous growth after many rounds of sub-culturing. Cell line CS 11 was used for further studies, i.e. determining the effect of elicitation on cell growth, biochemical change and gene expression.

In order to effectively study the biochemical changes (sesquiterpenes and chromones production) within the cells in cultures, it would first be necessary to devise suitable analytical methods which would enable the analysis of the effect of elicitation, and to study the chemical profile of each cell culture lines. Various analytical techniques were evaluated using agarwood oil extracts (as standards) and cell cultures as target material. Solid phase micro extraction (SPME) was found to be the most effective technique in detecting the presence of sesquiterpenes and chromones within the cells in cultures.

Four sesquiterpenes (alpha humulene, delta guaiene, beta caryophyllene, alpha guaiene) and four chromones (6-methoxy-2-(2-phenylethyl)- chromone, 5,8-dihydroxy-2-(2-(4-methoxyphenylethyl)-chromone, 7-hydroxy-2-(2-phenyl ethyl] chromone and 6-methoxy-2-[2-(3-methoxyphenyl)ethyl] chromones) were found to be produced in unstressed cell suspensions. However it was important to note that although chromones were detected there was no consistent production of any chromones in cell cultures.

Overall, the production of sesquiterpenes in cell suspension cultures was found to be higher following elicitation using methyl jasmonate, salicylic acid and ethanol. While salicylic acid was found to enhance cell growth, methyl jasmonate was found to suppress the growth of cells. Unexpectedly the addition of alcohol (0.17µM), the solvent used to dissolve methyl jasmonate was found to have an effect on the production of sesquiterpenes specifically when applied separately where, it was found to induce higher concentration of alpha guaiene and alpha humulene as compared to methyl jasmonate or salicylic acid treatments.

The correlation of increase in the production of sesquiterpenes in relation to sesquiterpene synthase expression was also explored in a preliminary study done using the ACL 154 primers whereby the increase in alpha humulene production was found to correlate with an increase in delta guaiene synthase activity suggesting that delta guaiene synthase may be responsible for alpha humulene production in *Aquilaria malaccensis*.

In summary, the combined results of the above studies led to the development of a series of analytical methods and the establishment of an *in vitro* model system for *Aquilaria malaccensis* using cell cultures. This represents the first study successfully examining the simulated effect of artificially induced wound (elicitation), in terms of its direct influence on sesquiterpenes profile expressed, and an insight to gene expression patterns which take place within cell cultures of *Aquilaria malaccensis.*

List of Abbreviations

%	: percentage
®	: Registered trademark
µg	: milligram
µM	: micromolar
2,4-D	: 2,4-dichlorophenoxyacetic acid
ANOVA	: Analysis of Variance
BAP	: 6-benzylaminopurine
BLAST	: Basic Local Alignment Search Tool
bp	: base pair
CAS	: Chemical Abstracts Service Registry
cDNA	: complementary deoxyribonucleic acid
CITES	: Convention on International Trade in Endangered Species of Wild Fauna and Flora
cm	: centimetre
CS	: Cell suspension
CTAB	: cetyltrimethylammonium bromide
CV	: coefficient of variance
DMRT	: Duncan multiple range test
DMSO	: Dimethyl sulfoxide
DNA	: deoxyribonucleic acidRNA
DNase	: deoxyribonuclease
dNTP	: deoxynucleotide triphosphates
EDTA	: Ethylenediaminetetraacetic acid
ESI	: Electrospray Ionisation
FDA	: Flourescien diacetate
g	: gram
GC	: Gas Chromatography

GC-O : Gas Chromatography olfactometry

GC-MŚ : Gas Chromatography coupled with mass spectometer

H_2O : water

HS : Headspace

IAA : indole acetic acid

IR : Infrared spectroscopy

IUCN : International Union for Conservation of Nature

K or Kin : Kinetin

LC : Liquid Chromatography

m : meter

M/Z : Mass over charge number

MEJA : methyl jasmonate

mg : milligram

$MgCl_2$: Magnesium chloride

ml : millilitre

mm : millimetre

MŚ : Mass Spectrometer

MS : Murashige and Skoog (1962)

MŚ/MŚ : Triple Quad Mass Spectrometer

MVA : Mevalonate (pathway)

MW : molecular weight

NAA : 1-naphthaleneacetic acid

NaCl : sodium chloride

NIST : National Institute of Standard and Technology

nm : nanometer

NMR : Nuclear Magnetic Resonance

^{o}C : degree Celsius

PCR : polymerase chain reaction

PDMS : Polydimethylsiloxane

PGR : Plant Growth Regulator

psi : pound-force per square inch

Rf : Retention factor

Rt : Retention time

RNA : ribonucleic acid

RNase : ribonuclease

rpm : revolutions per minute

SA : salicyclic acid

SCV : settled cell volume

SPME : Solid Phase Microextraction

SPSS : Statistical Package for the Social Sciences

TAE : buffer solution containing TRIS base , acetic acid & EDTA

TE : buffer solution containing TRIS base & EDTA

TLC : thin layer chromatography

UV : ultraviolet

V : Volt(s)

w/v : weight per volume

x g : centrifugal force

α : alpha

γ : gamma

δ : delta

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CHAPTER 1

General Introduction

1.0 Introduction

Agarwood, commonly known as Gaharu in Malaysia, refers to the high value resinous heartwood produced by species of the genera *Aquilaria* and *Gyrinops*. It is also known as Oud in Saudi Arabia, Kalambak in Indonesia, Jin Koh in Japan and Agaru in Nepal (Schadde, 2011). Historically, agarwood has been used intensively by Buddhist, Jewish, Christian, Muslim and Hindu communities for cultural, religious and medicinal purposes (Chua & Sumatra, 2008). However, since the 1970s, the demand for agarwood has expanded greatly causing demand to far exceed supply and agarwood production has only been able to meet about 40% of the global demand (Gratzfeld & Tan, 2008). The high demand for agarwood is mainly due to its unique aroma, which it has been almost impossible or not economically feasible to produce synthetic substitutes given the complexity and lack of understanding towards the aroma profile produced from the burning of wood or oil distilled from these agarwood.

Traditionally, agarwood is harvested from the wild and, due to the way in which it is formed within the trunk of a tree (signs of agarwood formation is not visible from the exterior), its detection is extremely difficult if not impossible (Persoon, 2007). The scarcity of agarwood is increased by the fact that not all *Aquilaria* trees contain agarwood, as it is only produced when the tree is subject to wounding and subsequent microbial infection. Gibson (1977 cited by Ng *et al*., 1997) estimated that only 10% of the total *Aquilaria* population may contain agarwood and the quality and quantity of agarwood produced varies from tree to tree depending on factors such as age of the tree, seasonal variation, environmental variation and genetic variation (Chakrabarty *et al*., 1994; Saeohartona & Mardiastuti, 1997; Ng *et al*., 1997).

Without a non-destructive means of determining the presence of agarwood in *Aquilaria* trees, trees are often chopped down indiscriminately causing *Aquilaria* populations in natural populations to decline rapidly. As a result of this, in the last decade eight of the fifteen *Aquilaria* species have been classified as threatened according to the IUCN Red List. One of the eight species, *Aquilaria malaccensis* (A. *malaccensis*), has been listed in Appendix II of the Convention on International Trade in Endangered Species of the Wild Fauna and Flora (CITES, 2005). The main purpose of listing agarwood species in Appendix II of the Convention was to control and verify agarwood international trade in the hope of deterring illegal harvesting and, in the long term, preventing *Aquilaria* species from facing extinction (Compton, 2004).

1.1 Distribution and Habitat

Aquilaria malaccensis has a wide distribution and has been found in 10 countries in South Asia and South East Asia (Figure 1.1), namely Bangladesh, Bhutan, India, Indonesia, Malaysia, Myanmar, the Philippines, Singapore and Thailand (Oldfield & Mackinven, 1998). Despite this wide distribution range, *Aquilaria* species often occur in low density and can be found throughout primary and secondary forests at altitudes between 0 to 1000m above sea level (Oguyen & Nguyen, 1999; Chua & Sumatra, 2008). The growth rate of *A. malaccensis* in native forests in Malaysia is quite low. La Frankie (1994) reported a mean increase in the diameter of tree trunks of 0.33 cm per year with some faster growing specimens achieving 0.8-1.0 cm per year.

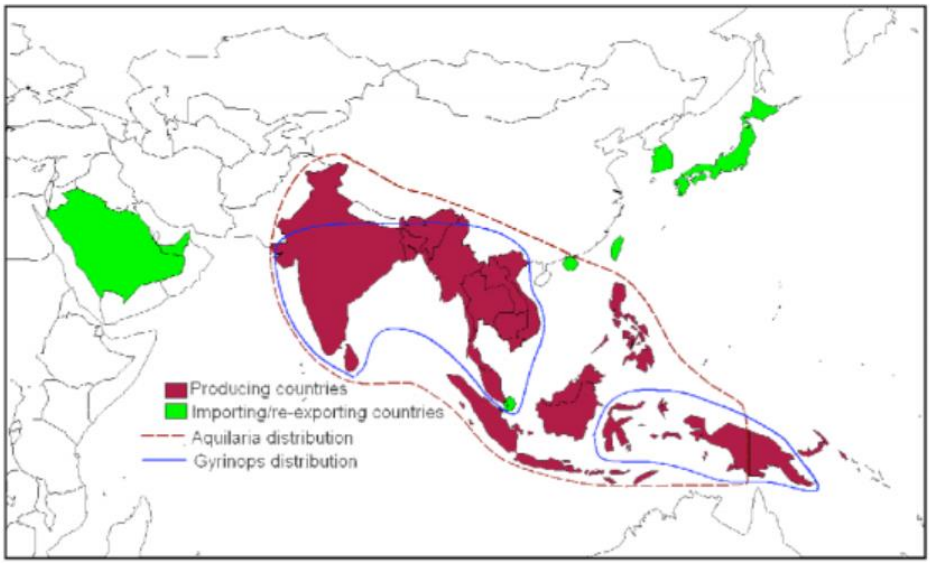


Figure 1.1. Distribution map of agarwood and importing/re-exporting countries (Lata, 2007).

1.2 Taxonomy and Biological Characteristics

The agarwood producing genus *Aquilaria* is comprised of 15 species, of which *A. hirta, A. beccariana, A. rostrata, A. malaccensis and A. microcarpa* occur in Malaysia (Whitmore, 1972; Chang *et al*., 2001). Of these 5 species, *Aquilaria malaccensis* (Table 1.1) is considered one of the most exploited species in Malaysia due to its high economic value.

Table 1.1 Classification of *Aquilaria malaccensis* (IUCN, 1998)

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Myrtales
Family	Thymelaeaceae
Genus	*Aquilaria* L.
Species	*Aquilaria malaccensis* L.

Aquilaria malaccensis is an evergreen tree that can grow up to 40 m in height with a trunk 60 cm in diameter. The wood of the species is typically white, lightweight and low in density (Oguyen & Nguyen, 1999). Once resin production is stimulated, the resin rich wood becomes dark, heavy and hard. The trees usually reach maturity after 6-7 years, whereby flowers start to form followed by seed production (Oguyen & Nguyen, 1999; Chua & Sumatra, 2008). The yellowish green or white flowers usually arise from younger branches (Adelina *et al*., 2004) and fruits are green and egg-shaped (Figure 1.1).

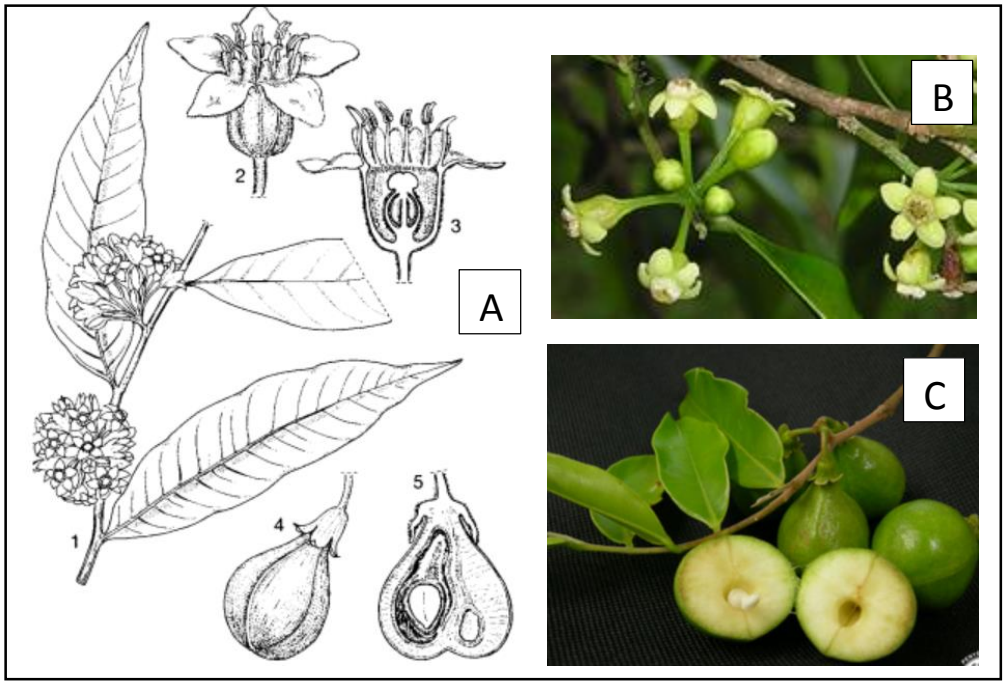


Figure 1.1. Biology of *Aquilaria malaccensis.* [A] Botanical illustration; 1 - twig, 2 - flower, 3 - longitudinal section of flower, 4 - fruit, 5 - longitudinal section of fruit (Oguyen & Nguyen, 1999). [B] Flowers (Lok & Zuhaidi, 1996). [C] Cross section of young fruit and leaves (Jalil, 2012).

1.3 Agarwood Production in Natural Populations

The formation of agarwood was first investigated in 1926 by Bose (as reported in Gibson, 1977). It is hypothesised that agarwood production is associated with a physiological disturbance, such as wounding (tree falling due to epiphyte load or a storm) or insect attack (Ng *et al*., 1997), which is followed by colonisation of the exposed tissues by naturally occurring microbes. As a response to the stress of wounding and microbial infection, *Aquilaria* species may activate its plant defence system triggering the production of a unique type of resin which is high in volatile organic compounds that aid in suppressing and localising the infected area (Nobuchi & Siripatanadilok, 1991).

Formation of agarwood can occur in the trunk or in branches where it usually appears as patches or streaks in the wood (Figure 1.2). While the unaffected wood is light in colour, the production of resin leads to a dramatic change in colour, mass and density of the wood, where the infected wood becomes dark brown, heavy and hard (Lata, 2007). Agarwood production in wild populations are highly unpredictable and the quantity and quality of the agarwood produced in each tree/species differs greatly. It is still not known as to whether the amount and quality of agarwood produced results from the severity of the infection, injury or other unknown factors (Chang *et al*., 2001).

Agarwood is comprised of a complex mixture of organic compounds and many studies have reported there to be two major constituents, namely sesquiterpenes and chromones (Ishihara *et al*., 1993; Ueda *et al*., 2006). These two compounds have been identified as the main chemicals responsible for the unique fragrance of agarwood. The first compound successfully isolated from agarwood was a sesquiterpene known as agarol, from *A. agallocha* in 1959 by Bhattacharuua and Jain (Jain *et al*., 1962). In the past 20 years, numerous

sesquiterpenes and chromones have been isolated and characterised in *Aquilaria* species, including *A. malaccensis* (Yoneda *et al*., 1984), *A. sinensis* (Yang & Chen, 1983; Yagura *et al*., 2003) and *A. crassna* (Yagura *et al*., 2005).

Although many compounds have been identified, the mechanism responsible for the onset and development of agarwood remains unclear. Understanding the mechanism behind agarwood production is deemed extremely important as it would enable us to differentiate agarwood composition in different species, establish an efficient grading system for agarwood trading, formulate efficient inoculation techniques to develop sustainable production of agarwood, and engineer the production of agarwood through synthetic or natural sources.

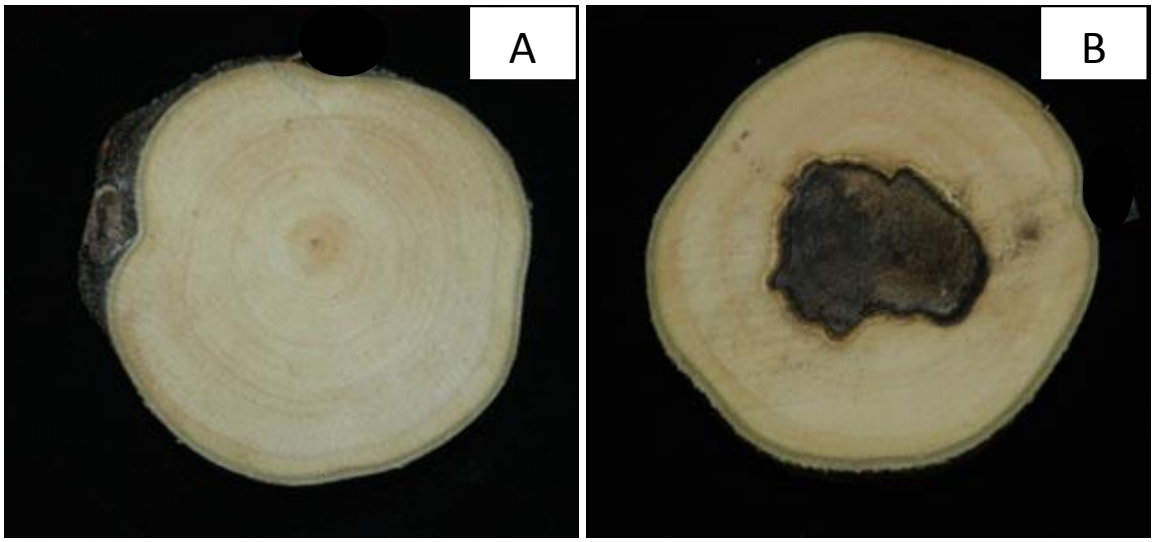


Figure 1.2. Cross section of Aquilaria tree trunk. [A] Healthy trunk wood of *Aquilaria malaccensis*. [B] Infected wood (agarwood formation in the middle of the trunk) of *Aquilaria malaccensis* (Blanchette, 2013).

1.4 Economic Value of Agarwood

Agarwood is the most expensive wood in the world with prices reaching US$30,000 per kilogram (Abdin, 2014). In the retail market, agarwood (Figure 1.3) is usually sold in solid form (wood chips/wood blocks/powdered) or as oil (obtained through distillation). The wood form is often used for carving into artifacts or religious figures, making beads (Persoon, 2007) and burned for its fragrance (Akter & Neelim, 2008). The distilled oil is used in aromatherapy and perfumes (Mamat *et al*., 2010).

The earliest record of international trade of agarwood occurred in India during the thirteenth century whereby it was traded from India and China to Persia, Egypt and the Roman Empire (Sultan Al-Salem, 2007). Agarwood is currently traded in large quantities throughout an increasing number of countries. On average, agarwood chips may sell for several hundreds to thousands of US dollars per kg while the price of oil distilled from agarwood usually ranges between US $ 5000 to 10,000 per litre (Akter & Neelim, 2008). One of the major flaws in agarwood trade is the lack of standardised or systematic grading system causing trading and pricing to be fixed on willing seller and buyer basis. This has created potential risk for resource owners and consumers to be exploited by agarwood merchants and traders (Lata, 2007). In order to regulate this industry in a more efficient manner and prevent the introduction of 'fake agarwood' which could potentially be harmful to consumers (Gao *et al*., 2009), a better understanding of the actual composition of each *Aquilaria* species should be pursued so that a universal grading system can be formulated.

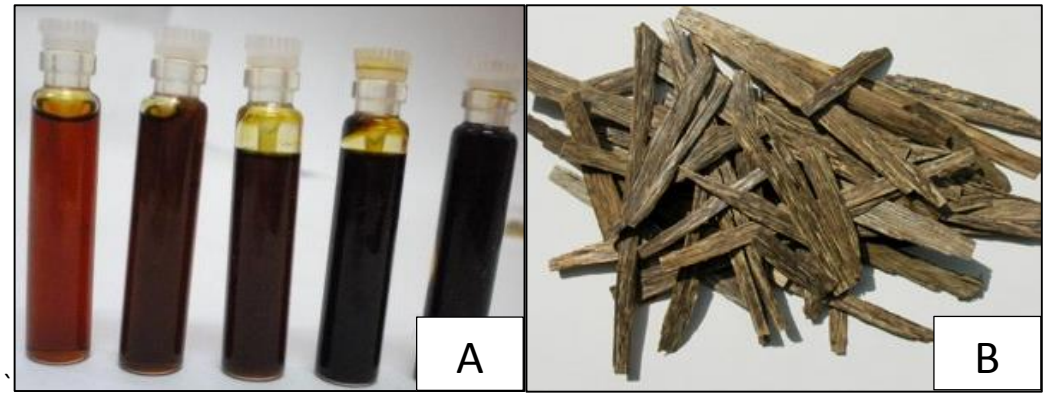


Figure 1.3. Different traded forms of agarwood. [A] Agarwood oil (SQB, 2012). [B] Agarwood chips (Blanchette, 2013).

1.5 Over Exploitation of Agarwood

Due to the high market value and demand for agarwood, there has been indiscriminate felling of *Aquilaria* trees in the past decade leading to the low number of mature trees surviving in the world today. Excessive exploitation of natural resources of agarwood has been reported in Laos (Jensen, 2007), China (Wang, 2007), Myanmar (Than, 2007), Indonesia (Soehartono & Newton, 2001) and Malaysia (Ibrahim *et al*., 2007). If these resources are not properly managed, overharvesting will lead to the depletion of agarwood in these countries in the next 5 – 10 years. Although, trading of agarwood today is regulated through CITES, most remaining stands of *Aquilaria* are decreasing as a result of illegal logging, gold mining operations and clearing of huge tracts of forests for agricultural purposes, particularly in Indonesia and Borneo (Soehartono & Newton, 2002). While it may be impossible to implement regulations and guidelines in all parts of the world, initiatives such as establishing cultivated agarwood resources should be explored to help satisfy international demand and relieve pressure on natural resources.

1.6 Cultivation

The preservation of natural *Aquilaria* populations to increase the supply of agarwood in the world can be assisted through commercial cultivation of *Aquilaria* species. The establishment of *Aquilaria* plantations is not new. *Aquilaria* plantations were established in India in the 1930s and 1940s, primarily by private growers across Assam (Quavi, 2007). Due to the dwindling supply of agarwood, plantations have also been established in Indonesia, Cambodia, Thailand (Figure 1.4), Vietnam and Malaysia (Barden *et al*., 2000) by planters hoping to establish their own green 'gold mine' (Mamat *et al*., 2010). Unfortunately, to date there have been no substantiated reports validating the success of these plantations as agarwood production is an extremely complex and lengthy process whereby planters usually have to wait for 3 to 4 years for the plant to mature (Liu *et al*., 2013). The planters then have to source ways to induce the production of agarwood in the mature trees in which, if the production of agarwood is low, they risk losing their time and investment.

Figure 1.4. Aquilaria plantation in Thailand. (Chiu *et al*., 2011)

Although the establishment of *Aquilaria* plantations seems a positive move towards conserving the species, setbacks, such as poor development of robust inoculation techniques and the lack of quality planting material, have hampered the development of this initiative. Traditionally, artificial induction of agarwood production has been done by deliberately

wounding the tree with large knives and hammering nails in the trunk, however this method usually yields low quality agarwood (Persoon, 2007). The most recent approach is more scientific, in which holes are drilled in the trunk and inoculants are inserted into these holes. These inoculation kits mainly comprise of a concoction of naturally occurring microbes which have been isolated from agarwood and are assumed to be responsible for triggering its production (CALLC, 2014).

Strains of fungi successfully isolated from agarwood include *Aspergillus* spp., *Botryodiplodia* spp., *Diplodia* spp., *Fusarium bulbiferum, F. laterium, F. oxysporum, F. solani, Penicillium* spp. and *Pythium* spp. (Soehartono and Mardiastuti, 1997; Mohamad *et al*., 2010; Tamuli *et al*., 2000; Lei *et al*., 2009) and some are used to formulate inoculation kits. To date there have been two noteworthy studies which seem to yield positive results. One is the Rainforest Project (study) which was established in the South of Vietnam in collaboration with Prof Robert Blanchette from the University of Minnesota, in which artificial inoculation was done using patented inoculation kits known as Cultivated Agarwood (CA) kits (Persoon, 2007; CALLC, 2014). In the second study by Liu *et al*. (2013), an inoculation kit named Agar-Wit kit was projected to be capable of increasing agarwood production by 28 times more as compared with traditional methods. Despite these advances, the success of these techniques when applied on a large scale remains unknown.

Planters have also started to look for alternative sources of *Aquilaria* seedlings. To date most plantations have been established using seeds obtained from the wild. However, due to the increasing scarcity of quality mature trees, there has been interest in exploring the potential of tissue culture for clonal propagation of *Aquilaria* spp. (Mongkolsook *et al*., 2007; He *et al*., 2005; Van Minh, 2001; Majid *et al*., 2010; Hassan *et al*., 2013).

1.7 Statement of Research Problem

The commercial production of agarwood remains unviable due to the lack of understanding of (i) the basic mechanism of agarwood induction and production and (ii) the chemical composition of agarwood originating from different species. The current study focuses on the development and application of plant tissue and cell culture techniques to increase our understanding of the effect of stress, specifically wound induced stress, on agarwood production. First, it was necessary to establish a model system using cell cultures before progressing to the development of analytical techniques to enable correlation of biochemical changes in the cells with the wound related stress and genetic expression profiles of the cells.

The objectives of this study are as follows:

1. To establish a cell culture establishment protocol from 'in vitro' propagated *Aquilaria malaccensis* plantlets.
2. To investigate the effect of wounding on *Aquilaria* cell suspensions using stress inducing chemicals such as methyl jasmonate and salicylic acid.
3. To develop a series of analytical techniques for studying the biochemical profile of 'stressed' cultures.
4. To develop molecular techniques to enable the correlation between biochemical expression and gene expression profiles.

The thesis work reported was carried out mainly at the Biotechnology Research Centre, University of Nottingham Malaysia. The biochemical analysis was carried out at the School of Chemistry, the University of Malaya, Malaysia.

CHAPTER 2

Development of plant cell culture system for *Aquilaria malaccensis*

2.0 Introduction

Plant tissue culture refers to the application of a collection of techniques which enable us to grow and maintain plant cells over an indefinite period of time using nutrient solutions in a controlled environment under aseptic conditions (Loyola-Vargas & Vazquez-Flota, 2006). The establishment of plant tissue culture protocols provides important biotechnology tools for large-scale propagation, generation of genetically modified or elite plant lines, cryopreservation of endangered species, metabolic engineering of chemicals and model systems for fundamental plant cell physiology / biochemical studies (Hall, 1999).

In general, propagation of plant material via tissue culture is divided into two pathways, direct regeneration or indirect regeneration. Direct regeneration usually entails using embryo or organ cultures in which whole plants are then regenerated from these explants (Figure 2.1). In contrast, indirect regeneration involves dedifferentiation of cells excised from leaves, shoots or roots using varying concentrations of PGR (Plant Growth Hormones) and subsequently using these undifferentiated cells to regenerate whole plants. Unlike animal cells, most plant cells are totipotent which means that each cell, no matter whether differentiated or undifferentiated, carries genetic information to enable it to regenerate into a whole plant (Osbourne & Mcmanus, 1986). Plant cell cultures, such as callus and cell suspensions, make ideal model subjects for studying biochemical reactions within plants in a controlled environment without the interference of tissue organization.

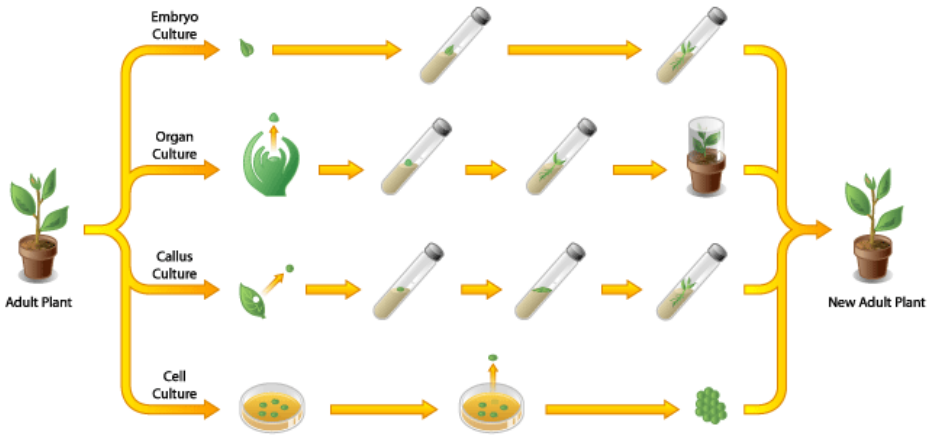


Figure 2.1 Methods of plant cell culture, i.e. embryo culture, organ culture, callus culture and cell culture (Pighin, 2006).

The development of plant cell culture technology started in the late 1960's and has since enabled us to develop and identify the dynamics of different metabolic pathways within the plant kingdom. One classic example is the development of the shikimate pathway through the suspension cultures initiated from plants such as mung bean, soy bean, buckwheat, rose, horseradish, potato and wax bean (Gamborg, 1966). Plant cell cultures start with the establishment of undifferentiated cell lines (callus) from differentiated tissue such as shoot, leaf or root. The transition from a differentiated to an undifferentiated state seldom occurs in nature, but commonly occurs when pieces of tissue are cultured *in vitro* and the cells are exposed to plant growth regulators in the growth medium (Skoog & Miller, 1957). Once stable callus lines have been established, the morphology (colour, texture) of each line is identified to ensure suitable cell lines (preferably friable callus) are selected to establish cell suspension lines. Although the use of callus culture has been reported in various physiological studies, drawbacks, such as the high variability within and among cell clusters and limited surface area, have restricted their development, thus favouring the development of cell suspensions as model plant systems (Domir *et al*., 1992). Main advantages of using cell suspensions include the rapid growth rate, high degree of uniformity between individual cells (enabling more reproducible

and reliable results) and, under optimal conditions, cultured cells of some plants can be maintained almost indefinitely without differentiation provided nutrients are replenished regularly through sub-culturing (Loyola-Vargas & Vazquez-Flota, 2006).

In this chapter, focus is placed on establishing callus cultures and subsequently cell suspension cultures for *Aquilaria malaccensis* with the aim of using the cell suspensions as a model system to study the fundamentals of agarwood production at biochemical and molecular levels. *Aquilaria* species are a unique group of agarwood producing tropical timber trees which take many years to grow and mature. Due to the long time to reach maturity, studies based on fresh plants (*ex vitro*) have been scarce. The first attempt to establish plant cell culture techniques for *Aquilaria* species was reported in 2005 by Ito and Qi. Qi *et al*. (2005) successfully established cell suspensions from root tissue obtained from *in vitro* germinated plantlets of *A. sinensis,* while Ito *et al*. (2005) established cell suspension cultures using leaf tissue from seedlings of *A. crassna* which had been germinated and grown in a greenhouses. To date, no studies have been reported on the establishment of cell cultures in *A. malaccensis*.

The aims of this part of the study are:

1. To establish a tissue culture system for *Aquilaria malaccensis* in which callus could be induced and maintained in *in vitro* culture from explants (shoot and leaf segments) perpetually.
2. To develop and characterize culture conditions required for the induction of synchronised cell suspension cultures.
3. To establish distinct cell lines in suspensions for use in the study of agarwood induction

2.1 Materials and Methods

2.1.1 Initiation of *Aquilaria* callus cultures

Shoot and leaf segments excised from *in vitro* shoot cultures of *A. malaccensis* clone PK25 (obtained from Forest Research Institute Malaysia) were used to induce callus (Figure 2.2) on MS medium (Murashige & Skoog, 1962) supplemented with 3% (w/v) sucrose, 0.3% (w/v) Phytagel® and a varying range of phytohormone concentrations and combinations (Table 2.1). Cultures were incubated under ambient conditions i.e. 27 ± 2˚C in continuous darkness for 8 weeks. For each treatment, 5 replicates were prepared and callus development was measured by scoring the callus size using a 3 x 3 mm grid (Figure 2.3). Based on the increase in size of the callus cultures (final size – initial size), a score (Table 2.2) was then assigned to each explant (with callus) and the mean score for each treatment calculated. The data obtained were analysed with two-way ANOVA and the post-hoc Duncan's Multiple Range test (to identify treatments suitable for callus initiation) using SPSS ®.

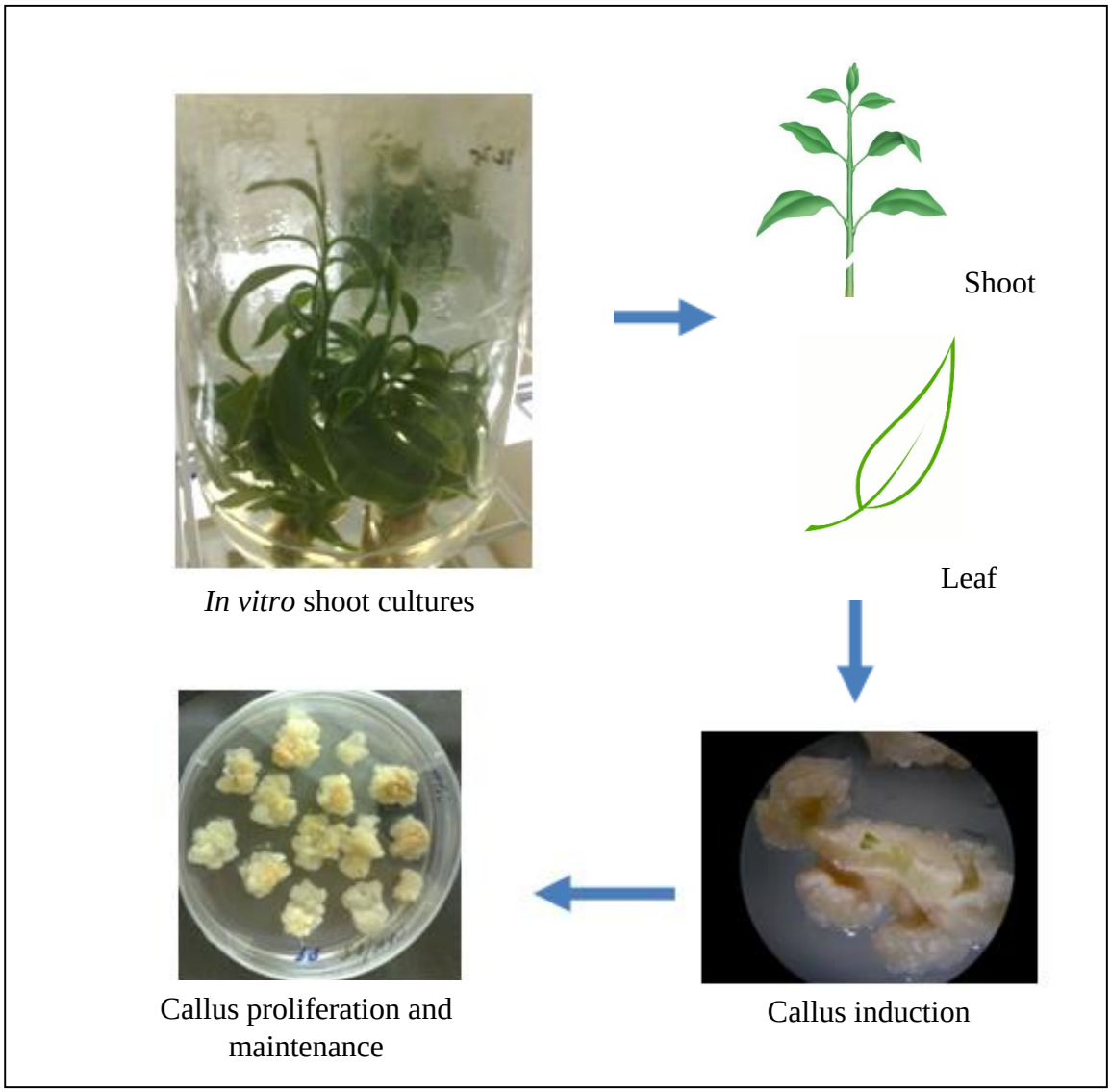


Figure 2.2 Callus induction from *in vitro* shoot and leaf segments and of *A. malaccensis.*

Table 2.1 Concentrations and combinations of plant growth regulators used for callus induction from shoot and leaf segments of *A. malaccensis*.

Phytohormone	Concentration (µM)	2,4-D (2,4-dichlorophenoxyacetic acid)				
		0 (D_0)	2.3 ($D_{2.3}$)	4.5 ($D_{4.5}$)	9.0 ($D_{9.0}$)	22.6 ($D_{22.6}$)
BAP (6-Benzylaminopurine)	0 (B_0)	D_0 B_0	$D_{2.3}$ B_0	$D_{4.5}$ B_0	$D_{9.0}$ B_0	$D_{22.6}$ B_0
	0.4 ($B_{0.4}$)	D_0 $B_{0.4}$	$D_{2.3}$ $B_{0.4}$	$D_{4.5}$ $B_{0.4}$	$D_{9.0}$ $B_{0.4}$	$D_{22.6}$ $B_{0.4}$
	2.2 ($B_{2.2}$)	D_0 $B_{2.2}$	$D_{2.3}$ $B_{2.2}$	$D_{4.5}$ $B_{2.2}$	$D_{9.0}$ $B_{2.2}$	$D_{22.6}$ $B_{2.2}$
	4.4 ($B_{4.4}$)	D_0 $B_{4.4}$	$D_{2.3}$ $B_{4.4}$	$D_{4.5}$ $B_{4.4}$	$D_{9.0}$ $B_{4.4}$	$D_{22.6}$ $B_{4.4}$
	8.9 ($B_{8.9}$)	D_0 $B_{8.9}$	$D_{2.3}$ $B_{8.9}$	$D_{4.5}$ $B_{8.9}$	$D_{9.0}$ $B_{8.9}$	$D_{22.6}$ $B_{8.9}$
	Concentration (µM)	NAA (1-naphthaleneactetic acid)				
		0 (N_0)	2.2 ($N_{2.2}$)	4.5 ($N_{4.5}$)	8.9 ($N_{8.9}$)	22.2 ($N_{22.2}$)
Kinetin	0 (K_0)	N_0 K_0	$N_{2.2}$ K_0	$N_{4.5}$ K_0	$N_{8.9}$ K_0	$N_{22.2}$ K_0
	0.5 ($K_{0.5}$)	N_0 $K_{0.5}$	$N_{2.2}K_{0.5}$	$N_{4.5}K_{0.5}$	$N_{8.9}K_{0.5}$	$N_{22.2}$ $K_{0.5}$
	2.2 ($K_{12.2}$)	N_0 $K_{2.2}$	$N_{2.2}K_{2.2}$	$N_{4.5}K_{2.2}$	$N_{8.9}K_{2.2}$	$N_{22.2}$ $K_{2.2}$
	4.6 ($K_{4.6}$)	N_0 $K_{4.6}$	$N_{2.2}K_{4.6}$	$N_{4.5}K_{4.6}$	$N_{8.9}K_{4.6}$	$N_{22.2}$ $K_{4.6}$
	9.3 ($K_{9.3}$)	N_0 $K_{9.3}$	$N_{2.2}K_{9.3}$	$N_{4.5}K_{9.3}$	$N_{9.3}K_{9.3}$	$N_{22.2}$ $K_{9.3}$

Table 2.2 Scoring system for measuring callus development

Callus Size – Total surface area (mm^2)	**Score**
0	1
1 – 45 mm^2	2
46 – 90 mm^2	3
91 - 135 mm^2	4
≥ 135 mm^2	5

Figure 2.3 Grading of callus size based on 3mm x 3mm grid box.

2.1.2 Maintenance of callus induced from shoot and leaf segments

Callus obtained from all explants (shoot and leaf segments) were excised and sub cultured at a 8 weeks interval on Petri dishes containing the same medium and conditions in order to identify suitable phytohormone combinations for the production and long term maintenance. Callus development was measured as described in Section 2.1.1. Due to unequal sample sizes (the number of replicates generated was dependent on the amount of callus formed on each explant type), data from 3 samples were selected randomly via Microsoft Excel® and analyzed using ANOVA and post-hoc test.

2.1.3 Initiation of cell suspensions

Cell suspensions were initiated by placing 2g of callus in a 50ml conical flask containing 20ml of liquid culture medium (callus proliferation medium; as described in Section 2.1.1), but without gelling agent, and placed on a rotary shaker (75rpm) in continuous darkness. The total volume of liquid medium was gradually increased to 50 ml over a period of 6 weeks. After 6 weeks, cell viability was tested using the Fluorescein Diacetate (FDA) method (Widholm, 1972) and viewed under UV light (with B filter) using an Olympus microscope. Once cell suspensions were established, they were sub cultured at 6 week intervals in which 5ml of cells (SCV) was transferred in to 100ml conical flasks containing 50ml of liquid MS medium.

2.1.4 Synchronization of cell suspensions.

During each subculture, large cell clumps were manually removed using sterile forceps and continuously sub cultured until a uniform cell suspension was established. Once a uniform cell suspension line had been established, the growth pattern of the cell suspension cultures were assessed by measuring the settled cell volume (SCV) biweekly with cell viability FDA test carried out at the end of each observation sub culture period (6 weeks). Growth curves were generated using Microsoft Excel® to determine the growth rate of cell suspensions over time.

2.2. **Results**

2.2.1 Induction of callus from shoot explants

Callus was only produced on media containing both cytokinin and auxin (Figures 2.4 & 2.5). Explants on the control medium (devoid of PGR) did not produce any callus.

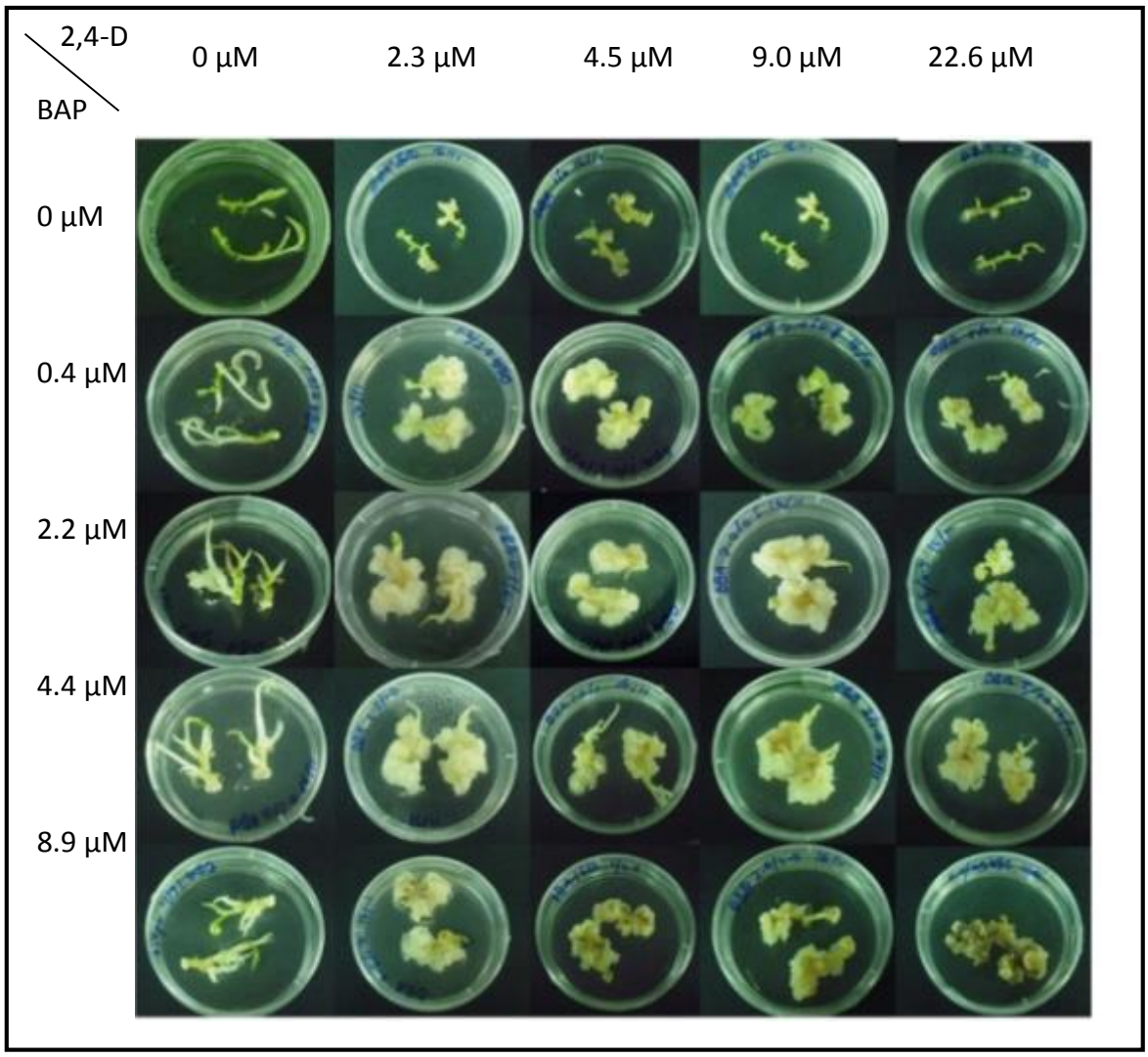


Figure 2.4 *In vitro* callus induction on shoot explants of *A. malaccensis* at varying concentrations of 2,4-D and BAP.

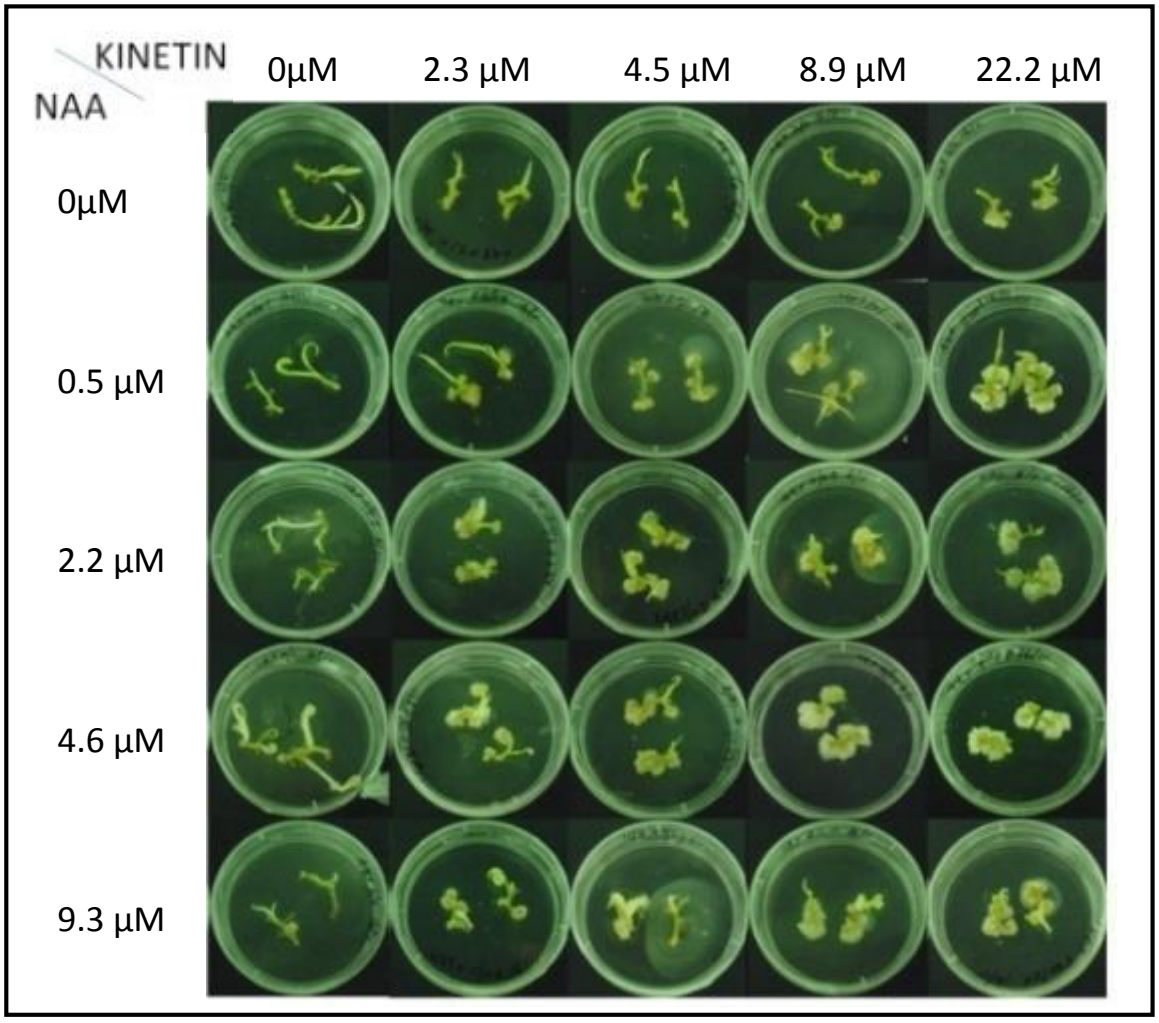


Figure 2.5 *In vitro* callus induction on shoot explants of *A. malaccensis* at varying concentrations of NAA and Kinetin.

Two-way ANOVA (Table 2.3 - Appendix A) revealed that PGR (Plant Growth Hormone) treatment had a significant effect (at 1% confidence level) on callus formation in shoot explants. Interaction between auxin and cytokinin in both treatments (A and B) was also found to be significant at α=0.05, therefore a DMRT analysis was conducted. Callus formation was found to be significantly better in treatments which included both auxin and cytokinin as compared to the control treatments (without auxin, cytokinin or both) (Figures 2.6 and 2.7). Overall, 2,4-D + BAP was observed to consistently (lower standard deviation across treatments) induce a higher callus score as compared to NAA + Kinetin.

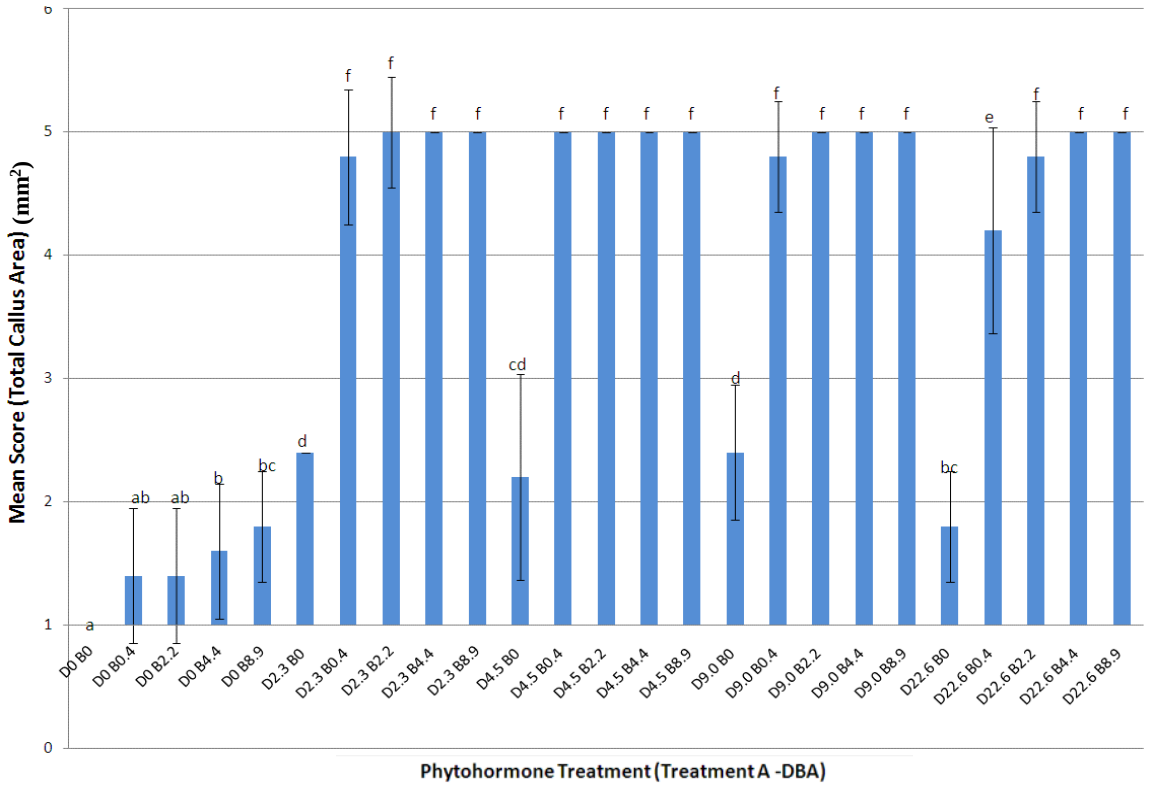


Figure 2.6 Mean score representing callus development (total callus area) in shoot cultures containing varying concentration of 2,4D and BAP. D = 2,4-D; B = BAP.

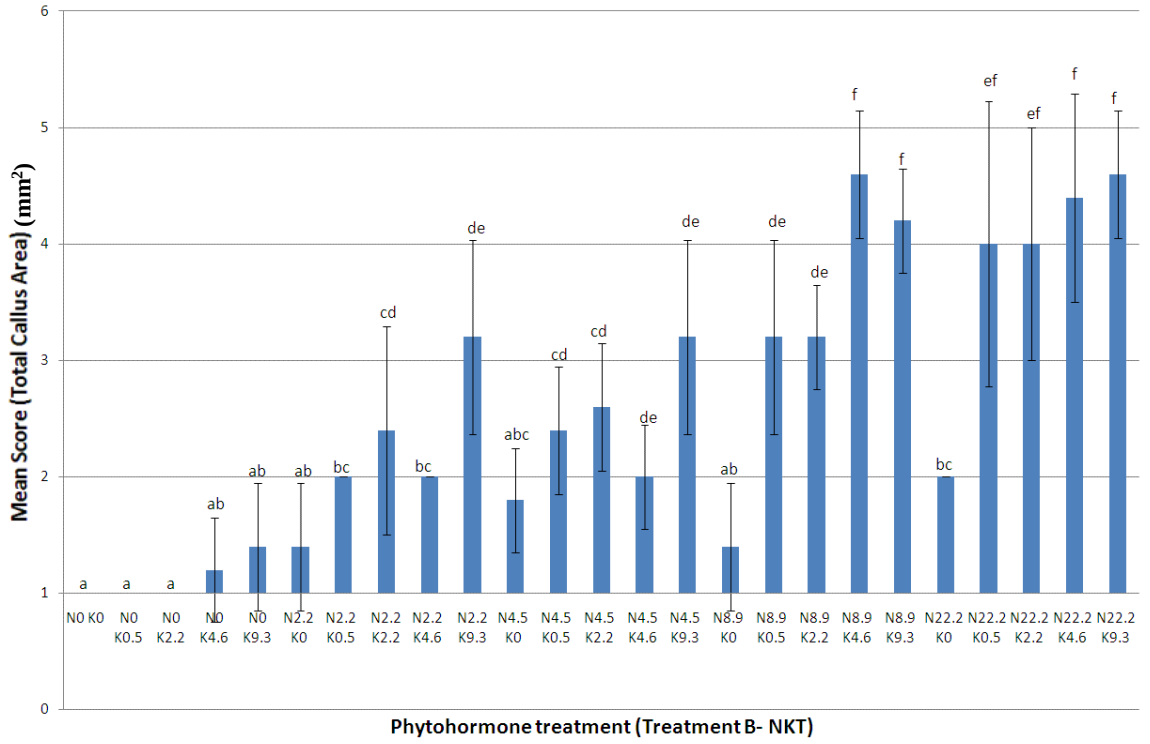


Figure 2.7 Mean score representing callus development (total callus area) in leaf segment cultures containing varying concentration of NAA and Kinetin. N = NAA; K = Kinetin.

2.2.2 Induction of callus from leaf explants

Callus was only produced in medium containing both cytokinin and auxin (Figures 2.8 & 2.9), where explants on the control media (devoid of PGR) produced little or no callus.

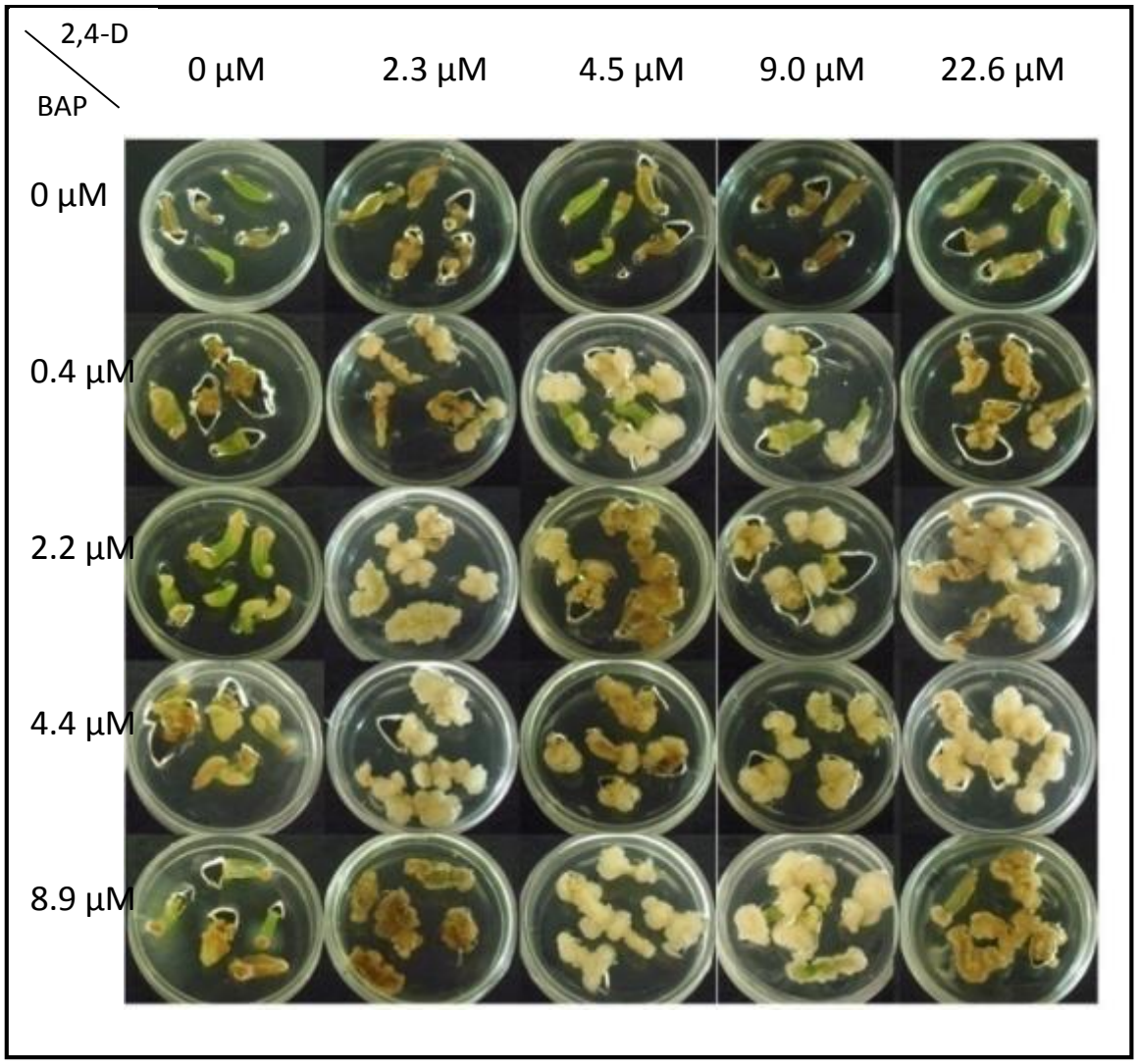


Figure 2.8 *In vitro* callus induction of leaf explants of *A. malaccensis* at varying concentrations of 2,4-D and BAP.

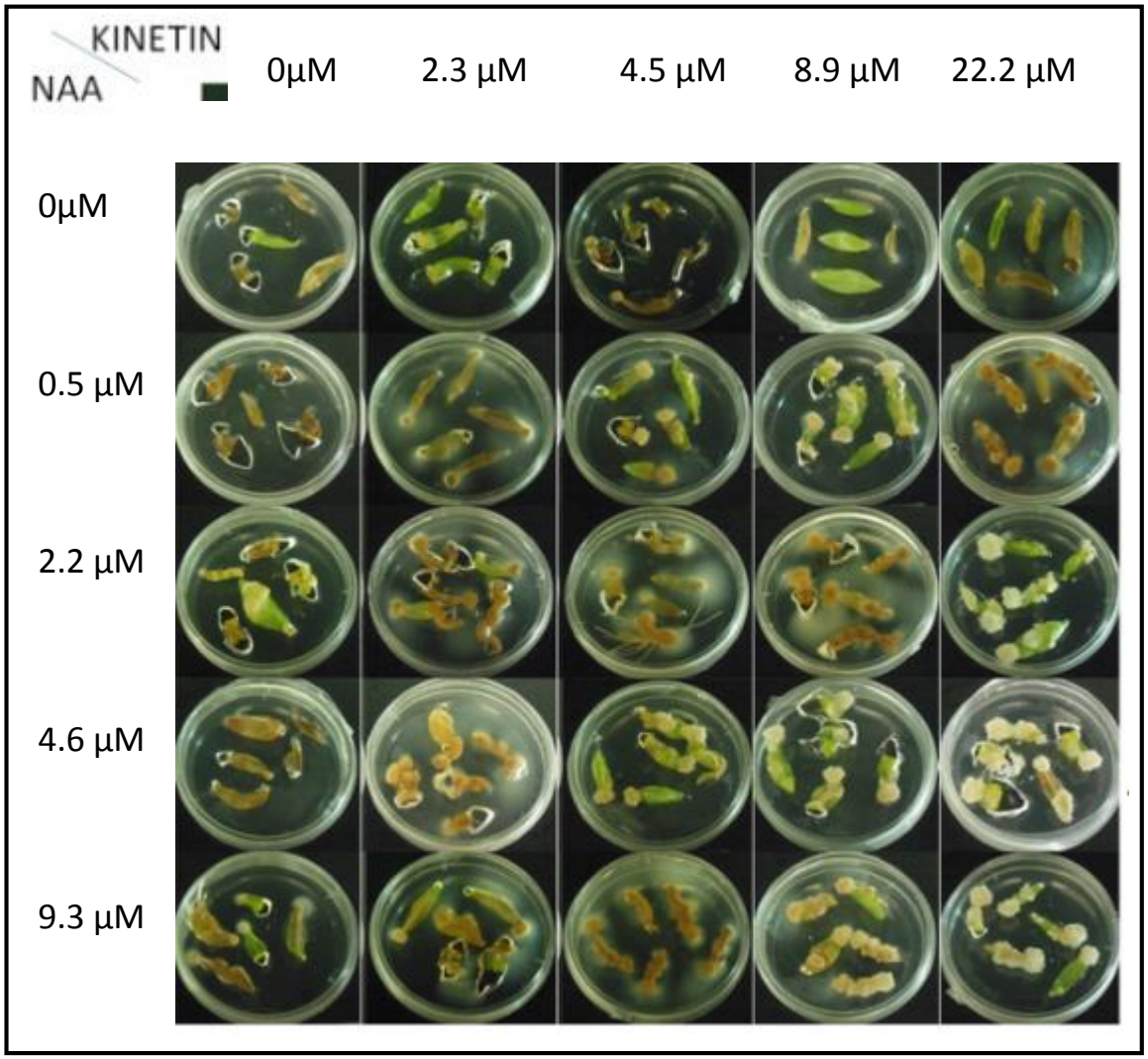


Figure 2.9 *In vitro* callus induction of leaf explants of *A. malaccensis* at varying concentrations of NAA and Kinetin.

Two-way ANOVA (Table 2.4 – Appendix A) revealed that the combination (auxin + cytokinin) at varying concentrations had a significant effect (at 1% confidence level) on callus formation in leaves. Interaction between auxin and cytokinin in both treatments (A and B) was also found to be significant at α=0.05, therefore a DMRT analysis was conducted (Figures 2.10 and 2.11). In general, callus formation was found to be significantly better in treatments which incorporated both auxin and cytokinin as compared to the control treatments. Consequently, callus obtained from the control group was not sub cultured into fresh medium due to its poor callus induction properties. As compared to shoot cultures, the standard deviation of callus

formation in leaf segments was significantly higher. This suggests that shoot explants might be more suitable as starting material for callus induction and proliferation.

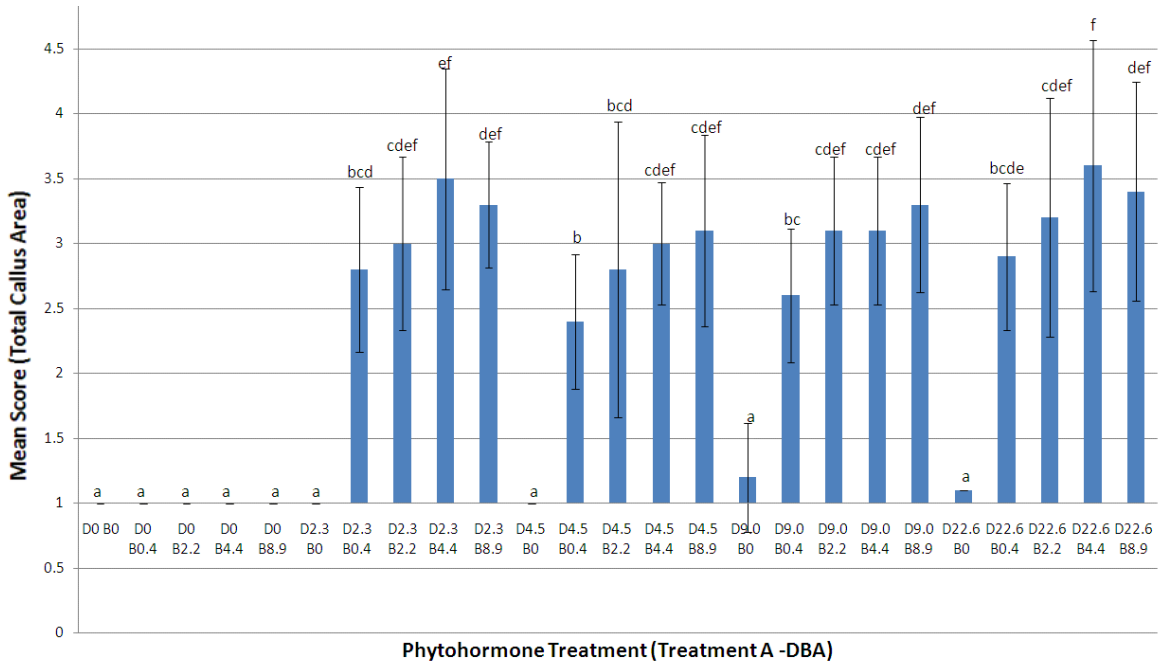


Figure 2.10 Mean score representing callus development (total callus area) in leaf segment cultures containing varying concentrations of 2,4-D and BAP. D= 2,4-D; B = BAP.

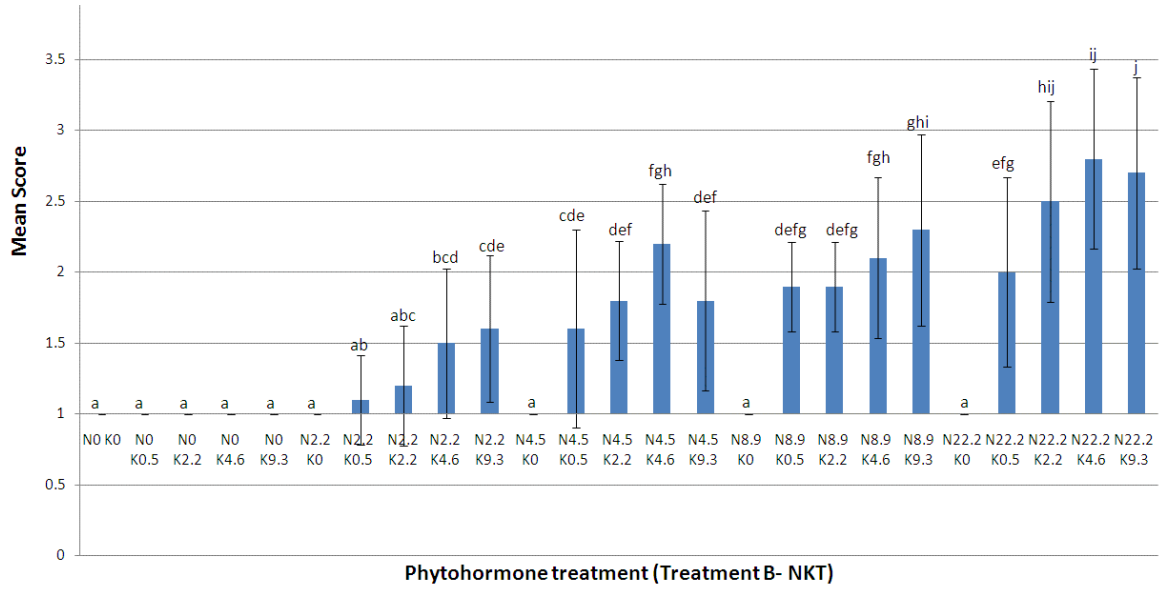


Figure 2.11 Mean score representing callus development (total callus area) in leaf segment cultures containing varying concentrations of NAA + Kinetin. N = NAA; K = Kinetin.

2.2.3 Maintenance of callus induced from shoot and leaf explants.

2.2.3.1 Maintenance of callus line obtained from shoot explants

The effect of different combinations of plant growth regulators (Kinetin + NAA and BAP + 2,4-D) at varying concentration on callus lines obtained from shoot explants (Figure 2.12) was analysed to identify the best proliferation and maintenance media after three consecutive sub culturing cycles.

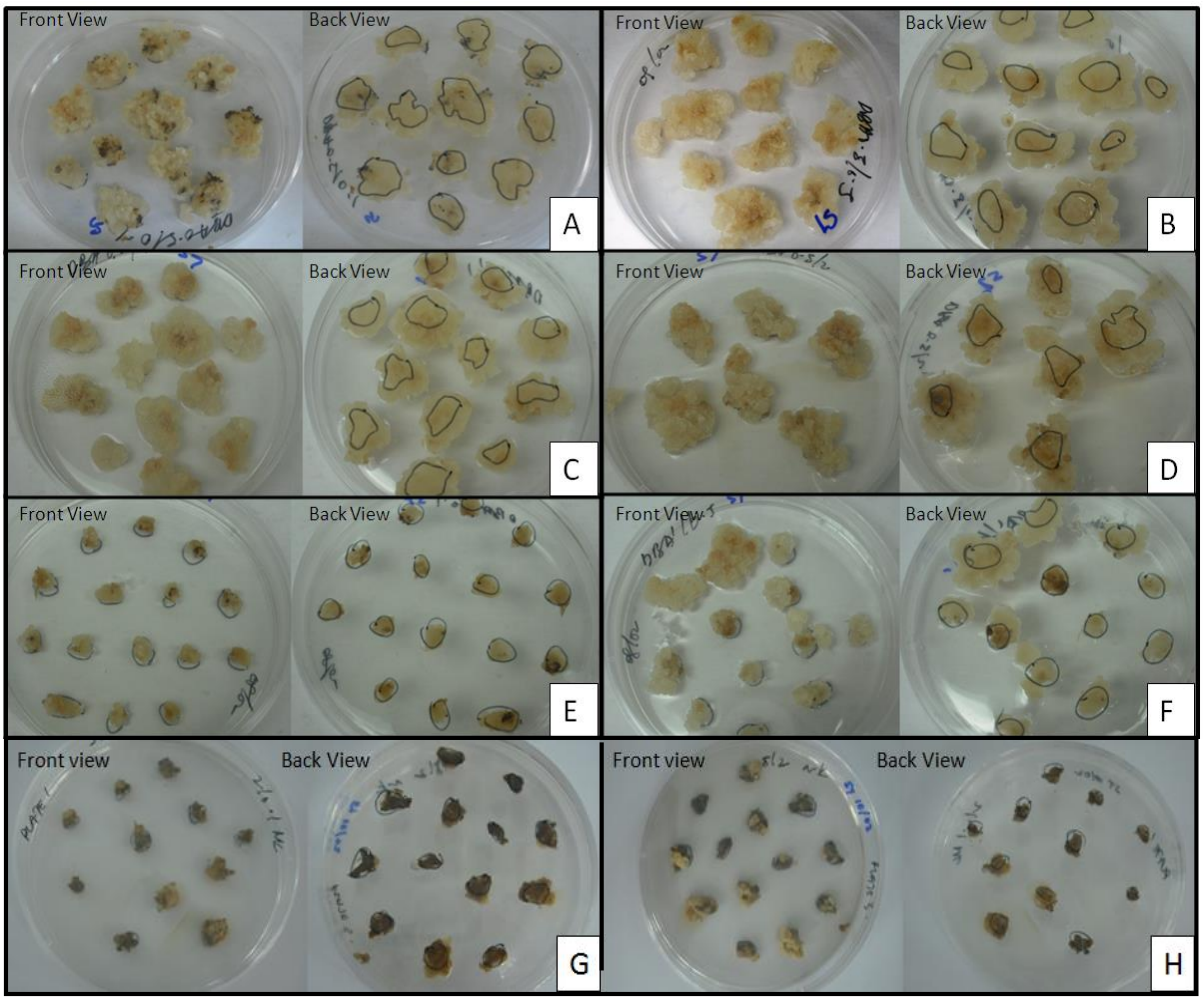


Figure 2.12 Shoot-derived callus cultures for identifying suitable proliferation medium. [A&B] 2.3µM 2,4-D + 2.2µM BAP; [C] 2.3µM 2,4-D + 0.4µM BAP; [D] 2.3µM 2,4-D + 8.9µM BAP; [E] 9.0 µM 2,4-D + 8.9 µM BAP; [F] 4.5µM 2,4-D + 2.2µM BAP; [G] 8.9µM 2,4-D + 4.6µM BAP; [H] 22.2µM 2,4-D + 9.3µM BAP.

One-way ANOVA (Table 2.5 - Appendix A) was used to determine the effect of PGR treatment on callus proliferation. PGR treatment was found to significantly affect callus proliferation in the first and third subculture cycle, but not in the second subculture cycle. Due to the absence of any significant growth of cultures in the second subculture cycle, callus in all treatments were sub cultured onto fresh medium (no callus was discarded even though some callus lines did not display any signs of growth). DMRT analysis identified the best proliferation of callus at the end of the third subculture cycle on 5 sets of media (highlighted in yellow in Table 2.6). These callus lines were maintained and introduced into liquid medium. Cultures with a mean value of 1.0000 represented non-responsive callus. The amount of callus produced in each culture plate was found to vary from one replicate to another.

Table 2.6 DMRT analysis comparing all possible pairs of treatment means.

Treatment	First Subculture cycle		Third subculture cycle	
	Mean[a]	DMRT [c]	Mean[b]	DMRT [c]
$D_{4.5}\,B_{8.9}$	1.0000*	a	-	-
$D_{9.0}\,B_{2.2}$	1.0000*	a	-	-
$D_{9.0}\,B_{4.4}$	1.0000*	a	-	-
$D_{9.0}\,B_{8.9}$	1.0000*	a	-	-
$D_{22.6}\,B_{2.2}$	1.0000*	a	-	-
$D_{22.6}\,B_{4.4}$	1.0000*	a	-	-
$D_{22.6}\,B_{8.9}$	1.0000*	a	-	-
$D_{4.5}\,B_{4.4}$	1.4533	ab	2.3333	ab^{+}
$D_{4.5}\,B_{0.4}$	1.8667	ab	1.0000*	a
$D_{9.0}\,B_{0.4}$	1.8667	ab	1.0000*	a
$D_{22.6}\,B_{0.4}$	2.1667	ab	1.0000*	a
$D_{2.3}\,B_{2.2}$	2.3967	ab	4.2960	c^{+}
$D_{2.3}\,B_{0.4}$	2.4167	ab	2.9345	bc^{+}
$D_{4.5}\,B_{2.2}$	2.6233	b	3.6204	bc^{+}
$D_{2.3}\,B_{8.9}$	2.7667	b	1.0000*	a
$D_{2.3}\,B_{4.4}$	2.8200	b	3.6204	bc^{+}

[a] Uses Harmonic Mean Sample Size = 48.000.
[b] Uses Harmonic Mean Sample Size = 27.000.
[c] Any two means having a common letter are not significantly different at $\alpha = 0.05$
[+] Culture introduced into liquid cultures
* No growth – non responsive callus

2.2.3.2. Maintenance of callus lines obtained from leaf segment explants

None of the callus from treatments with NAA + Kinetin (Figure 2.13) survived the first round of sub culturing, in which all callus turned brownish black after subculture (premature senescence). In treatments with 2,4-D + BAP (Figure 2.13), despite very little or no growth, some callus remained whitish yellow indicating that the cells were still viable. Subsequently, callus lines that did not show browning were continuously sub cultured in the hope that the callus would eventually start growing.

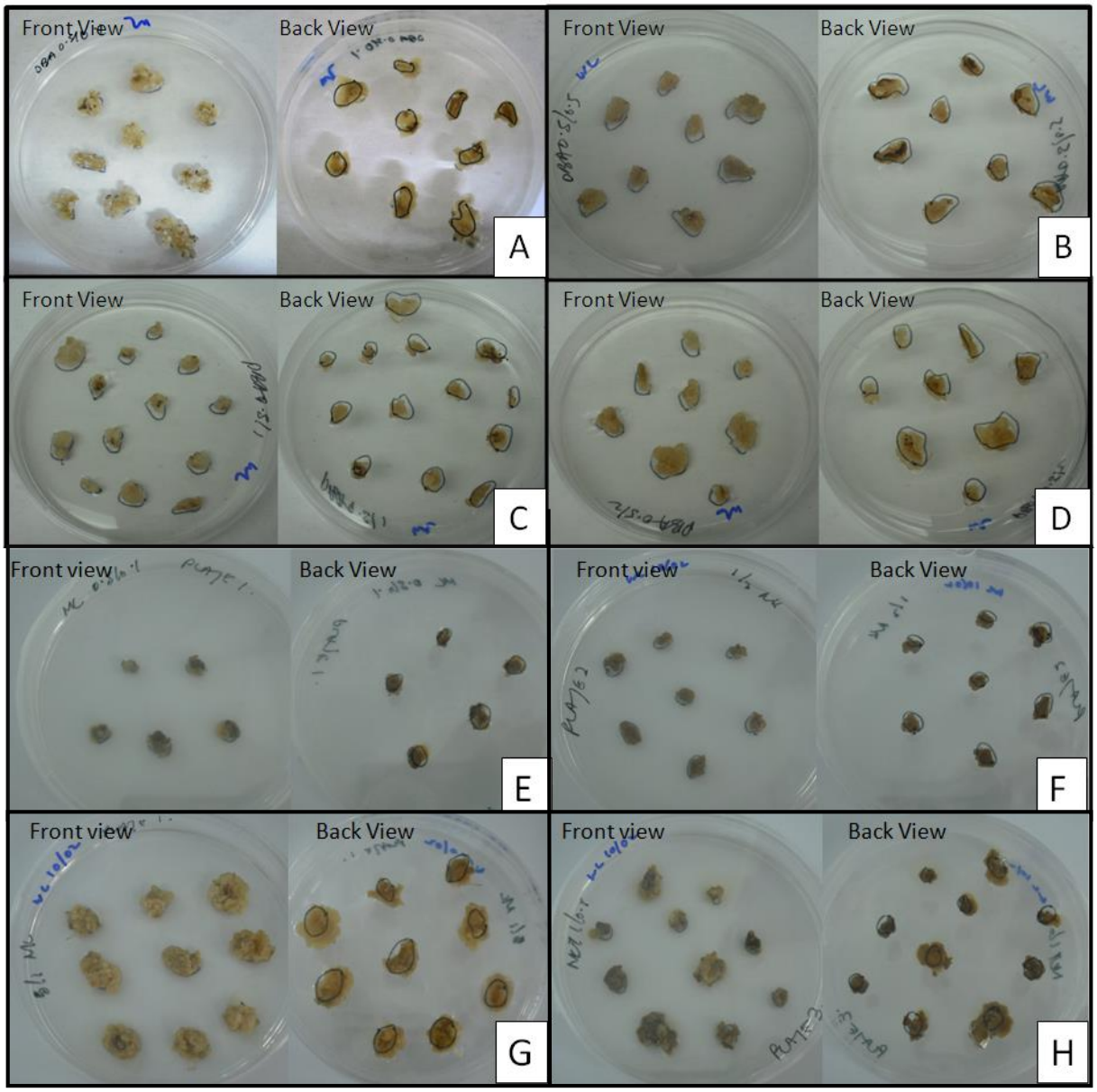


Figure 2.13 Leaf-derived callus grown on media containing different concentrations of PGR. [A] 2.3µM 2,4-D + 0.4µM BAP; [B] 2.3µM 2,4-D + 2.2µM BAP; [C] 2.3µM 2,4-D + 4.4µM BAP; [D] 2.3 µM 2,4-D + 8.9 µM BAP; [E] 2.2µM NAA + 0.5µM Kinetin; [G] 22.2µM NAA + 4.6µM Kinetin; [H] 4.5µM NAA + 2.2µM Kinetin.

After 5 cycles of sub culturing, some of the callus from treatment $D_{2.3}$ $B_{2.2}$ (2.3µM 2,4-D + 2.2µM BAP) (Figure 2.14) started to proliferate and was continuously sub cultured and eventually introduced into liquid medium. It is important to note that not all callus from the same treatment or plate exhibited growth upon subculture (Figure 2.14), callus which had turned brown (not viable - died) was discarded while only friable yellowish-white callus was maintained.

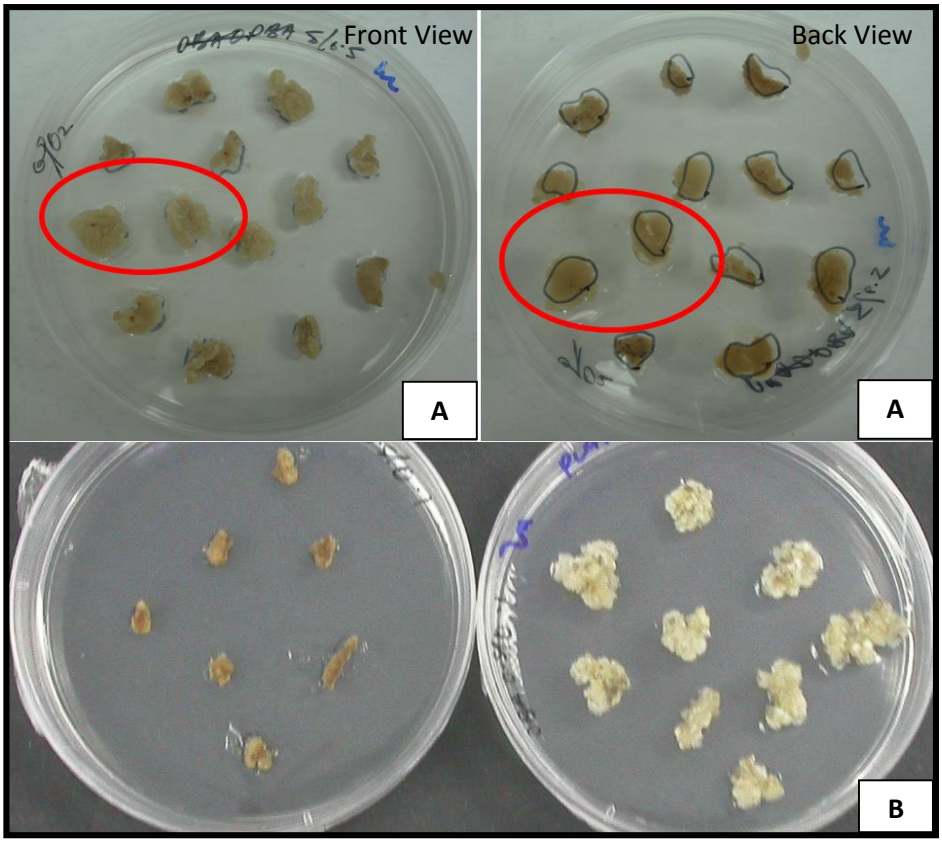


Figure 2.14 Callus initiated from leaf explants grown on 2.3µM 2,4-D + 2.2µM BAP ($D_{2.3}$ $B_{2.2}$) medium. [A] Yellowish – white callus (circled in red) exhibiting minimal growth after 4 rounds of subculture. [B] Callus subcultured from plate A in the 5th round of subculture where some callus died prematurely and some callus, which exhibited minimal growth, started to proliferate.

2.2.4 Initiation, optimisation and maintenance of cell suspension cultures

2.2.4.1 Initiation of *A. malaccensis* cell suspension cultures

Attempts were made to introduce 5 types of callus obtained from shoot and leaf explants using different phytohormone treatments (4.5µM 2,4-D + 4.4µM BAP; 2.3µM 2,4-D + 2.2µM BAP; 2.3µM 2,4-D + 0.4µM BAP; 4.5 µM 2,4-D + 2.2 µM BAP; 2.3µM 2,4-D + 4.4µM BAP) into cell suspension (Figure 2.15). Viable cell suspensions could only be established in 2.3µM 2,4-D + 2.2µM BAP medium regardless of origin of callus (shoot or leaf-derived). Using callus obtained from this medium, several cell lines were established where each plate of callus was considered as an individual cell line.

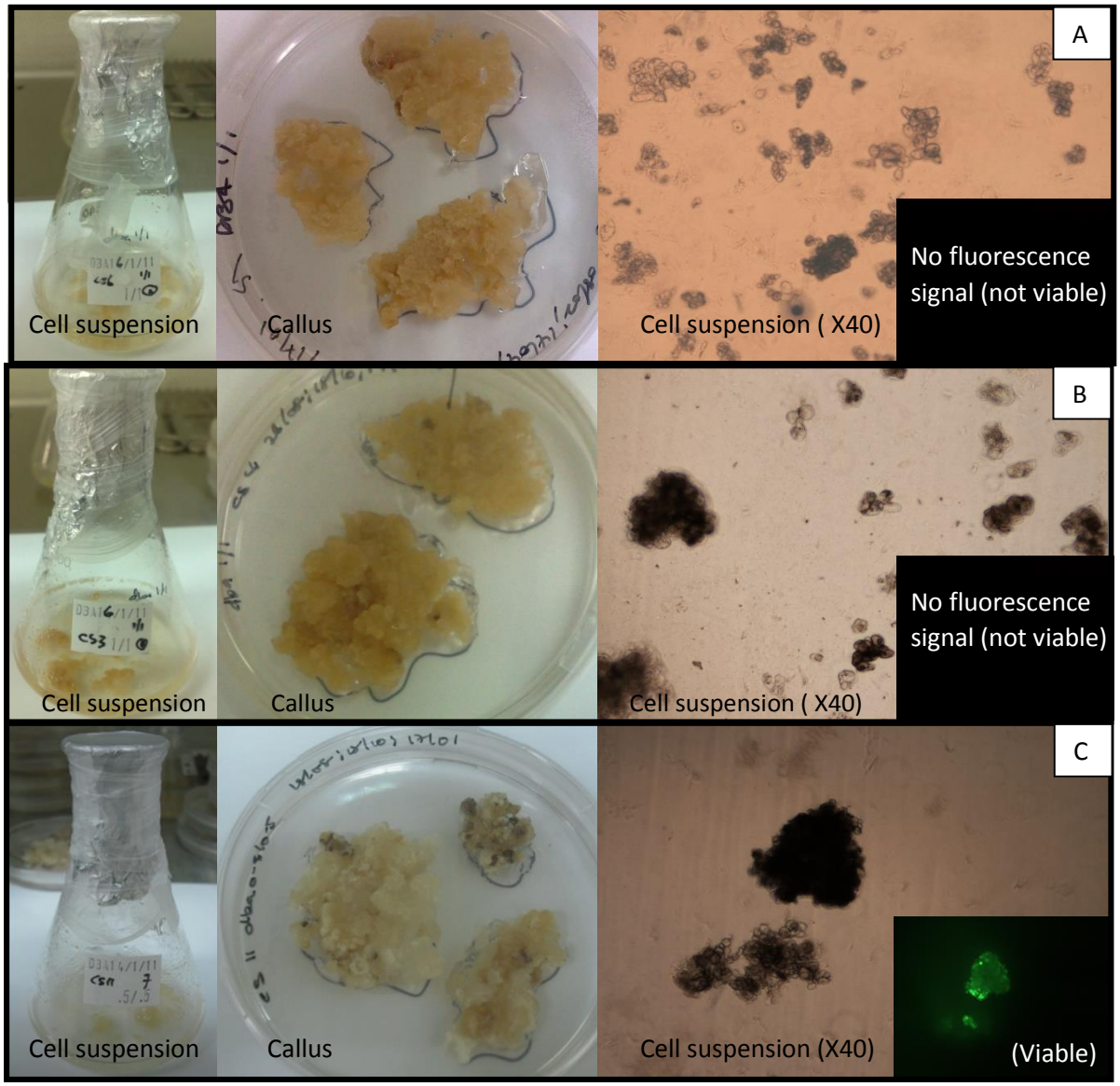


Figure 2.15 Cell suspension of *A.malaccensis* intiated from callus originating from shoot and leaf explants. [A] CS 6 (shoot-derived) 2.3µM 2,4-D + 0.4µM BAP; [B] CS 3 (shoot-derived) 4.5µM 2,4-D + 4.4µM BAP; [C] CS 11 (leaf-derived) 2.3µM 2,4-D + 2.2µM BAP.

Out of ten shoot explants-derived callus lines and one from leaf explant-derived callus that were initiated, only 3 lines (CS5 – shoot-derived; CS4 – shoot-derived; CS11 – leaf-derived) proliferated actively (Figure 2.16). The successful initiation of cell suspensions was found to rely highly on the morphology of the callus e.g. in the case of shoot-derived callus, there were two distinct types of callus i.e. opaque white and yellow (Figure 2.17). Based on FDA viability test and growth patterns, viable cell suspensions could only be initiated from the opaque white callus type.

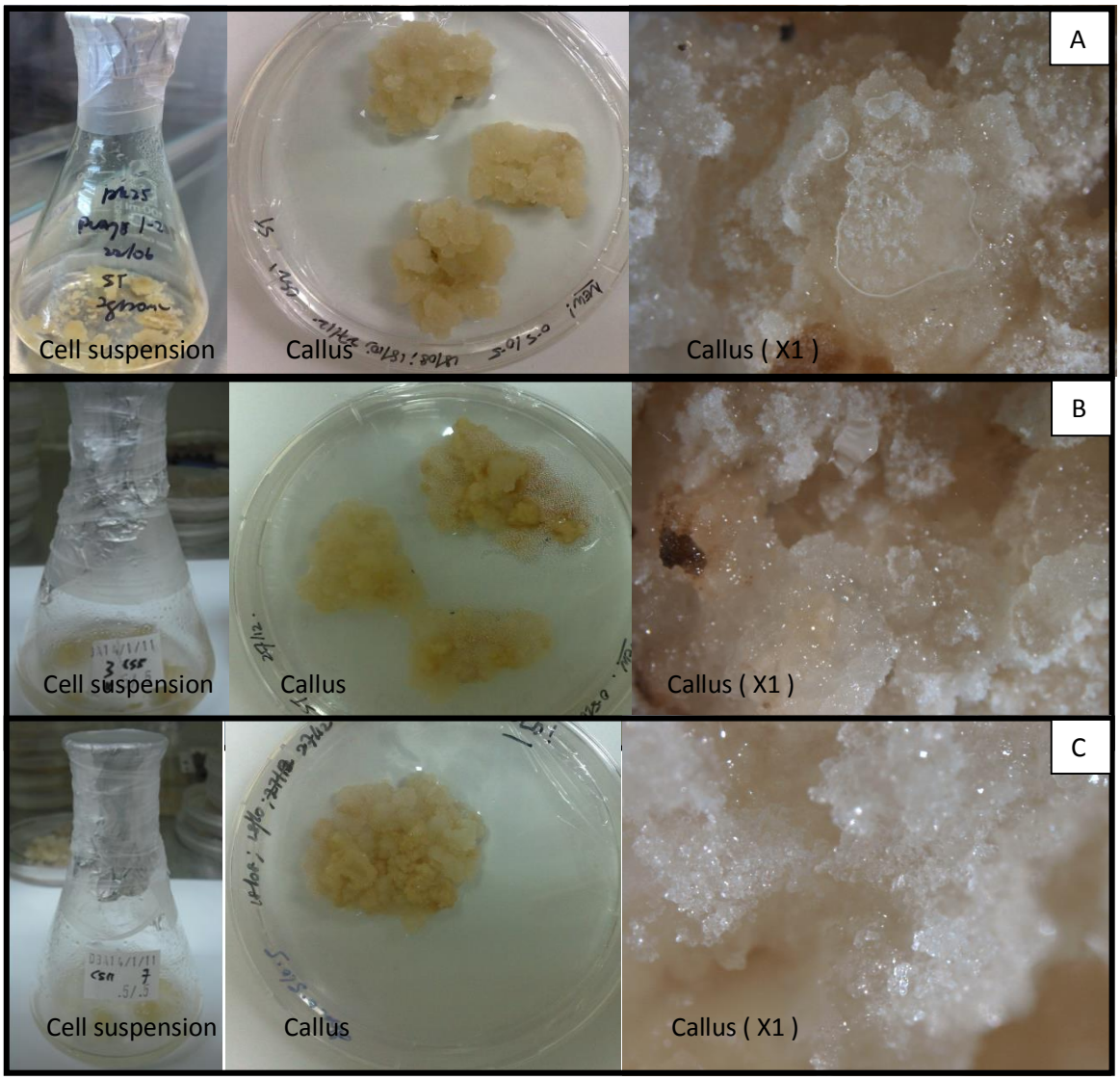


Figure 2.16 Opaque white callus originating from 2.3µM 2,4-D + 2.2µM BAP treatments and the cell suspensions initiated. [A & B] CS4 and CS5 – cell suspension initiated from shoot-derived callus. [C] CS11 – cell suspension initiated from leaf-derived callus.

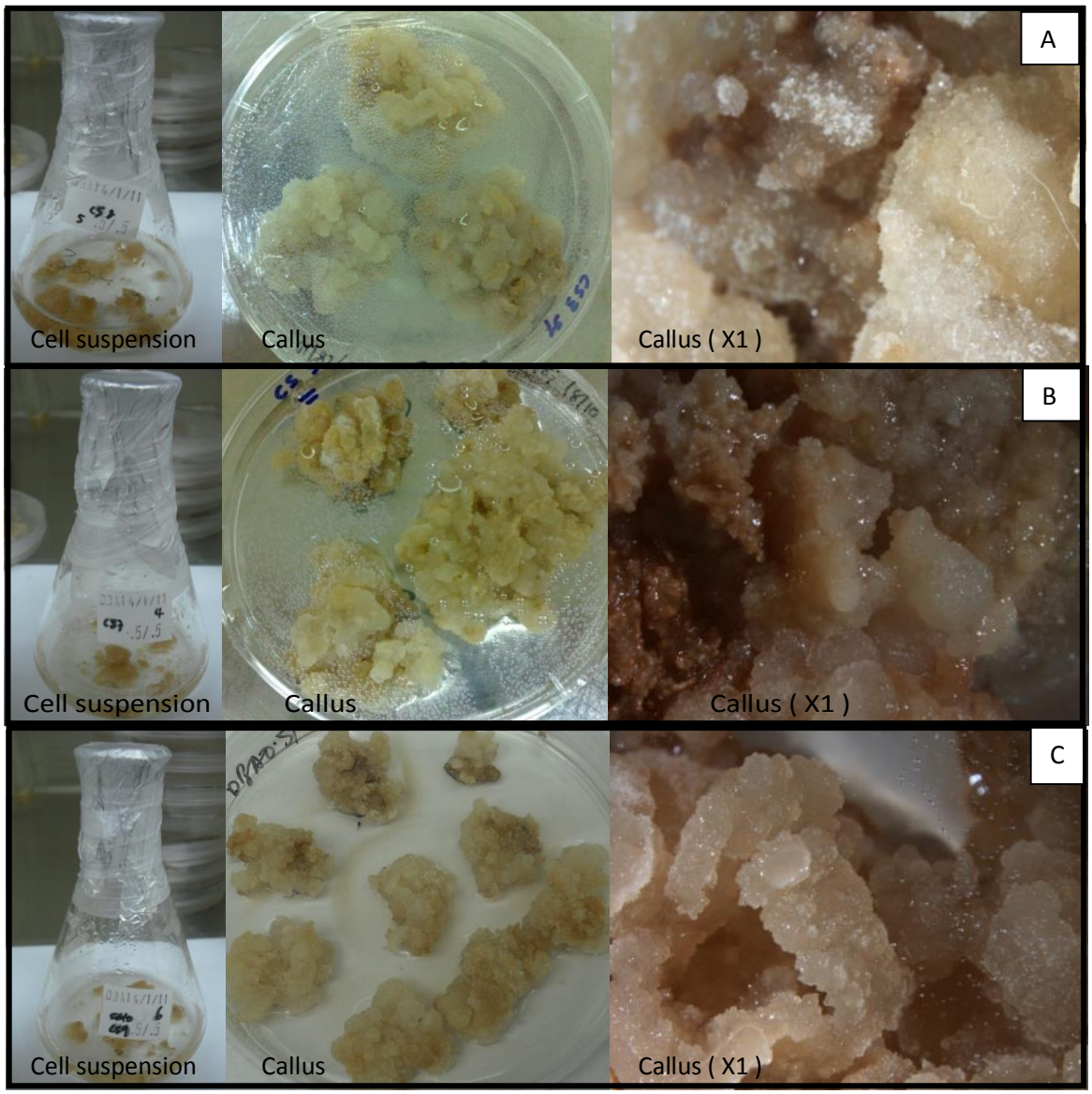


Figure 2.17 Yellow callus originating from 2.3µM 2,4-D + 2.2µM BAP treatment and the cell suspensions initiated. [A–C] CS2, CS7 and CS10 respectively – cell suspensions initiated from shoot-derived callus.

2.2.4.2 Optimisation of culture conditions for *A. malaccensis* cell suspension cultures

The composition of the liquid medium used with an inoculum of 2g callus per 20ml of liquid medium was found to be suitable for initiating cell suspension cultures, however the speed of the rotary shaker had to be reduced from 100 rpm to below 75 rpm because at 100 rpm, *A. malaccensis* cells tended to shear and die leading to a high amount of debris in cell suspensions (Figure 2.19).

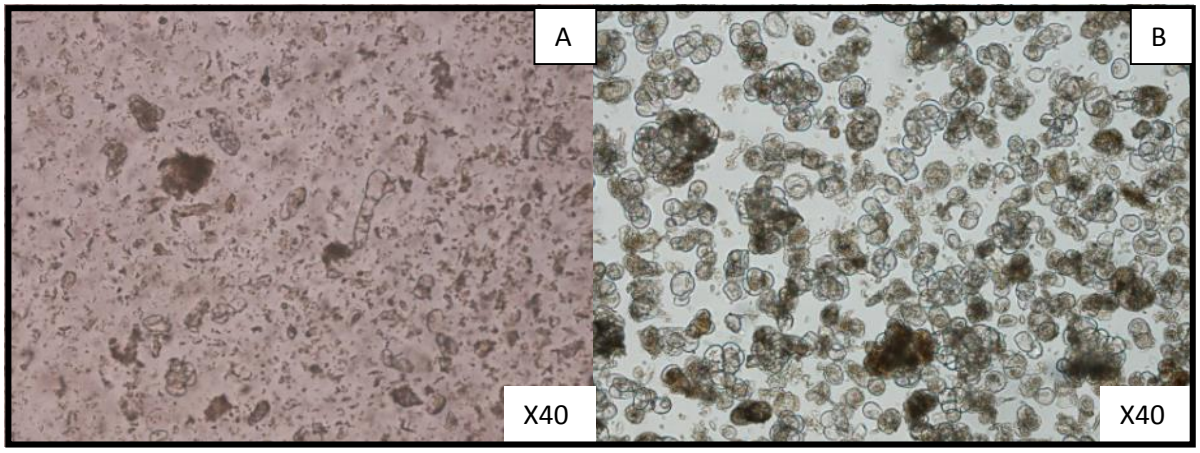


Figure 2.19 *Aquilaria malaccensis* cell line CS11. [A] Cells cultured at 100 rpm. [B] Cells cultured at 75 rpm.

2.2.4.3 Establishment of *A. malaccensis* cell suspension lines

Cell line CS11 (Figure 2.20 A) was found to be more uniform and had a high proliferation rate as compared to CS5 and CS4 (Figure 2.20 B & C) indicating that cell lines established from leaf explant-derived callus performed better than callus originating from shoot explants. By contrast, cell lines CS5 and CS4 formed cell suspensions consisting of large cell aggregates and lacked the uniformity displayed by CS11 therefore based on these observations, it was decided that only CS5 and CS11 cell lines were to be maintained.

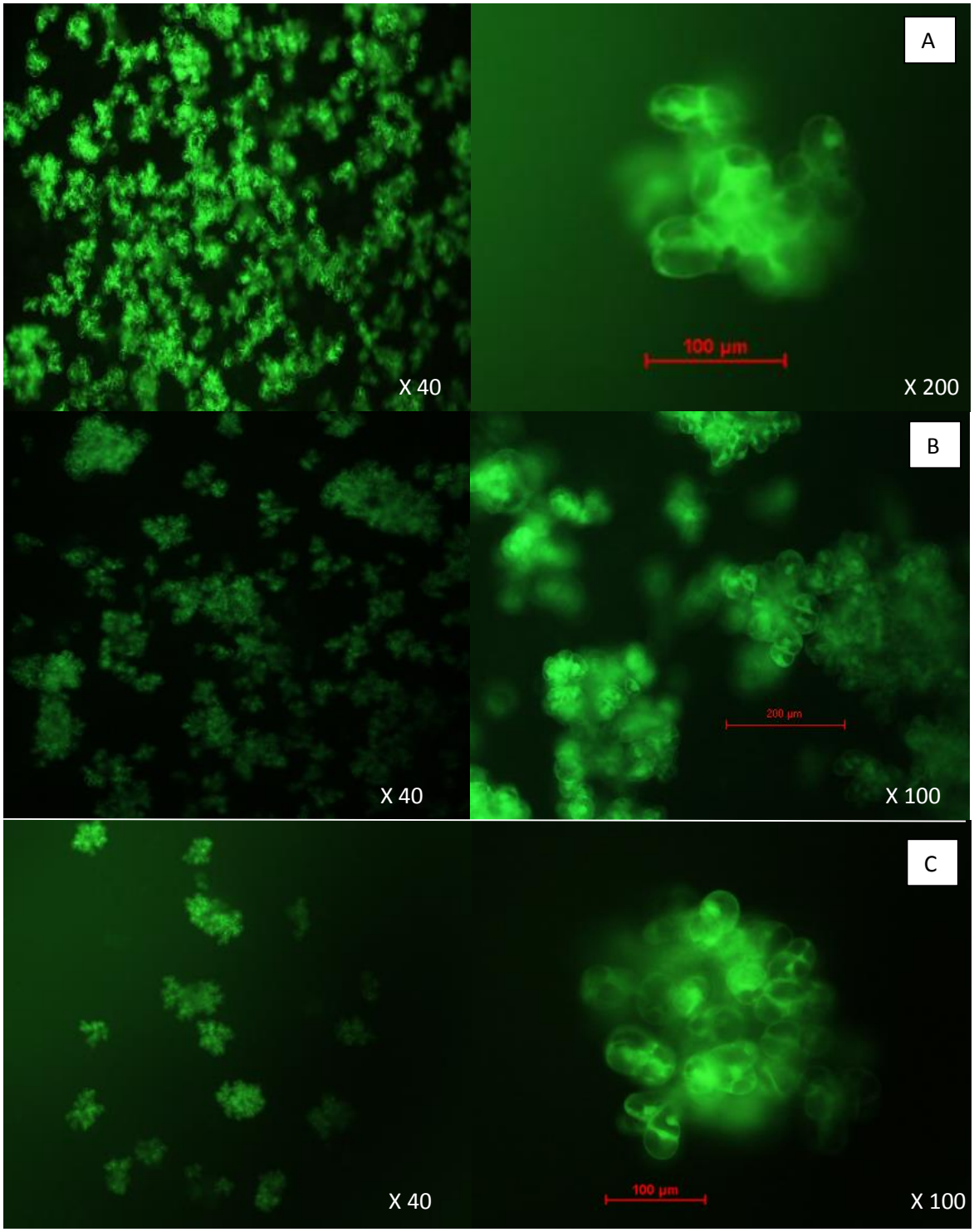


Figure 2.20 FDA test for viability of *A. malaccensis* cell suspension lines to confirm viable dividing cell suspensions of [A] CS11 (leaf-derived), [B] CS5 (shoot-derived) and [C] CS4 (shoot-derived).

2.2.5. Characterisation of growth and development of cell suspensions cultures

The proliferation of cell suspension lines CS5 and CS11 was assessed by measuring the settled cell volume (SCV) which showed a sigmoid increment pattern for both cell lines (Figure 2.21). Cell viability was found to be higher in CS11 as compared to CS5 (Figure 2.22).

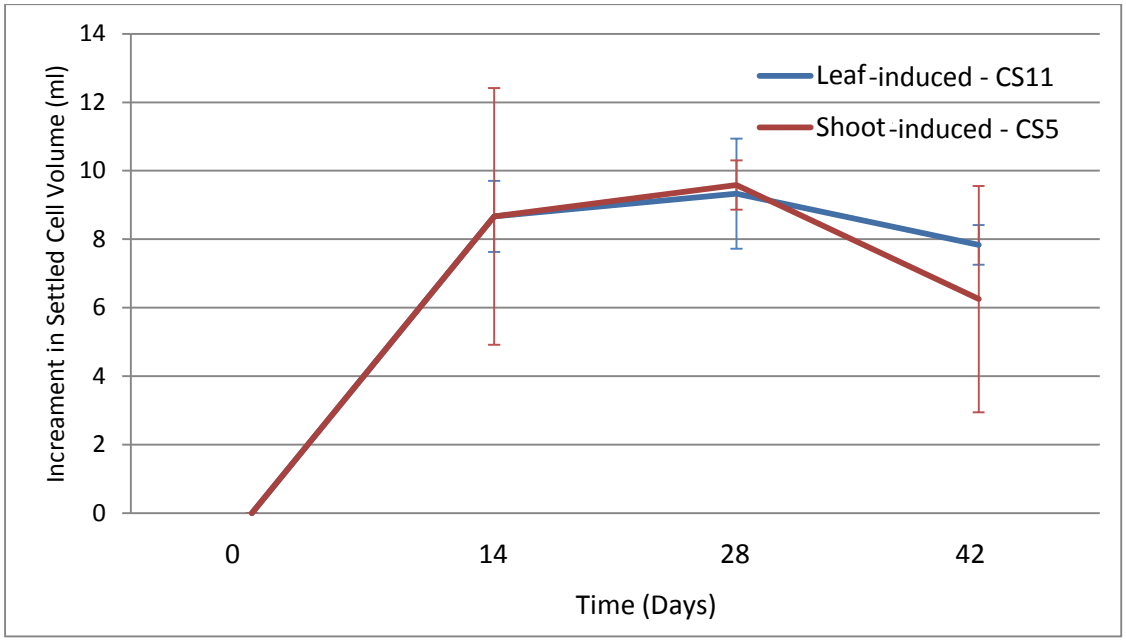


Figure 2.21 Growth curve for cell suspensions of cell lines CS11 and CS5.

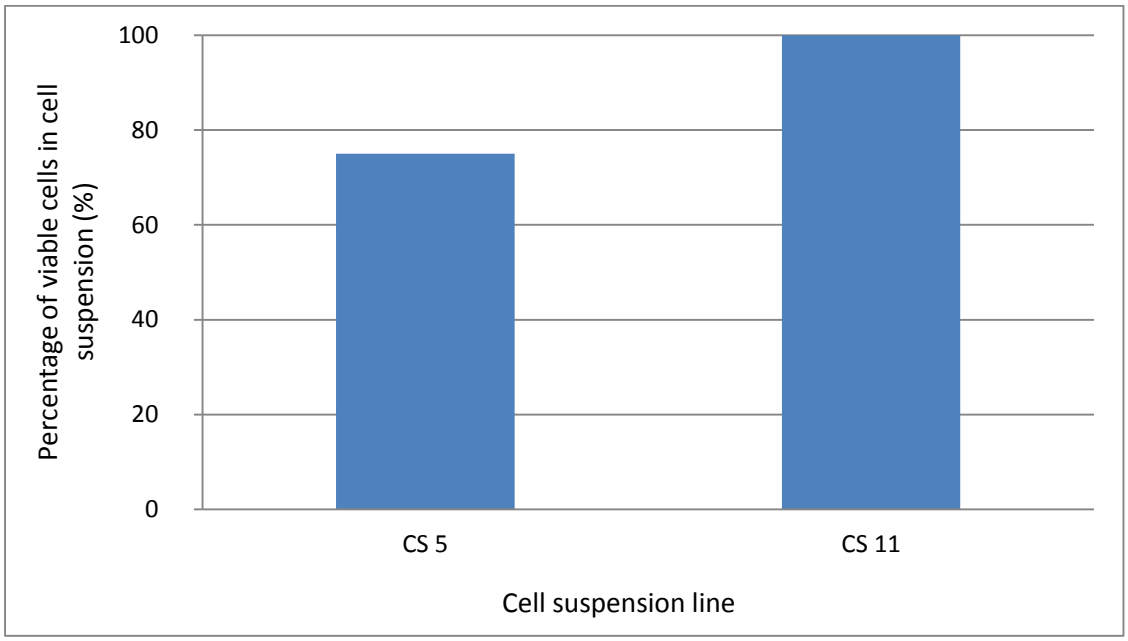


Figure 2.22. Percentage of viable cells in suspension following subculture.

2.3 Discussion

The selection of auxin and cytokinin type, concentration and combination used in this study was based on a review of relevant literature. Although no cell suspensions of *A. malaccensis* have been reported, similar work has been reported in species such as *A. sinensis* (Qi *et al*., 2005) and *A. crassna* (Ito *et al*., 2005) where NAA, BA and 2,4-D were used to induce callus and cell suspension cultures.

In this study, callus was initiated using two combinations of auxin and cytokinin. The inclusion of both an auxin and a cytokinin was found to be necessary as media which contained only one phytohormone did not produce much callus. Following this observation, treatments which did not produce sufficient callus were discarded while responsive callus was sub-cultured onto fresh media. Generally, callus obtained from NAA + Kinetin treatments (Figures 2.5 & 2.9) was more compact as compared to callus from 2,4-D + BAP treatments which was more friable (Figures 2.4 & 2.8). The friable callus from the 2,4-D + BAP treatments seemed to be a more suitable source for cell suspension initiation, however further investigation on callus proliferation was done to ensure that callus development was sustainable.

It was noteworthy that callus development in most treatments was confined to the cut edges of the explants suggesting that callus formation could be further enhanced if explants were cut into smaller pieces or sliced in longitudinal sections. In addition, root formation occurred in the NAA + Kinetin treatments, such as 22.2µM NAA + 4.5µM Kinetin ($N_{22.2}K_{4.5}$) and 22.2µM NAA + 0.5µM ($N_{22.2}K_{0.5}$), which suggests that media with high NAA and low Kinetin concentrations are more likely to undergo root organogenesis. This observation was interesting as NAA could potentially be used to sustain (or support) root formation in *in vitro* propagated shoots which usually take up to 10 weeks to root before plants are transferred to a greenhouse. Since organogenesis was not a part of this study, it was not pursued further.

Shoot explants were clearly capable of producing a more consistent (low deviation) and higher amount (high mean value) of callus as compared to leaf explants (Section 2.2.1). The 2,4-D/BAP treatments seemed to be a good source of callus because morphologically it was more suitable for cell suspension initiation. However, further investigation on callus proliferation was required to ensure that the callus development was sustainable.

In general, callus growth/proliferation was observed to be better in callus derived from shoots explant which were subject to the 2, 4-D/BAP treatment (Figure 2.12) as the growth rate and the amount of callus generated was higher than leaf-derived callus lines. After three consecutive sub-culture cycles (February 2011), sufficient callus was generated from the shoot-derived callus, for the use in initiating suspension cultures. Contrarily it took double the time to establish callus lines from leaf tissue which may be related to the slow growth of leaf callus during the initiation stage. A direct comparison of the diameter of leaf-derived callus with the shoot-derived callus showed that that shoot-derived callus was at least double the size of leaf-derived callus given that both cultures had been subject to three sub-culture cycles upon initiation (Figure 2.13 & 2.14). However it was interesting to note that once a substantial amount of leaf-derived callus had established (Approximately 80mm^2 in size) the growth rates were comparable.

After a series of subculture cycles, 5 out of the 15 phytohormone treatments ($D_{4.5}B_{4.4}$, $D_{2.3}B_{2.2}$, $D_{2.3}B_{0.4}$, $D_{4.5}B_{2.2}$, $D_{2.3,}B_{4.4}$) were found to produce sufficient callus that would be suitable for introduction into liquid medium for cell suspension culture initiation. Out of 5 of the phytohormone treatments (Figure 2.15), the $D_{2.3}B_{2.2}$ (2.3µM 2,4-D + 2.2µM BAP) treatment was identified to most suitable whereby, regardless of explant source (leaf or shoots), the callus produced were friable (suitable for cell suspensions cultures; other treatments produced a more compact type of callus which did not respond well in liquid treatment). In addition, the amount of callus generated was also substantially more when compared to the

other treatments. The establishment of a stable high yielding homogenous callus line plays an important role in ensuring a continuous supply of viable cells to facilitate the establishment of cell suspensions (Franklin & Dixon, 1994).

One of the major challenges, in the establishment of a consistent callus line was that, over time, aging callus (yellowish brown) started to form within the callus clump (as shown in Figure 2.17). This callus must be removed before transferring the callus into fresh medium, as the old browning tissue would affect the morphology / quality of the callus whereby it would cause friable and translucent callus to transition into a more compact and yellow-brown form. Although, growth could still be observed after the first subculture cycle, it was not sustainable. After 2 to 3 cycles, the callus started to show signs of premature senescence, and eventually died. The formation of yellow-brown callus is most likely linked to cell aging in which, as cell proliferation accelerates, older cells tend to die off. As these older cells undergo senescence, ethylene gas is produced. Subsequently, in a closed culture environment, ethylene build-up will lead to compromised growth rate and would eventually cause cells to undergo premature senescence (Burg, 1988; Proft *et al*., 1985; Thomas & Murashige, 1979; LaRue & Gamborg, 1971). This observation suggests that *Aquilaria* cells are extremely sensitive to stress. This could be associated with early stages of agarwood production in which, once cells are injured, the plants natural defence system would react by killing the injured cells to localize infection. Further research is required to validate this.

After initiating multiple cell suspension cultures using callus originating from both leaf and shoot explants, three lines of cell suspension cultures were established (Figure 2.18). Out of these three cell suspension lines two originated from shoot-derived callus (CS7 and CS5) and the third from leaf-derived callus (CS11). All these cell suspension lines comprised of friable cell aggregates. Unlike microbial (bacteria) cell suspension cultures which exist as single cells, plant cells in suspension tend to form clumps due to the failure of new cells to

separate after division or due to adherence of free cells (Tanaka *et al*., 1988). This tendency may also be due to the secretion of extra-cellular polysaccharides which increase adhesion among cells (Kato *et al*., 1994). Generally, plant cell suspensions consisting of aggregates of 5-200 cells are considered as acceptable and may be used in batch cultures or experiments (Loyola-Vargas & Vazquez-Flota, 2006). The establishment of single plant cell suspensions is not practical as it can only be achieved by using cell wall degrading enzymes or sieves and, once the cell density has increased, the presence of a high concentration of polysaccharides will cause the clumps to reform (Fowler, 1982).

In this study, the formation of large aggregates of cells was also a consequence of the slow agitation speed of 75 rpm compared to the commonly used agitation used in plant cell culture of 100-120 rpm. During initial attempts, the cells in suspensions were agitated at 100 rpm but most suffered severe shearing (Figure 2.19) resulting in cell death probably caused by excessive agitation speed (Tanaka *et al*., 1988) thus, the speed was reduced to 75 rpm. The establishment of cell suspension cultures from woody plants is usually difficult mainly due to the inflexible cell walls and their large size (compared to mammalian or bacterial cells), which cause plant cells to be extra shear sensitive (Merchuk, 1991; Zhong, 2001). It is important to determine the optimal agitation speed for cell growth while maintaining the uniformity of the cell suspension.

Overall, the shoot explant-derived cell suspension cultures formed larger aggregates (almost double the size) compared to leaf explant-derived cell suspension cultures (Figure 2.18). A comparison amongst the shoot explant-derived cell suspension cultures showed that CS5 was much more prolific, homogenous and stable (high viability following subculture) than the CS7 line . Based on these observations, only CS5 and CS11 were maintained for further standardisation. Growth of the homogenous cell suspensions was assessed using the settled cell volume (SCV) over time. The determination of packed cell volume, which is commonly used

in plant cell suspension studies, was deemed unsuitable possibly due to the high possibility of shearing during centrifugation.

From the growth curves, the growth of CS11 and CS5 were similar (Figure 2.21), however viability (FDA) analysis revealed that CS11 had more viable cells as compared to CS5 (Figure 2.22). Based on this observation, it was concluded that CS11 would be bulked up and used in stress related experiments later in the study.

2.4 Conclusion

From this part of the study, it can be concluded that callus lines of *Aquilaria malaccensis* can be established and maintained by culturing leaf segments from '*in vitro*' grown shoot cultures, on MS medium supplemented with 3% sucrose, 0.3% phytagel, 2.2µM of 2,4-D and 2.3 µM of BAP, and cultured at 28 ± 2 ºC under continuous dark conditions. Once callus lines were established, cell suspensions could be initiated using 5ml of cells (SCV) in 50 ml of liquid medium which comprise of the same medium composition, without the gelling agent and placed on a rotary shaker at 75 rpm.

CHAPTER 3

Chemical analysis of fragrant compounds of *Aquilaria malaccensis*

3.0 Introduction

Agarwood, commonly known as gaharu in Malaysia, refers to the highly valuable resinous heartwood produced by some species of the genera *Aquilaria* and *Gyrinops* (Naef, 2011). It is commercially available in its raw or resin form, where the oil is embedded in the wood, and as a processed oil. Both of these products are used intensively by Asian and Middle East communities for cultural, religious and medicinal purposes (Chakrabarty *et al*., 1994). The formation of resin (agarwood) in *Aquilaria* species is a result of the production of secondary metabolites initiated by the plant defence response to external stimuli such as wounding, mechanical forces and pathogen attack (Nobuchi & Siripatanadilok, 1991). Not all *Aquilaria* trees contain resin as the production of agarwood only takes place when the immune system of the plant is compromised. The formation of agarwood occurs over a long period under optimal conditions (which are not well understood) and the amount produced varies greatly between plants. This has hindered efforts to understand the mechanism behind agarwood formation.

Agarwood oil and extracts consist of a highly complex mixture of volatiles and semi-volatiles. The first study of the chemical content of agarwood was in 1935 when Kafu and Inchikawa reported the presence of sesquiterpene alcohol in agarwood (Ng *et al*., 1997). Since then, agarwood oil and extracts have been found to consist mainly of sesquiterpenes and phenyl-ethyl chromones. To date, 66 sesquiterpene derivatives and 39 phenyl-ethyl chromones have been identified in the aromatic resin (agarwood) produced by the *Aquilaria* genus (Naef, 2011). Prior to analysing the composition of agarwood, it is necessary to extract the oil from the resinous material which is embedded in the wood. This is usually done through hydro-distillation, supercritical fluid extraction or using solvents such as pentane, acetone, methanol, ethanol and diethyl ether. Once extracted, the oil is analysed using techniques such as infrared spectroscopy (IR), gas chromatography coupled with a mass spectrometer (GC-MS), liquid chromatography coupled with a mass spectrometer (LC-MS), gas chromatography

olfactometry (GC-O), X-ray and nuclear magnetic resonance technology (NMR) (Maheshwari *et al.*, 1963a, 1963b; Nakanishi & Yamagata, 1984; Yang *et al*., 1989; Ishihara *et al*., 1991a; Xu *et al*., 1988; Nat *et al.*, 1993). A comprehensive knowledge of the constituents of agarwood is important in order to obtain a better understanding of agarwood formation within *Aquilaria* species and to develop methods to differentiate the essential oil produced by different species (important in ensuring CITES protected species are not illegally traded).

3.0.1 Sesquiterpenes

Terpenoids represent the largest class of secondary metabolites present in the plant kingdom in which, to date, there have been over 36,000 compounds identified with approximately 1000 new structures being discovered annually (Ashour *et al., 2010*). The classification of terpenoids (Table 3.1) is based on the number of isoprenoid units present in the structure. Terpenoids have well-established roles in almost all basic plant processes ranging from growth, development, reproduction, to defence.

Table 3.1 Classification of terpenoids (Sell, 2003)

Number of isoprenoid units	Classification
2	Monoterpenes
3	Sesquiterpenes
4	Diterpenes
5	Sesterpenes
6	Triterpenes
8	Tetraterpenes

In plant cells, terpenes are produced through two distinct pathways. The cytosolic mevalonate (MVA) pathway produces sesquiterpenes, triterpenes and monoterpenes and the choloroplastic 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway produces monoterpenes, diterpenes and isoprenes (Figure 3.1). Both pathways utilise isopentyl pyrophosphate (IPP) and

its isomer dimethylallyl diphosphate (DMADP) as building blocks for the production of terpenes (Vickers *et al*., 2009).

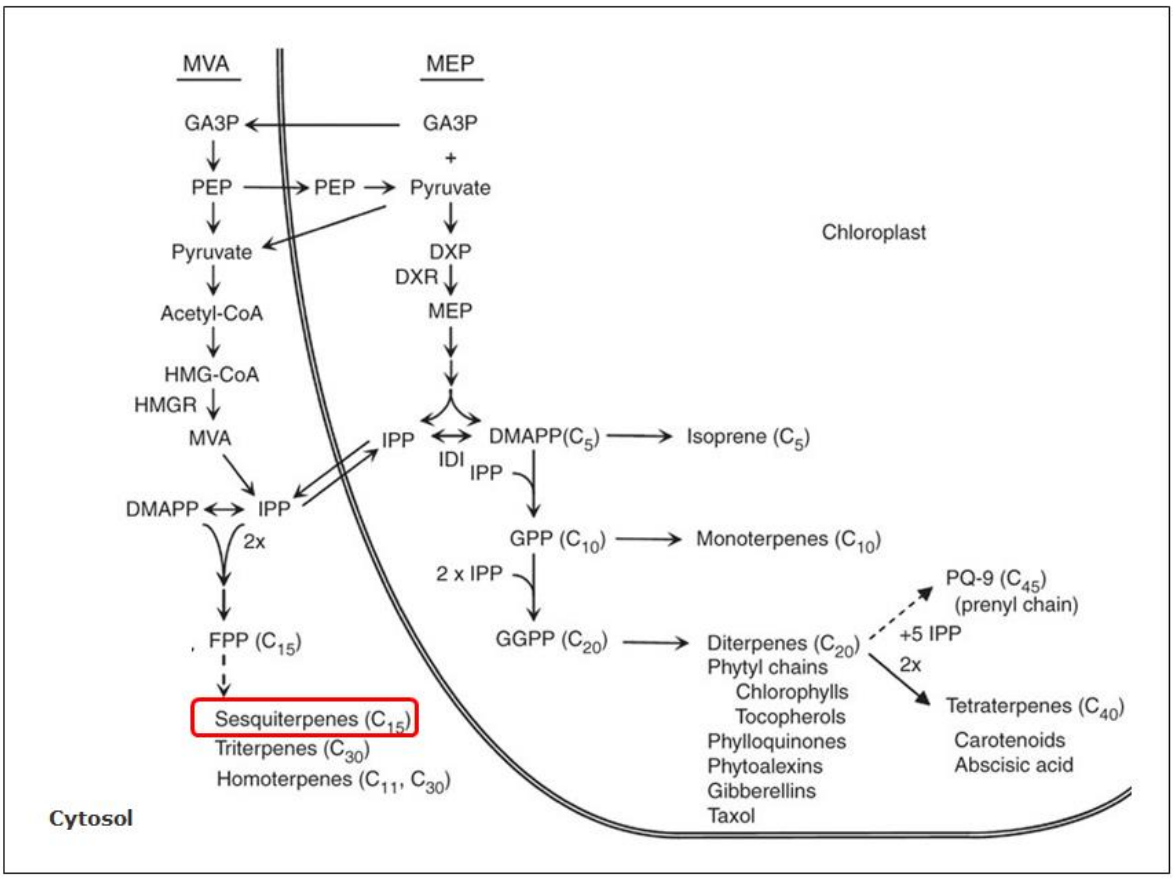


Figure 3.1 Terpene biosynthetic pathway (Vickers *et al*., 2009).

Since 1990, many types of sesquitepenes have been isolated and identified from *Aquilaria* oil and extracts (Table 3.2). They are produced in the cytosolic MVA pathway via farnesyl diphosphate – FPP (Figure 3.1) before being converted into different types of sesquiterpenes (Figure 3.2). Pathways which involve sesquiterpene synthesis include germacrane, humulane, caryophylane, cadinane, bisabolene, carotene and drimane (Limberger *et al*., 2002). Sesquiterpenes play a variety of roles in plant cells, which include being a plant growth regulator (Kalsi *et al*., 1988; Chen *et al*., 1990; Gross *et al*., 1994), allelopathic agent (Macias, 1995; Macias *et al*., 1996; Abdelgaleil & Hashinaga 2007), phytoalexin (Banerjee *et*

al., 2006), allomone (Mayer *et al*., 2008) and phytotoxin (Mancini *et al*., 2009; Alarcon *et al*., 2007). Many sesquiterpenes are of economic interest, particularly in the flavour and fragrance industries and in the nutraceutical, pigment and agrochemical sectors.

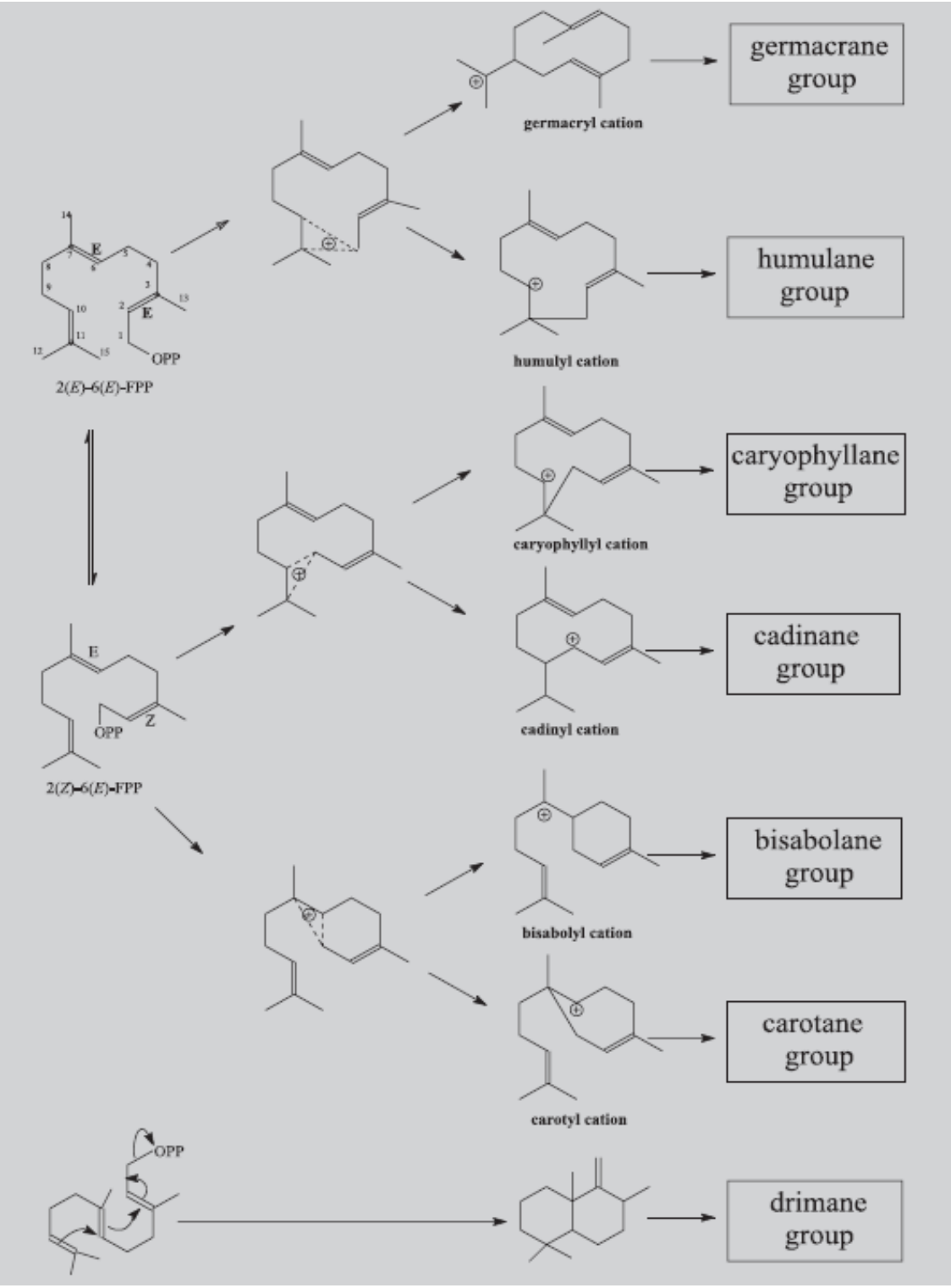


Figure 3.2 Biosynthetic pathways for cyclic sesquiterpenes (Limberger *et al*., 2002).

Table 3.2 Types of sesquiterpenes extracted from *Aquilaria* species.

No.	Compound Name	CAS No.	Isolation and analytical technique used	Plant source	Citation
1.	α-Agarofuran	5956-12-7	IR, 1H-NMR (60 MHz)	*A. agallocha* *A. sinensis* *A.malaccensis*	Maheshwari *et al.* (1963a); Yang *et al*. (1989); Nakanishi *et al*. (1984)
2.	β-Agarofuran	6040-08-0	IR, 1H-NMR (60 MHz)	*A. agallocha* *A. crassna* *A. sinensis*	Maheshwari *et al.* (1963a); Wetwitayaklung *et al*. (2009); Mei *et a*l. (2008)
3.	Dihydro-β-agarofuran	20053-66-1	IR, 1H-NMR (60 MHz)	*A. agallocha* *A. sinensis*	Maheshwari *et al.* (1963a); Ishihara *et al*. (1991a); Xu *et al.* (1988)
4.	4 (1R,2R,6S,9R)-6,10,10-Trimethyl-11-oxatricyclo[7.2.1.01,6] dodecane-2-spiro-2′-oxirane (epoxy-b-agarofuran)	154855-32-0	Not Available	*A. agallocha*	Nat *et al.* (1993)
5.	4-Hydroxy-dihydro-agarofuran	15052-76-3	IR, 1H-NMR (60 MHz)	*A. agallocha* *A. sinensis*	Nat *et al.* (1993; Mei *et al*. (2008)
6.	3,4-Dihydroxydihydroagarofuran	97805-32-8	IR, 1H-NMR (60 MHz)	*A. agallocha*	Maheshwari *et al.* (1963b)
7.	Baimuxinol	105013-72-7	IR, 1H-NMR, MŚ	*A. sinensis*	Yang *et al*. (1986); Mei *et al*. (2008)
8.	Isobaimuxinol	105013-72-7	IR, 1H-NMR, MŚ, X-Ray	*A. sinensis*	Yang *et al*. (1986)
9.	Dehydrobaimuxinol	105013-74-9	IR, 1H-NMR, MŚ	*A. sinensis*	Yang *et al*. (1989)
10.	(1S,2S,6S,9R)-6,10,10-Trimethyl-	154855-33-1	MŚ	*A. agallocha*	Nat *et al.* (1993)

	11-oxatricyclo[7.2.1.01,6] dodecane-2-carbaldehyde				
11.	Baimuxifuranic acid	147362-51-4	IR, MŚ, 1H-, 13C-NMR (500 MHz)	*A. sinensis*	Yang *et al*.(1983); Mei *et al*.(2008)
12.	(1R,6S,9R)-6,10,10-Trimethyl-11-oxatricyclo[7.2.1.01,6]dodecane	147807-98-5	MŚ, 1H-, 13C-NMR (360MHz)	*A. agallocha* *A. sinensis*	Nat *et al.* (1993); Mei *et a*l. (2008)
13.	(1R,2R,6S,9R)-6,10,10-Trimethyl-11-oxatricyclo[7.2.1.01,6] dodecan-2-ol	147807-99-6	MŚ	*A. agallocha* *A. sinensis*	Nat *et al.* (1993); Mei *et al*. (2008)
14.	nor-Keto-agarofuran	5986-25-4	IR, 1H-NMR (60 Mz)	*A. agallocha* *A. crassna*	Maheshwari *et al.* (1963a); Nat *et al.* (1993);Wetwitayaklung *et al*. (2009)
15.	Agarol	5956-13-8	IR, UV, 1H-NMR	*A. agallocha*	Pant & Rastogi (1980)
16.	Gmelofuran	70863-78-4	IR, UV, 1H-NMR	*A. agallocha*	Pant & Rastogi (1980)
17.	(5S,7S,10S)-(-)-Selina-3,11-dien-9-one	117212-69-8	EIMŚ, HRMŚ, IR, 1H-NMR (500 MHz), 13C-NMR (126 MHz)	*A. agallocha* *A. crassna*	Ishihara *et al*. (1993); Wetwitayaklung *et al*. (2009)
18.	(5S,7S,9S,10S)-(+)-Selina-3,11-dien-9-ol	133593-96-1	EIMŚ, HRMŚ, IR, 1H-NMR (500 MHz), 13C-NMR (126 MHz)	*A. agallocha* *A. crassna*	Ishihara *et al*. (1993); Wetwitayaklung *et al*. (2009)
19.	Selina-3,11-dien-14-ol	150034-04-1	EIMŚ, HRMŚ, IR, 1H-NMR (500 MHz),	*A. agallocha*	Ishihara *et al*. (1993);

			13C-NMR (126 MHz)		
20.	Selina-3,11-dien-14-al	150034-03-0	EIMS, HRMS, IR, 1H-NMR (500 MHz), 13C-NMR (126 MHz)	*A. agallocha* *A. crassna*	Ishihara *et al*. (1993); Wetwitayaklung *et al*. (2009)
21.	Selina-3,11-dien-14-oic acid (as methyl ester)	150034-06-3	EIMS, HRMS, IR, 1H-NMR (500 MHz), 13C-NMR (126 MHz)	*A. agallocha* *A. crassna*	Ishihara *et al*. (1993); Wetwitayaklung *et al*. (2009)
22.	Selina-4,11-dien-14-al	150034-05-2	EIMS, HRMS, IR, 1H-NMR (500 MHz), 13C-NMR (126 MHz),	*A. agallocha* *A. crassna*	Ishihara *et al*. (1993); Wetwitayaklung *et al*. (2009)
23.	Selina-4,11-dien-14-oic acid (as methyl ester)	150071-58-2	EIMS, HRMS, IR, 1H-NMR (500 MHz), 13C-NMR (126 MHz),	*A. agallocha* *A. crassna*	Ishihara *et al*. (1993); Wetwitayaklung *et al.* (2009)
24.	9-Hydroxy-selina-4,11-dien-14-oic acid(as methyl ester)	150034-07-4	EIMS, HRMS, IR, 1H-NMR (500 MHz), 13C-NMR (126 MHz),	*A. agallocha*	Ishihara *et al*. (1993)
25.	Agarol (11(13)-Eudesmen-12-ol)	1460-64-6	UV, IR, derivatization, degradation	*Aquilaria sp.*	Jain & Battacharrya (1959)

26.	10-epi-g-Eudesmol	15051-81-7	IR, EIMŚ, HRMŚ, 1H-NMR (400 MHz),	*A. agallocha* *A. crassna* *A.malaccensis* *A. sinensis*	Wetwitayaklung *et al*. (2009); Nakanishi *et al*. (1984); Naf *et al.* (1995), Mei *et al*. (2008)
27.	(S)-4a-Methyl-2-(1-methylethylidene)-1,2,3,4,4a,5,6,7-octahydronaphthalene	147807-97-4	MŚ, Rt	*A. agallocha* *A. sinensis*	Naf *et al.* (1995); Mei *et al*. (2008)
28.	(S)-4a-Methyl-2-(1-methylethyl)-3,4,4a,5,6,7-hexahydronaphthalene	147807-96-3	MŚ, Rt	*A. agallocha* *A. sinensis*	Naf *et al.* (1995); Mei *et al*. (2008)
29.	(2R,4aS)-4a-Methyl-2-(1-methylethenyl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	147853-17-6	MŚ, Rt	*A. agallocha*	Naf *et al.* (1995)
30.	(2R,4aS)-2-(4a-Methyl-1,2,3,4,4a,5,6,7-octahydronaphthyl)-propan-2-ol (4-nor-epi-g-eudesmol)	147853-18-7	1H-NMR (360 MHz), 13C-NMR (90 MHz), MŚ	*A. agallocha* *A. sinensis*	Naf *et al.* (1995); Mei *et al*. (2008)
31.	(+)-(4S,5R)-Dihydrokaranone	19598-45-9	MŚ, IR, 1H-NMR (90 MHz)	*A. agallocha* *A. crassna* *A. sinensis*	Naf *et al.* (1995); Wetwitayaklung *et al*. (2009); Xu *et al.* (1988)
32.	(+)-(4S,5R)-Karanone	91466-22-7	MŚ, IR, 1H-NMR (90 MHz)	*A. agallocha*	Naf *et al.* (1995);
33.	Eremophila-9,11-dien-8-one (Neopetasane)	13902-42-6	MŚ, IR	*A. agallocha* *A. crassna* *A. sinensis*	Naf *et al.* (1995); Wetwitayaklung *et al*. (2009); Mei *et al*. (2008)
34.	rel-(2R,8R,8aS)-2-(1,2,3,5,6,7,8,8a-Octahydro-8,8a-dimethyl-2-	168099-18-1	MŚ, 1H-NMR (360 MHz),	*A. agallocha*	Naf *et al.* (1995)

	naphthyl)-prop-2-en-1-ol (Eremophila-9,11(13)-dien-12-ol		13C-NMR (90 MHz)		
35.	rel-(3R,7R,9R,10S)-9,10-Dimethyl-6-methylene-4-oxatricyclo[7.4.0.03,7] tridec-1-ene, (8,12-Epoxyeremophila-9,11(13)-diene)	168099-19-2	MŚ, 1H-NMR (360 MHz), 13C-NMR (90 MHz)	*A. agallocha*	Naf *et al.* (1995)
36.	(-)-(4R,5S,7R)-Jinkoh-eremol	86747-08-2	IR, HRMŚ, 1H-NMR, CD	*A. agallocha* *A. crassna* *A. sinensis*	Wetwitayaklung *et al*. (2009); Naf *et al.* (1995); Mei *et al*. (2008)
37.	Dehydro-jinkoh-eremol	150034-02-9	EIMŚ, Rt,	*A. agallocha* *A. sinensis*	Ishihara *et al.,* (1993); Mei *et al*. (2008)
38.	(+)-(4R,5S,7R)-Kusunol (= Valerianol)	20489-45-6	IR, 1H-NMR, CD	*A. agallocha* *A. sinensis*	Naf *et al.* (1995); Ishihara *et al.* (1993); Mei *et al*. (2008)
39.	rel-(2R,8S,8aS)-2-(1,2,6,7,8,8a-Hexahydro-8,8a-dimethyl-2-naphthyl)-propan-2-ol (Valenca-1(10),8-dien-11-ol)	168099-20-5	MŚ,1H-NMR (360 MHz), 13C-NMR (90 MHz)	*A. agallocha* *A. crassna*	Nat *et al.* (1995); Wetwitayaklung *et al*. (2009)
40.	2,t-3-Dimethyl-r-2-(3-methyl-2-butenyl)-1-cyclohexanone	154855-35-3	NA	*A. agallocha*	Naf *et al*. (1995)
41.	α-Guaiene	88-84-6	EIMŚ, Rt	*A. agallocha*	Ishihara *et al*. (1991b)
42.	α-Bulnesene	3691-11-0	EIMŚ, Rt	*A. agallocha* *A. crassna*	Ishihara *et al*. (1991a); Wetwitayaklung *et al*. (2009)
43.	(-)-Epoxyguai-11-ene (epoxybulnesene)	133647-70-8	-IR, EIMŚ, 1H-NMR	*A. agallocha*	Ishihara *et al*. (1991b)

44.	(-)-Guaia-1(10),11-dien-15-ol	138529-08-5	HRMŚ, IR, 1H-NMR, 13C-NMR, COSY	*A. agallocha*	Ishihara *et al*. (1991b)
45.	(-)-Guaia-1(10),11-dien-15-al	133593-95-0	HRMŚ, IR, 1H-NMR, 13C-NMR,	*A. agallocha* *A. crassna*	Ishihara *et al*. (1991b); Wetwitayaklung *et al*. (2009)
46.	(-)-Guaia-1(10),11-diene-15-carboxylic acid	138529-09-6	HRMŚ, 1H-NMR, 13C-NMR	*A. agallocha* *A. crassna*	Ishihara *et al*. (1991a); Wetwitayaklung *et al*. (2009)
47.	Methyl guaia-1(10),11-diene-15-carboxylate	138529-10-9	HRMŚ, 1H-NMR, 13C-NMR	*A. agallocha*	Ishihara *et al*. (1991b)
48.	(-)-Guaia-1(10),11-dien-15,2-olide	138529-12-1	HRMŚ, 1H-NMR, 13C-NMR, COSY	*A. agallocha*	Ishihara *et al*. (1991b)
49.	(-)-2a-Hydroxyguaia1(10),11-dien-15-oic acid	146201-43-6	Not available	*A. agallocha*	Ishihara *et al*. (1991b)
50.	(+)-Guaia-1(10),11-dien-9-one	138529-11-0	IR, EIMŚ, HRMŚ, 1H-NMR, 13C-NMR,	*A. agallocha* *A. crassna*	Ishihara *et al*. (1991b)
51.	Rotundone	18374-76-0	IR, 1H-NMR, 13C-NMR	*A. agallocha* *A. crassna*	Ishihara *et al*. (1991b); Wetwitayaklung *et al*. (2009)
52.	(+)-1,5-Epoxy-nor-ketoguaiene	150034-01-8	IR, EIMŚ, HRMŚ, 1H-NMR, 13C-NMR,	*A. agallocha* *A. crassna*	Ishihara *et al*. (1991b); Wetwitayaklung *et al*. (2009)
53.	epi-Ligulyl oxide	18680-81-4	MŚ	*A. sinensis*	Mei *et al*. (2008)
54.	Sinenofuranol	122739-17-7	MŚ, IR, 1H-NMR,	*A. sinensis*	Xu *et al*. (1988); Mei *et al*. (2008)
55.	Sinenofuranal	122739-16-6	MŚ, IR, 1H-NMR,	*A. sinensis*	Xu *et al*. (1988)

56.	Jinkohol	66512-57-0	IR, HRMŚ, 1H-NMR (400 MHz), 13C-NMR (100 MHz),	*Aquilaria sp.*	Nakanishi *et al*. (1984)
57.	Jinkohol II	86703-03-9	IR, HRMŚ, 1H-NMR (400 MHz), 13C-NMR (100 MHz	*Aquilaria sp.*	Nakanishi *et al*. (1984)
58.	Agarospirol, (2R,5R,10R)-a,a, 6,10-tetramethyl-spiro [4,5]dec-6-ene-2-methanol	1460-73-7	IR, derivatization, degradation HRMŚ, IR, 1H-NMR (200 MHz), EIMŚ, Rt	*A. agallocha* *A. crassna* *A. sinensis*	Wetwitayaklung *et al*. (2009); Yang *et al*. (1983); Nat *et al*. (1993)
59.	Isoagarospirol	-	Not Available	-	Nakanishi *et al*. (1981)
60.	Oxo-agarospirol = Baimuxinal	93133-69-8 or 86408-21-1	MŚ, IR, 1H-NMR, degradation	*A. agallocha* *A. crassna* *A.malaccensis* *A. sinensis*	Ishihara *et al*. (1991a); Wetwitayaklung *et al*. (2009); Nakanishi *et al*. (1984); Mei *et al*. (2008)
61.	Baimuxinic acid = Bai Mu Xiang acid	84210-00-4	MŚ, IR, 1H-NMR X-ray	*A. sinensis*	Yang *et al*. (1983)
62.	rel-(5R,10R)-2-Isopropylidene-10-methyl-spiro[4.5]dec-6-ene-6-carbaldehyde (Vetispira-2(11), 6-dien-14-al)	168099-21-6	MŚ, 1H-, 13C-NMR	*A. agallocha*	Naf *et al*. (1995)
63.	rel-(1R,2R)-9-Isopropyl-2-methyl-8-oxatricyclo[7.2.1.01,6]dodec-5-ene (2,14-Epoxy-vetispir-6-ene)	168099-23-8	MŚ, 1H-, 13C-NMR,	*A. agallocha*	Naf *et al*. (1995)

64.	rel-(1R,2R)-9-Isopropyl-2-methyl-8-oxatricyclo[7.2.1.01,6]dodeca-4,6-diene (2,14-Epoxy-vetispira-6(14),7-diene)	168099-24-9	MŚ, 1H-, 13C-NMR,	*A. agallocha*	Naf *et al*. (1995)
65.	rel-(5R,7S,10R)-2-Isopropylidene-10-methyl-6-methylene-spiro [4.5]decan-7-ol(Vetispira-2(11),6(14) -dien-7-ol)	168099-22-7	MŚ, 1H-, 13C-NMR,	*A. agallocha*	Naf *et al*. (1995)
66.	(-)-(4R,5R,7R)-11-Hydroxy-vetispir-6-en-8-one	887781-95-5	HRMŚ, EIMŚ, IR, UV, 1H-, 13C-NMR,	*Aquilaria sp.*	Ueda *et al.* (2006)

Most of the pioneering work on the isolation and identification of sesquiterpenes listed in Table 3.2 was done using mass spectrometry and nuclear magnetic resonance spectroscopy. After positive identification, a compound would be assigned a Chemical Abstracts Service Registry (CAS) number and be incorporated into the National Institute of Standard and Technology (NIST) database enabling subsequent research to be done through Gas Chromatography – Mass Spectrometry (GC-MS). Compounds could then be identified based on their standard fragmentation pattern. GC-MS was developed in the late 1950s to separate and identify chemical compounds (Sparkman *et al*., 2011). The gas chromatograph separates the analytes present in a mixture into individual molecules in the column, which are then channelled into a mass spectrometer which acts as a detector. In the mass spectrometer the molecules are ionised to form charged molecules or molecule fragments. The difference in masses of the fragments allows the mass analyser to sort the ions based on their mass-to-charge ratio and subsequently the detector calculates the abundance of each ion present. The mass spectra generated can be compared with a pure compound or compared to the NIST database to check on the possible identity of the molecule. In this way it is possible to identify the types of compounds in a complex mixture without the need to isolate and identify each compound (Hubschmann, 2009).

In addition to the development of GC-MS, the coupling of modern analytical techniques, specifically solid phase micro-extraction (SPME) with GC-MS, has further enhanced agarwood analysis by overcoming limitations such as extensive sample preparation, low sample yield, lack of sensitivity in detecting trace level components and high variability among samples (Pripdeevech *et al*., 2011; Wetwitayaklung *et al*., 2009; Kumeta & Ito, 2010). SPME was first developed by Pawliszyn and associates in the early 1990s in an attempt to simplify sample preparation (Pawliszyn, 1997). The technique uses coated fused-silica fibers to isolate and concentrate analytes before transferring the analytes to analytical instruments for

separation and quantification. SPME requires only a fraction of the amount of sample and solvent used in the conventional method while still maintaining high reproducibility and sensitivity (Lord *et al*., 2004). Due to the low amount of extraction solvent compared to the sample volume, equilibrium is easily achieved between the sampling matrix and the extracting phase. Today SPME is applied across various types of research, including environmental, industrial, clinical, forensic, plant, food and drug analysis (Pawliszyn, 1997).

SPME is mainly used in gas chromatography analysis whereby fibers were designed for sampling of volatile or semi-volatile compounds with thermal desorption as the means of introducing the samples into gas chromatographic columns (Shirley & Aurand, 2010). Coating materials, such as polydimethylsiloxane (PDMS for non-polar compounds), polyacrylate (PA for polar compounds) and polyethyleneglycol (PEG for polar compounds), are used to coat the fused-silica fiber housed in a syringe-like holder (Figure 3.3) or coupled with a CombiPal auto-sampler before injecting into the GC column (Pawliszyn, 1997). GC phase SPME fibers have been used in the analysis of volatile and semi-volatile compounds, particularly sesquiterpenes, in agarwood from *Aquilaria malaccensis*, *A. crassna* and *A. sinensis* (Pripdeevech *et al*., 2011; Wetwitayaklung *et al*., 2009; Kumeta & Ito, 2010). In this chapter, we attempt to optimize the analytical conditions, such as GC program setup and SPME conditions, using agarwood oil derived from *A. malaccensis* in Malaysia. Optimization of GC and SPME conditions is prerequisite for the positive identification (based on retention times together with the built in NIST database in GC-MŚ systems) of volatiles and semi-volatiles thought to be produced during elicitation of cell suspensions of *A. malaccensis* (see Chapter 4).

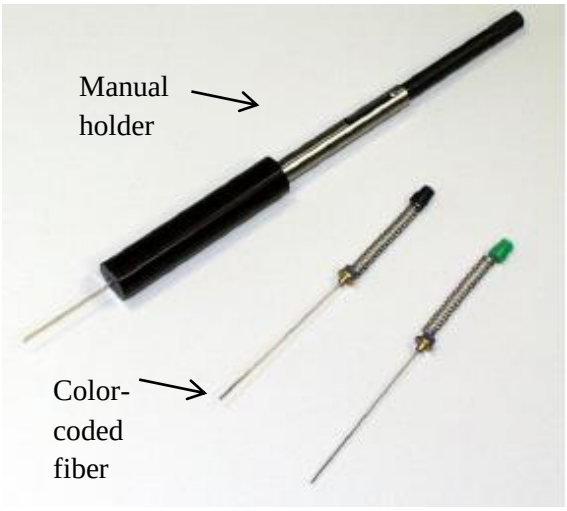


Figure 3.3 SPME - GC fiber probes and manual holder. (Sigma Aldrich, 2012)

3.0.2 Chromones (Phenyl-ethyl chromones)

Chromones belong to the flavonoid group of compounds that occur naturally in the plant kingdom. They have been successfully extracted from plant parts such as bark, leaves, nuts, seeds and fruits. The term chromone refers to their chemical structure which is comprised of a 1-benzopyran-4-one ring (Dyrager, 2012) (Figure 3.4).

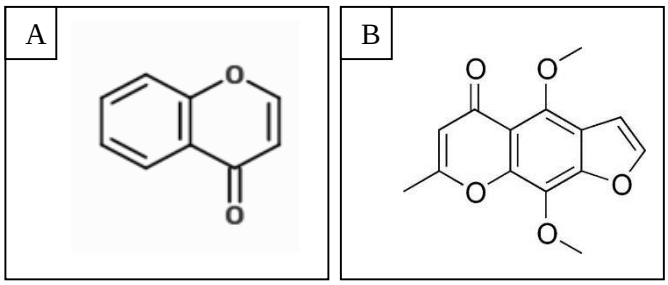


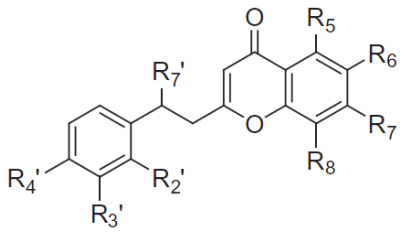
Figure 3.4 Chemical structure of chromones. [A] General structure of chromones and [B] Chemical structure of Khellin. (Dyrager, 2012).

One of the first chromones to be isolated and purified in clinical practice was a furanochromone known as khellin. Khellin (Figure 3.4B), originating from the seeds of *Ammi visnaga*, was first studied pharmacologically by Samaan, as decoctions were commonly used in Mediterranean communities as a diuretic and muscle relaxant (Edwards & Howell, 2000). In the 1950s, Khellin was prescribed as a muscle relaxant for the treatment of angina pectoris and asthma. However, today, use of this compound focuses on the treatment of vitiligo, a pigmentation disorder (Dyrager, 2012).

In recent years, chromones have been investigated for their unique chemical and biological properties as potential pharmaceuticals, including anti-HIV, antioxidant, anti-inflammatory, antiviral, insecticidal, antibacterial, antifungal, anti-allergy and anticancer (Wang *et al*., 2009a; Huang *et al*., 2007). Chromones can be classified into numerous groups, such as furanochromones, chromone alkaloids, alkoxychromones and ketonic chromones, depending on the dominant or unique functional group in the structure. To date a total of 39 different chromones (Tables 3.3 and 3.4) have been identified in various types of agarwood (originating from different species), and all are classified as 2-(2-phenylethyl)-chromones (Naef, 2011). The function of chromones in agarwood formation remains unclear but it is believed to be relevant to the quality of the agarwood. Hence, more attention should be placed on this group of chemicals in the hope of gaining a better understanding on the formation of agarwood in *Aquilaria* species. Although agarwood extracts have been shown to possess anticancer (Liu *et al*., 2008b; Xu *et al*., 2010; Cui *et al*., 2011), antioxidant (Miniyar *et al*., 2008), anti-inflammation (Zhou *et al*., 2008; Kumphune *et al*., 2011) and antimicrobial (Cui *et al*., 2011; Novriyanti *et al*., 2010) properties, more studies are necessary to link the effect of agarwood based chromones with its potential pharmaceutical properties. In addition, studies focusing on agarwood based chromones are important as some of the highly oxidized 5,6,7,8, tetrahydro-2-(2-phenylethyl)-chromones, which are only found in aloeresin from *Aquilaria*

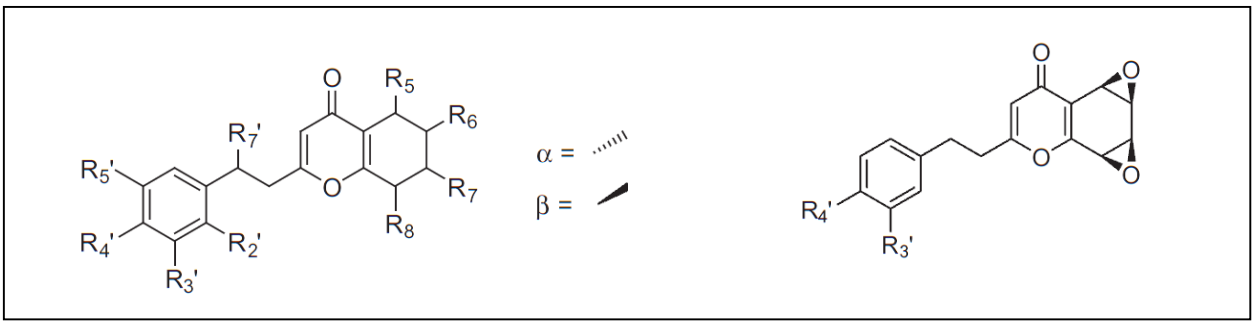
species, could potentially be used as markers for grading, authentification and species identification of agarwood (Qi *et al*., 2005; Naef, 2011).

Table 3.3 2-(2-Phenyl-ethyl)-chromones identified in agarwood (Naef, 2011)



Group A 2-(2-phenyl-ethyl)-chromones									
N°	**R5**	**R6**	**R7**	**R8**	**R2'**	**R3'**	**R4'**	**R7'**	**CAS RN**
1									61828-53-3
2			OH						449728-44-3
3		OH							84294-90-6
4		OCH3							84294-89-3
5							OCH3		92911-82-5
6		OH					OH		449728-42-1
7		OH			OH				449728-43-2
8		OH		OH					449728-41-0
9	OH			OH					69809-24-1
10	OH	OCH3							626235-06-1
11		OH					OCH3		125092-36-6
12		OH	OCH3						449728-45-4
13		OCH3					OCH3		111286-05-6
14		OCH3				OCH3			84294-88-2
15		OCH3	OCH3						84294-87-1
16	OH			OH			OCH3		128922-70-3
17		OCH3				OCH3	OH		449728-40-9
18		OCH3	OCH3				OCH3		117596-92-6
19	OCH3			OCH3		OCOCH3			863771-27-1
20		OH						OH	626235-07-2

Table 3.4 5,6,7,8-Tetrahydro-2-(2-phenylethyl)-chromones and diepoxy-tetrahydro-2-(2-phenylethyl)-chromones identified in agarwood (Naef, 2011)



Group B Group C

Group B 5,6,7,8-Tetrahydro-2-(2-phenylethyl)-chromones									
N°	**R5**	**R6**	**R7**	**R8**	**R2′**	**R3′**	**R4′**	**R7′**	**CAS RN**
21			b-OH	b-OH					626236-07-5
22	a-OH	a-OH	b-OH		OH				117596-93-7
23	a-OH	a-OH	b-OH			OH	OCH3		Not yet
24	a-OH	a-OH	a-OH			OH	OCH3		Not yet
25	a-OH	b-OH	b-OH	a-OH					69809-22-9
26	a-OH	b-OH	a-OH	b-OH					104060-61-9
27	b-OH	b-OH	a-OH	b-OH					123363-31-5
28	a-OH	b-OH	a-OH	b-OH	OH				104926-77-4
29	a-OH	b-OH	a-OH	b-OH	OH				135308-83-7
30	a-OH	b-OH	b-OH	a-OH			OCH3		104901-11-3
31	a-OH	b-OH	a-OH	b-OH			OCH3		104901-10-2
32	a-OH	a-OH	b-OH	a-OH		OH	OCH3		1236273-32-7
33	a-OH	b-OH	b-OH	a-OCH3					135308-81-5
34a	a-OH	b-OH	a-OH	b-OH				(*R*)-OH	142628-23-7
34b	a-OH	b-OH	a-OH	b-OH				(*S*)-OH	142628-22-6
35	a-OH	a-OH	a-OH	b-Cl					626236-06-4
36	a-OH	a-OH	a-OH	b-Cl		OH	OCH3		1058130-00-9
Group C Diepoxy-tetrahydro-2-(2-phenylethyl)-chromones									
	R3	**R4**							**CAS RN**
37	H	H							858937-82-9
38	OH	OCH3							858927-83-0
39		OCH3							858927-84-1

Shimada and coworkers dedicated almost two decades (1982-2002) to the isolation and identification of 2-(2-phenylethyl)-chromones from agarwood extracts. They identified more than twenty types of chromones from solvent extracts of *A. malaccensis* originating from Kalimantan, Indonesia. Qi *et al*. (2005) undertook one of the first studies in pursuit of a better understanding of chromone development at the cell level. They attempted to induce the formation of chromones in artificially stressed cell suspensions initiated from seeds of *A. sinensis.* They managed to induce 4 types of chromones, namely 6,7-dimethoxy-2-(2-phenylethyl)-chromones, 6,7-dimethoxy-2-[4'-methoxyphenyl)ethyl]-chromones, 6-methoxy-2-[4'-methoxyphenyl)ethyl]-chromones and 6-methoxy-2-(2-phenylethyl)-chromones. This success was repeated by Okudera & Ito (2009) who artificially induced the formation of four types of 2-(2-phenylethyl)-chromones, namely 6-hydroxy-2-(2-phenylethyl)-chromones, 6-methoxy-2-(2-phenylethyl)-chromones, 6-methoxy-2-[2-3-methoxyphenyl)ethyl]-chromones and 6,7-dimethoxy-2-(2-phenylethyl)-chromones, in leaf derived cell cultures of *A. crassna*.

Various analytical and fractionation techniques have been developed to identify and isolate chromones from agarwood, however most of these techniques rely heavily on the use of large amounts of solvents, such as acetone, methanol, ethanol, diethyl ether and hexane, which are flammable/toxic. A conventional extraction process usually requires a large volume of sample, which impedes agarwood research as the supply of agarwood is limited due to its high economic value. In this study, the development of analytical procedures to positively detect the presence of chromones in different fractions of *A. malaccensis* oil using Triple Quad Mass Spectrometry (LC-ESI-MŚ/MŚ) has been investigated. The main objective was to develop procedures to circumvent the lack of commercial standards (chromones) as most of the chromones that have been reported in the literature were isolated and purified on a research scale making their availability confined to individual laboratories. Such a procedure, after

optimization, would then be used to detect the presence of chromones in cell suspensions of *A. malaccensis* before and after elicitation.

In addition to LC-MŚ, the application of SPME in the analysis of cell suspensions before and after elicitation was also explored. This was mainly to overcome the limitation of cell mass associated with tissue culture based studies and the chromones usually being present in trace amounts. The concept of SPME is similar to the GC application described in Section 3.1, however the procedures and type of fiber used differ. This is mainly due to the chemistry of chromones which are considered less volatile as compared to sesquiterpenes thereby impeding the use of gas chromatography based analytical techniques.

The introduction of LC fibers and procedures was aimed at overcoming the limitation of GC based fibers and expanding the use of SPME to liquid chromatography application enabling the micro-extraction of non-volatile analytes (polar or non-polar) using solvent desorption. The development of LC fibers has been driven mainly by the demand from clinical research to analyse small molecules such as drug molecules without the interference of phospholipids and proteins (Verbruggen *et al*., 2000; Xie *et al.*, 2011; Vuckovic *et al*., 2012; Zhou *et al*., 2007). The design of the C18 LC fiber was mainly influenced by its clinical application in which the fiber is sealed into a hypodermic needle with an attached hub to allow movement of the fiber for exposure and retraction into the needle (Figure 3.5). C18 LC fibers offer alternative methods for the analysis of non-volatile analytes of agarwood, such as chromones, which are usually extracted using conventional time consuming sample preparation methods including solvent extraction, distillation and fractionation by column chromatography (Nakanishi *et al.*, 1986; Yagura *et al.*, 2003; Qi *et al.*, 2005; Okudera & Ito, 2005; Zhang *et al*., 2011) .

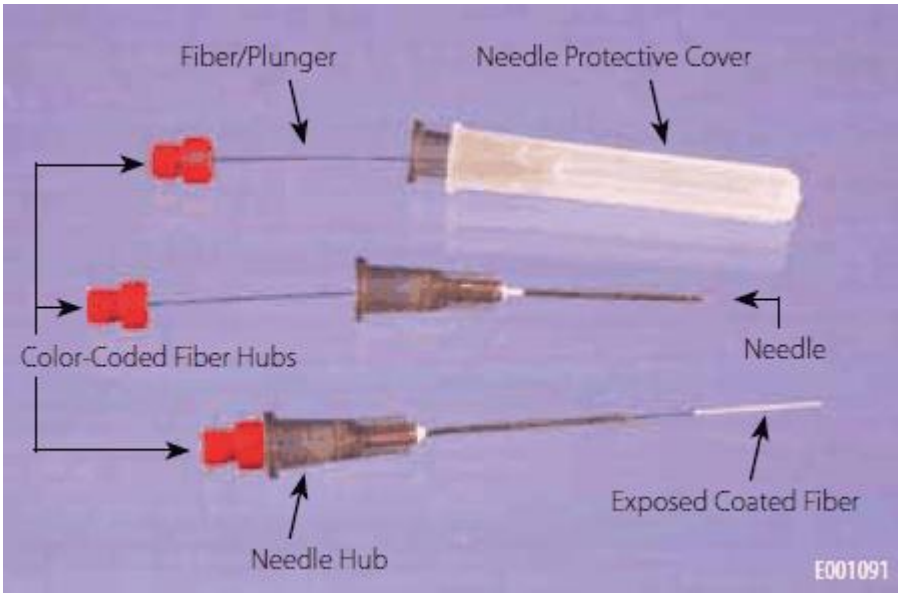


Figure 3.5 SPME - LC fiber probes (Shirley & Aurand, 2010)

The aims of this part of the study are to:

1. Develop a series of analytical techniques which would enable the extraction, detection and analysis of chromones and sesquiterpenes from cell suspension cultures
2. Identify potential chemical marker namely sesquiterpenes and chromones which could be used later to study the effect of exerting artificial stress / elicitation on the chemical profile of cell suspensions

3.1 Materials and methods

3.1.1 Detection and analysis of sesquiterpenes

3.1.1.1 SPME sampling for the analysis of three standard terpenes via GC-MŚ

Three standard terpenes (linalool, pinene and limonene from Sigma Aldrich) were each diluted to 100 micromolar. One ml was placed in 20ml headspace vials and sampled using a CTC auto-sampler where a 100µm polydimethylsiloxane (PDMS) fiber was inserted into the headspace of the sampling vial which was maintained at 80ºC (in an incubator) and agitated at 300 rpm. Active sampling was done for 40 minutes before injecting the fiber (containing the analytes) into the injector port of a Shimadzu (QP2010) GC-MŚ equipped with Agilent ® DB-5ms column (30m x 0.25mm ID x 0.25µm thickness). The injector temperature was set at 270ºC. The column oven program was started at 60ºC and held for 2 minutes before being progressively increased to 180ºC at 30ºC per minute. The temperature was then increased by 1.5ºC per minute until it reached 200ºC. The column temperature was further increased to 280ºC at a rate of 20ºC per minute and held for 15 minutes. Based on these settings, the total program time was 38 minutes per sample analysed. Analytes were identified by cross referencing retention times and mass fragmentation patterns with the database. Repeatability of the three standard terpenes was calculated by obtaining the mean peak area for three consecutive repetitions. From this value, the standard deviation and coefficient of variance (%) was calculated.

3.1.1.2 SPME sampling for the analysis of *Aquilaria* oil via GC-MŚ

Oil of *A. malaccensis,* which had been extracted by hydro-distillation (courtesy of Dr Kodiswaran) was used in an experiment to optimise the analytical conditions. HPLC grade methanol was used to dilute the oil extract (x10,000) prior to the analysis. Samples were analysed in the GC-MŚ (as described in Section 3.2.1.1), following their introduction by direct injection into the GC-MŚ after dilution and by using the SPME technique. Once the GC-MŚ analysis was complete, analytes were identified by cross referencing retention times and mass fragmentation patterns with the database. Due to the high number of analytes in the mixture, results obtained from GC-MŚ analysis were screened against a list of molecules known to contribute to the aromatic profile of *Aquilaria* (Table 3.2). Screening was done using Adobe Reader professional software to highlight and mark targeted analytes before entering the data [Mean peak area (%)] in an Excel spreadsheet for analysis.

3.1.1.3 Effect of solvent on sampling and analysis of *Aquilaria* oil via HS-SPME-GC-MŚ

HPLC grade solvents (n-pentane, ethyl acetate, methanol and ultra-pure water) were used to dilute (x1000) the oil extract (Section 3.1.1.2) prior to analysis. Samples were introduced into the GC-MŚ using the SPME technique and analysed as described in Section 3.1.1.1. The analytes detected were screened as described in Section 3.1.1.2. During the analysis, emphasis was placed on detecting the presence of alpha guaiene, delta guaiene and alpha humulene which would subsequently act as chemical markers throughout the method development and validation.

3.1.1.4 Development of HS-SPME-GC-MŚ technique for the analysis of sesquiterpenes using '*in vitro*' grown *A. malaccensis* shoots

Shoots with approximately 3 internodes were excised from shoot clumps (in multiplication stage) and was subsequently crushed (not ground) using a mortar and pestle. The crushed shoots were transferred to a 20ml headspace vials containing 5ml of solvent (water, pentane or methanol) before sampling by SPME and analysis by GC-MŚ (Section 3.2.1.1). Screening of the GC-MŚ output for the presence of alpha guaiene, delta guaiene and alpha humulene was done using Adobe Reader professional software (highlighted targeted analytes) before entering the data (% Mean peak area) in an Excel spreadsheet for analysis. In the experiment, two types of shoots were used, namely senescing shoot tips (browned) and actively growing shoot tips

3.1.1.5 Application of the HS-SPME-GC-MŚ technique on cell suspensions of *A. malaccensis*

Cell suspensions were sampled by pipetting 10 ml of suspensions into 15ml Falcon tubes and cells were allowed to settle for 30 minutes. The settled cell volume (SCV) was then recorded and cell suspensions (including medium) were transferred into 20ml headspace vials for SPME sampling and subsequently GC-MŚ analysis.

Prior to sampling, conical flasks containing cell suspensions were gently swirled to ensure homogeneity and 5ml pipettes were used with the tip shortened by 10mm to reduce possible shearing of the cells during pipetting. SPME sampling and the conditions for GC-MŚ analysis were as described in Section 3.1.1.1. Cell suspensions in MS medium and medium without cells were analysed separately. Fresh MS medium was used as a control to ensure that the starting medium did not contain any of the targeted markers.

3.1.1.6 Optimisation of HS-SPME-GC-MŚ technique for the identification of selected sesquiterpenes in *A. malaccensis* cell suspensions

Further optimisation of SPME conditions (including the size of the sampling vial and the sampling volume) was required to ensure that accurate and reproducible results could be obtained, taking into consideration two major limitations of the study:

a) Limited number of samples analysed per session as GC-MŚ was done at the University of Malaya.
b) A limited volume of cell suspensions was available for analysis because the study was done over a period of eight weeks. It was important to identify optimal sampling points and volume without compromising the viability of the cell suspensions.

3.1.1.7 Further Optimisation - Effect of vial and sample size (total volume) on analysis of cell suspensions by HS-SPME-GC-MŚ

Sampling of cell suspensions was done as described in Section 3.1.1.5. For SPME sampling, 20ml and 10ml Sigma Aldrich ® headspace vials were used to analyse 10ml and 5 ml of cell suspension samples (Figure 3.6).

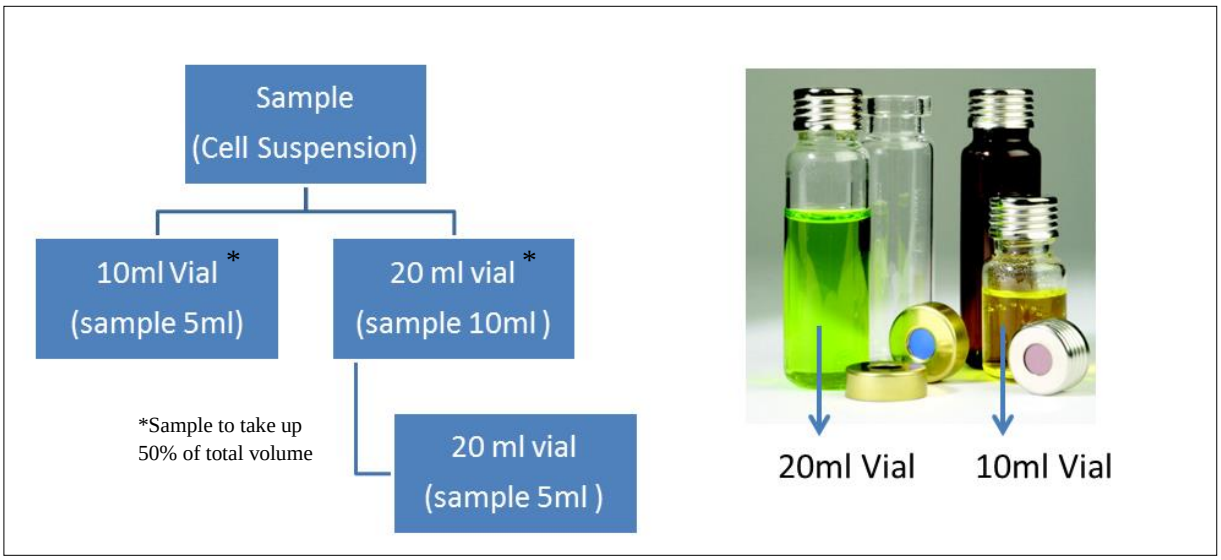


Figure 3.6. SPME sampling of cell suspensions to study the effect of vial and sample size (total volume) on sample analysis.

For sampling a CTC auto-sampler was used, where a 100µm PDMS fiber was inserted into the headspace of the sampling vial which was maintained at 60ºC (in an incubator) and agitated at 300 rpm. Active sampling was done for 30 minutes before injecting the fiber (containing the analytes) into the injector port of the GC-MŚ. The operating conditions of the GC-MŚ were as described in Section 3.1.1.1. On the chromatogram of each sample, the chemical markers (alpha guaiene, delta guaiene and alpha humulene) were identified by cross referencing retention times and mass fragmentation patterns with the database. Once the 'optimal' type of vial was identified the volume of the sample was then optimised to ensure accurate and reproducible results.

3.1.2 Detection and analysis of chromones (phenyl-ethyl chromones)

3.1.2.1 Detection and analysis of chromones in fractions of *A. malaccensis* oil

Sample extraction and preparation

Locally sourced wood chips of *A. malaccensis* (supplied by Dr Kodiswaran) were ground in a commercial blender. The powdered wood chips were extracted 3 times with diethyl ether under reflux for 3 hours and the combined filtrate was concentrated using a Buchi Rotavapor® R-210. The concentrated filtrate was mixed with silica gel and subsequently loaded into a pre-packed glass column (n-hexane:ethyl acetate 1:1 v/v and silica gel) and left overnight to settle. The column was then flushed with n-hexane:ethyl acetate (1:1 v/v) and 20ml fractions were collected in scintillation vials. A total of 100 fractions were collected and left in a desiccator overnight to concentrate. Samples from each fraction (per vial) were then spotted on a thin layer chromatogram (TLC) sheet and placed in a TLC chamber containing varying ratios of hexane:ethyl acetate (50:50 and 60:40). The spots were visualized using short and long UV light and the characteristic of each fraction was observed. Fractions containing the same characteristics were combined and subsequently concentrated before re-dissolving the sample in 1ml of methanol. Samples were then further diluted to x10 and 1ml of each diluted fraction was placed in 2ml sample vials.

Sample analysis by LC-ESI-MŚ/MŚ

Identification of analytes was carried out using a Varian-320 LC-ESI-MŚ/MŚ equipped with a Prostar 410 auto-sampler, two Prostar 210 pumps and a 1200L electrospray source tandem mass spectrometer. All the samples were analyzed using an Agilent® Pursuit XRs 3C18 column (100mm x 0.2mm) and the mobile phase of methanol:water (80:20 v/v) at a flow rate of 200µl/min with the column set at room temperature and injection volume of 15µl. The

electrospray capillary potential was set at 60V. The drying gas pressure was 35psi at 400ºC and the nebulizing gas pressure was 50psi. Spectra over the mass range of *m/z* 200-800 were collected. Compounds were then screened manually to identify chromones molecules. Targeted molecules were reanalysed using the conditions described above, but were subjected to 20V of collision energy to fragmentise the molecules into ions.

3.1.2.2 Detection and analysis of chromones in *Aquilaria* cell suspensions

Cell suspensions were filtered and dried in an oven at 60ºC. The cells were then crushed in a mortar and the cell powder (0.1-0.5g) was extracted with ethanol at room temperature overnight. The extract was concentrated to dryness, dissolved in 500µl of methanol and filtered prior to LC-ESI-MŚ/MŚ analysis using parameter outlined in Section 3.1.2.1

3.1.2.3 Application of LC-SPME-LC-ESI-MŚ/MŚ technique on the detection and analysis of chromones in *A. malaccensis* cell suspensions

The C18 LC fibers were preconditioned in a mixture of 50% HPLC grade methanol and 50% ultra-pure water for one hour. Conditioned fibers were then injected in 10ml headspace vials containing 5ml of cell suspension. Active sampling was done for 30 minutes with the sample being continuously agitated using a magnetic stirrer (100 rpm). After 30 minutes, the fiber was retracted into its protective sheath before being released into 200µl of HPLC grade methanol (in auto-sampler vials containing inserts) to facilitate desorption. The vials were then transferred to the LC-ESI-MŚ/MŚ and the samples analysed using the LC-ESI-MŚ/MŚ parameters described in Section 3.2.2.1

3.2 Results

3.2.1 Detection and analysis of sesquiterpenes

3.2.1.1 Detection and analysis of aromatic compounds in *Aquilaria* oil via GC-MŚ

The GC-MŚ results for linalool, pinene and limonene analysed using HS-SPME possessed a low coefficient of variation (CV) value of 0.05 indicated a low spread of the data and that the method has high repeatability (Table 3.5).

Table 3.5. Repeatability of headspace SPME method: peak areas of three representative terpenes.

GC Peak Area	Repetition			Mean ± SD	CV
	1	2	3		
Alpha pinene (13.62mg/l)	14.58	14.35	15.75	14.89 ± 0.75	0.05
Linalool (15.43mg/l)	21.46	22.43	22.43	22.11 ± 0.56	0.02
D-limonene(13.62mg/l)	11.96	10.84	12.73	11.84 ± 0.95	0.08
				Average CV value	0.05

Compared with direct injection, the HS-SPME technique resulted in the detection of a higher number of analytes (Figure 3.7). The sampling technique did not affect the mean peak area (%) of analytes, specifically alpha humulene (one of the identified chemical markers) (Figure 3.8).

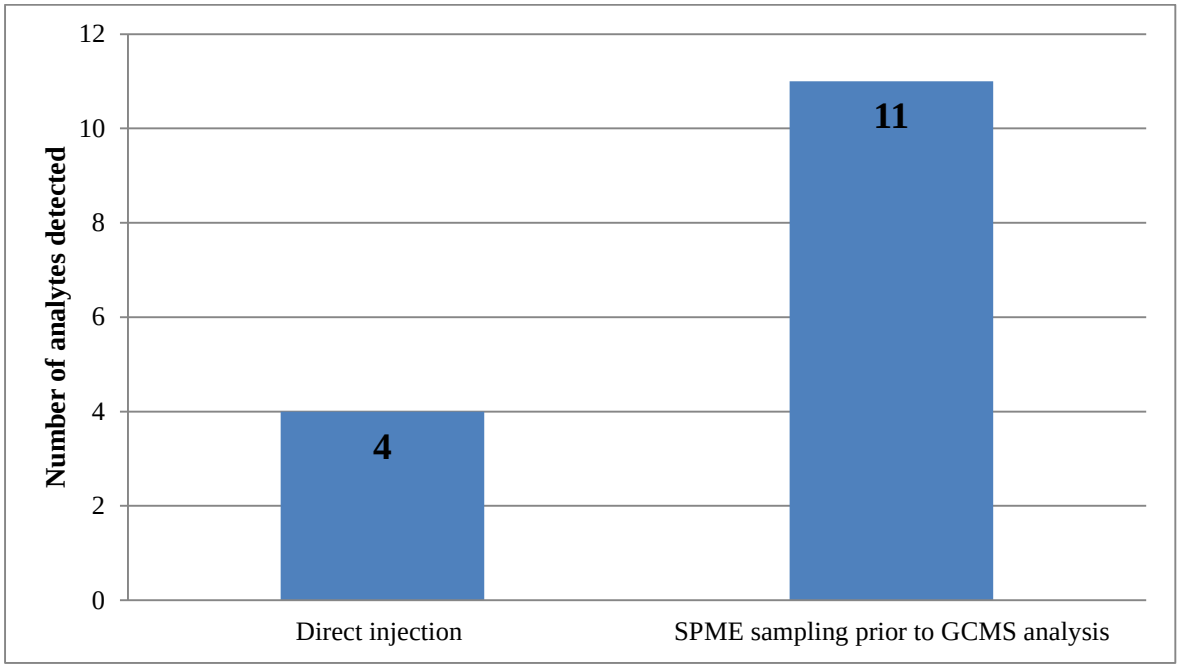


Figure 3.7. Number of analytes detected by GC-MŚ using sampling by direct injection and by HS-SPME.

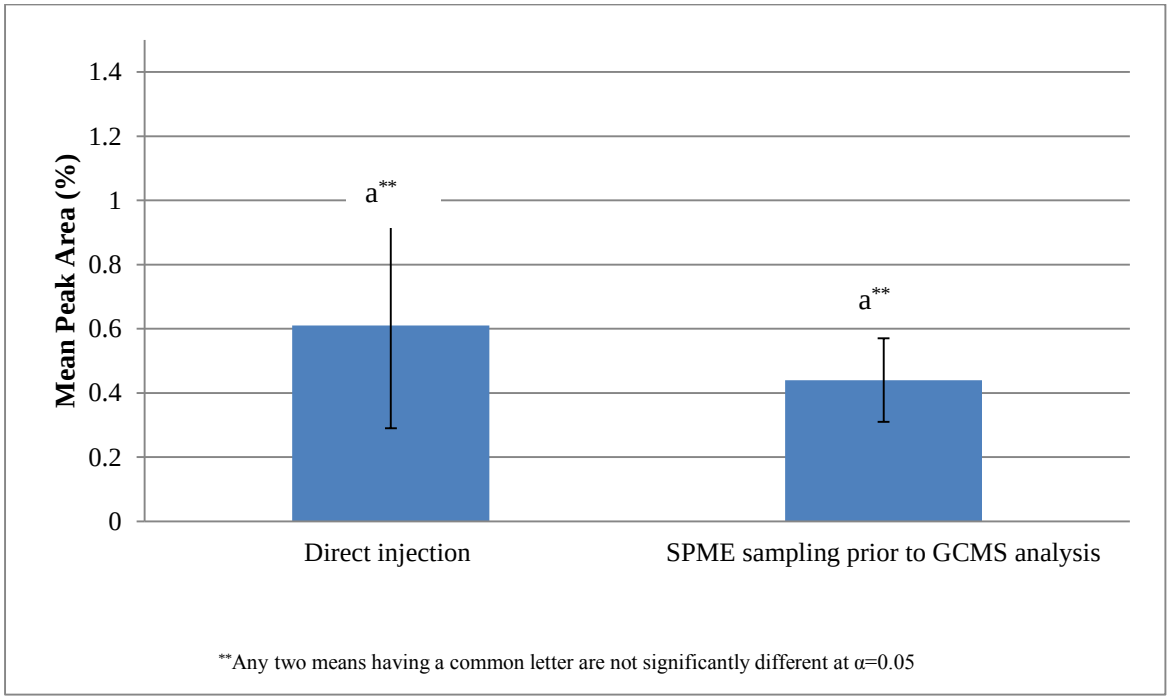


Figure 3.8 Mean peak area (%) of alpha humulene detected by GC-MŚ using sampling by direct injection and by HS-SPME.

3.2.1.2. Effect of solvent on the analysis and detection of aromatic compounds in *Aquilaria* oil via HS-SPME-GC-MŚ.

Water (H_2O) was identified as the most efficient solvent for detecting fragrant analytes in the oil extract of *A. malaccensis* because it resulted in the detection of the highest number of analytes (Table 3.6). The target molecules (chemical markers) alpha guaiene and delta guaiene could be detected with all four solvents, whereas alpha humulene was only detected in water, pentane and methanol (Table 3.7). Based on the one-way ANOVA (Table 3.8), the solvent used was found to affect significantly the detection of the targeted molecules. Mean peak area (%) of all three markers was significantly better in water samples as compared with other solvents (Table 3.9).

Table 3.6 Number of fragrant analytes detected in the oil extract of *A. malaccensis* using different solvents.

Solvent	**Number of analytes detected**
Pentane	21
Ethyl acetate	5
Methanol	11
Water	22

Table 3.7 Compounds detected in the oil extract of *A. malaccensis* using different solvents.

		Mean Peak Area (%)			
		Water	**Ethyl Acetate**	**Pentane**	**Methanol**
3691-12-1*	Alpha Guaiene	0.86	0.12	0.57	0.33
3691-11-0*	Delta Guaiene	3.04	0.69	1.75	1.93
1209-71-8	Gamma Eudesmol	1.74	0.83	5.125	2.87
1460-73-7	Agrospirol	2.44	1.06	1.79	2.05
6831-16-9	Aristolene	0.10	3.02	2.79	8.95
184705-51-9	Gamma.-Gurjunenepoxide	1.84	-	1.57	0.52
17066-67-0	Beta Selinene	0.79	-	0.46	0.32
3856-25-5	Copaene	0.335	-	0.2625	0.33
473-15-4	β-epi-eudesmol	-	-	0.71	0.79
6753-98-6*	Alpha humulene	1.16	-	-	1.04
104-76-7	1-Hexanol	0.05	-	0.16	-
2550-27-7	2-Butanone	0.48	-	1.55	-
515-13-9	Beta elemene	0.28	-	0.23	-
469-61-4	Alpha Cedrene	0.19	-	0.58	-
515-17-3	Gamma selinene	0.7	-	0.58	-
Humulane-1,6-dien-3-ol	Humulane-1,6-dien-3-ol	1.24	-	0.98	-
11031-45-1	Santalol	1.25	-	0.92	-
91416-23-8	Nootkatone	0.46	-	0.98	-
19598-45-9	2(3H)-Naphthalenone	0.44	-	0.43	-
639-99-6	Alpha elemol	-	-	0.68	-
112-30-1	1-Decanol	0.02	-	-	-
112-12-9	2-undecanone	0.02	-	-	-
112-31-2	Decanal	0.02	-	-	-
25246-27-9	Allo-aromadendrene	0.425	-	-	-
88-84-6	Guaiene	-	-	0.89	-
111-27-3	1-hexanol	-	-	0.195	-
	Total	17.88%	5.72 %	24.24 %	19.13 %

*Highlights the chemical markers which will be used to optimise analytic parameters.

Table 3.8 Analysis of variance to show the effect of solvent on the detection (Mean peak area (%)) of three chemical markers (alpha humulene, delta guaiene and alpha guaiene) in *A. malaccensis* oil.

Analyte / Chemical marker	Type III Sum of Squares	df	Mean Square	F	Sig.
Alpha humulene	3.022	3	1.007	1099.067	0.000**
Alpha guaiene	0.975	3	0.325	6.201	0.018*
Delta guaiene	9.025	3	3.008	27.991	0.000**

** significant at 1% level; * significant at 5% level

If significant, Duncan's test was performed.

Table 3.9 DMRT analysis comparing all possible pairs of treatment means.

Solvent	Mean Peak Area ± SD **/ Analytes of interest		
	Alpha humulene*	Delta guaiene*	Alpha guaiene*
Pentane	0^{b}	2.26 ± 0.45^{c}	0.65 ± 0.33^{c}
Methanol	1.02 ± 0.05^{a}	1.93 ± 0.23^{ab}	0.32 ± 0.07^{b}
Water (H_2O)	0.98 ± 0.04^{a}	3.04 ± 0.33^{a}	0.86 ± 0.31^{a}
Ethyl acetate	0^{b}	0.54 ± 0.26^{bc}	0.12 ± 0.30^{b}

* Uses harmonic mean sample size = 3.00

** Any two means having a common letter are not significantly different at $\alpha = 0.05$

3.2.1.3. Optimisation of HS-SPME-GC-MŚ technique for the analysis of sesquiterpenes in '*in vitro*' grown *A. malaccensis* shoots

Water (H_2O) was the most efficient solvent for detecting chemical markers in shoots of *A. malaccensis* (Table 3.10). In contrast, using pentane as the solvent permitted only alpha humulene to be detected. In the analysis, beta caryophyllene was found to be consistently

present in water samples, thus the number of chemical marker's was expanded to four. The concentration of the chemical markers appeared to be higher in 'senescing' shoots as compared to 'actively growing' shoots with exception of beta caryophyllene. The detection of analytes, i.e. signal detection and resolution, was suppressed more by pentane and methanol than by water as solvent (Figure 3.9).

Table 3.10 Signal strength of selected chemical markers in *in vitro* grown *A. malaccensis* shoots using different solvents.

	Mean peak area (%) of analytes of interest			
Explant / solvent	**Alpha humulene**	**Delta guaiene**	**Alpha guaiene**	**Beta Caryophyllene**
a) ' Actively growing' shoot tips				
i) Pentane	6.42 ± 1.20	-	-	-
ii) Methanol	-	-	-	-
iii) Water (H_2O)	0.22 ± 0.05	0.08 ±0.01	1.16± 0.10	0.34± 0.08
b) 'Senescing' shoot tips				
i) Pentane	-	-	-	-
ii) Methanol	-	-	-	-
iii) Water (H_2O)	0.61 ± 0. 17	0.86 ±0.10	3.91 ± 0.60	0.36 ± 0.12

- indicates absent of analytes

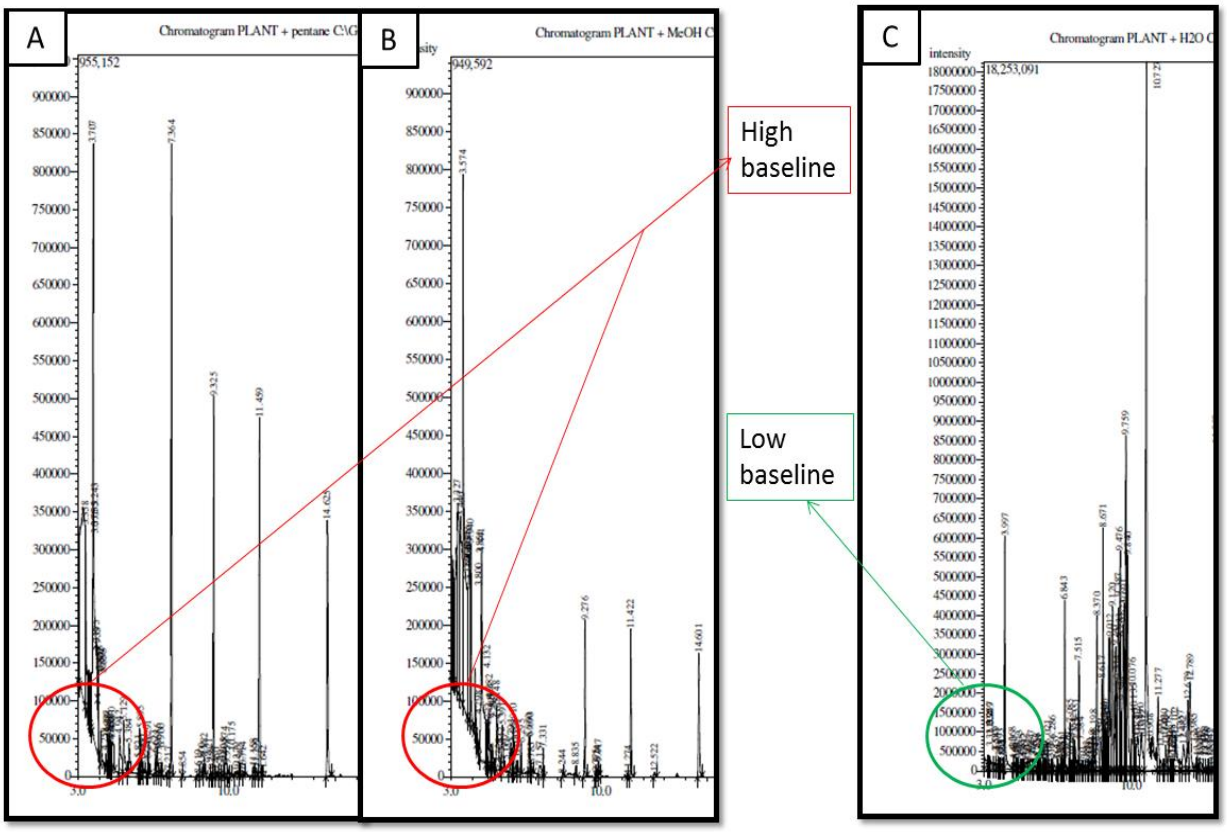


Figure 3.9 Chromatograms illustrating the effect of solvent on GC-MŚ analysis (signal resolution). [A & B] Chromatograms from samples dissolved in pentane and methanol respectively. Both chromatograms show a high baseline which supresses the detection of analytes. [C] Chromatogram from sample dissolved in water. Note the low baseline which minimises the suppression of analyte detection.

3.2.1.4 Application of HS-SPME-GC-MŚ method to cell suspensions of *A. malaccensis*

All four of the target compounds were found only in samples where the plant cells were present (Table 3.11), indicating that the molecules were produced and stored within the cells.

Table 3.11 Mean peak area of selected chemical markers in *in vitro* cell suspensions.

Explant / solvent	**Mean peak area (%) of analytes of interest**			
	Alpha humulene	**Delta guaiene**	**Alpha guaiene**	**Beta caryophyllene**
1) Cell Suspension in MS medium – subject to agitation	6.46 ± 2.06	25.45 ± 4.22	23.81 ± 3.97	0.56 ±0.14
2) Cell Suspension in 3) MS medium – not subject to agitation	-	-	-	-
4) MS medium ONLY (cells removed)	-	-	-	-
5) Fresh MS Medium (Control)	-	-	-	-

- indicates absence of analytes

3.2.1.5 Further Optimisation - Effect of vial and sample size (total volume) on analysis of cell suspensions by HS-SPME-GC-MŚ

The 20 ml vial was found to be better than 10 ml due to the low baseline with the former (Figure 3.10). Varying the sample volume between 10 ml or 5 ml did not have any major effect on the detection or mean peak area (%) of the chemical markers (Figure 3.11).

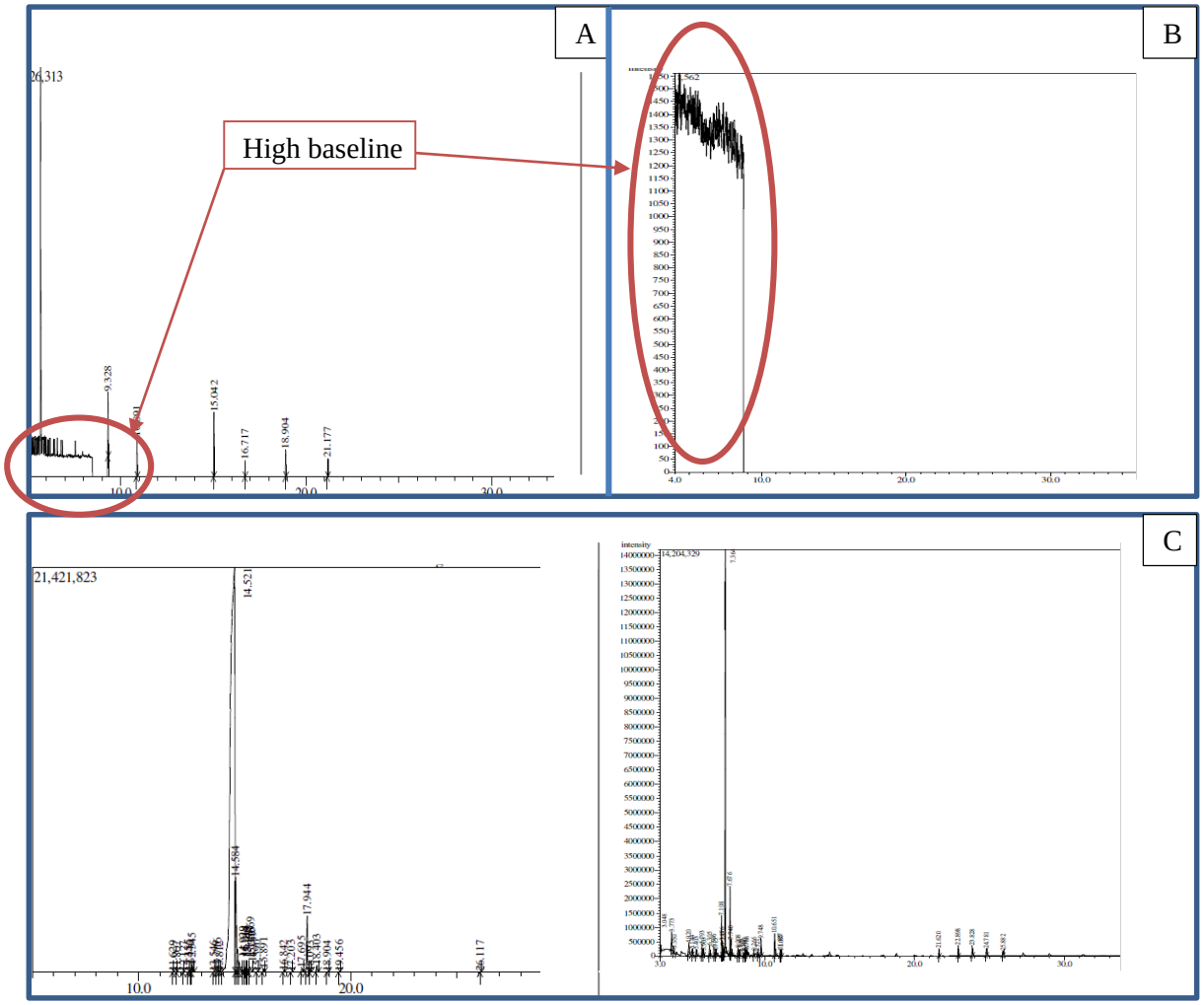


Figure 3.10 Chromatograms illustrating the effect of vial size on GC-MŚ analysis (signal resolution) of cell suspensions. [A] 10 ml sample placed in 20 ml headspace vial. [B] 5ml cell sample placed in 10 ml headspace vial. [C] 5 ml cell sample placed in 20 ml headspace vial. Note that the chromatograms in A and B exhibit a higher baseline which supresses the detection of analytes.

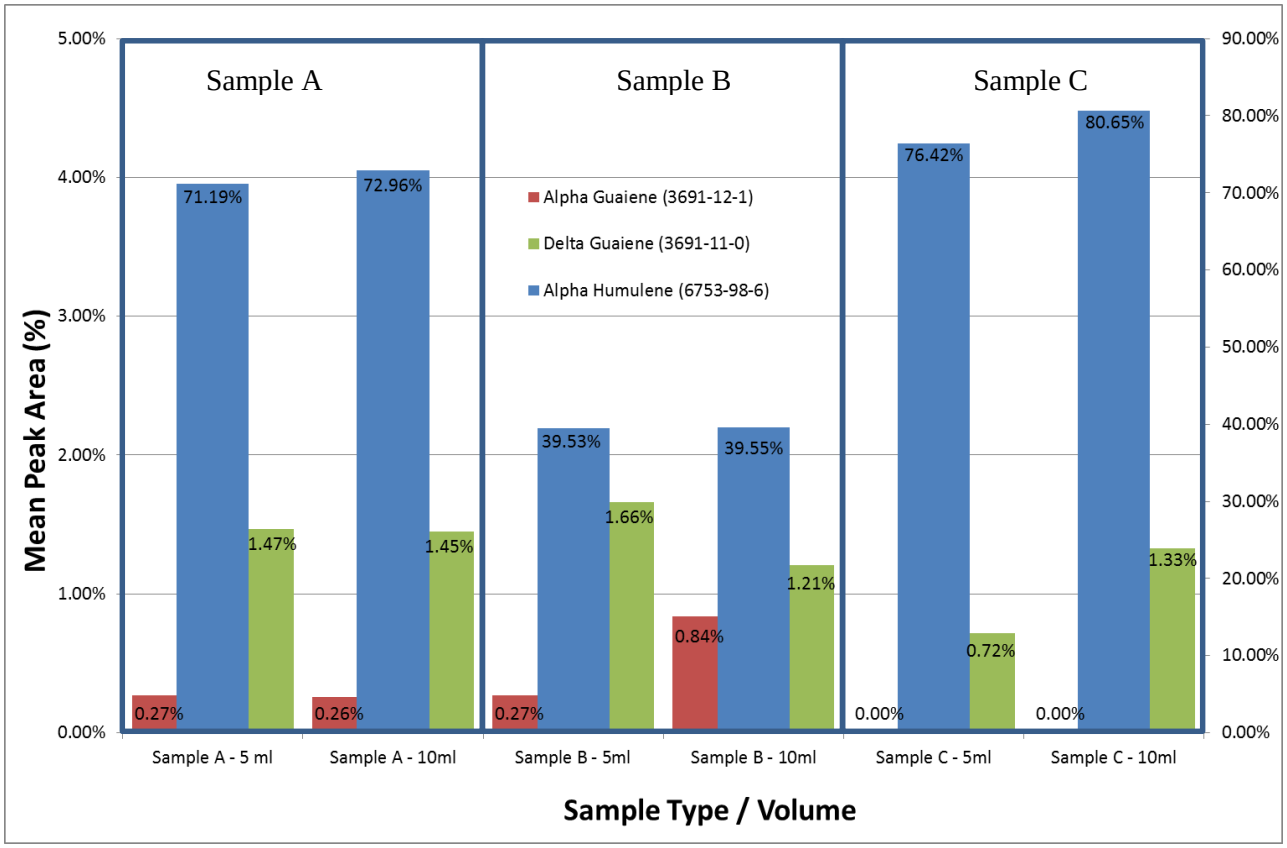


Figure 3.11 Mean peak area (%) of three chemical markers (alpha guaiene, delta guaiene and alpha humulene) analysed in 3 cell suspension samples initiated from different cell lines (A, B & C) using 2 volumes (5 ml or 10 ml). All samples were analysed in 20 ml headspace vials.

3.2.2. Detection and analysis of chromones (phenyl-ethyl chromones)

3.2.2.1 Detection and analysis of chromones in fractions of *A. malaccensis* oil

Using column chromatography, 20 fractions were isolated from the agarwood extract (Table 3.12 and Figure 3.13). Based on the results of thin layer chromatography (Figure 3.13), fractions with similar content (compounds with same retention factor [Rf] and chromophore properties) were combined and concentrated before analysing by LC-ESI-MŚ/MŚ.

Table 3.12 Separation of *Aquilaria* oil sample by column chromatography using hexane:ethyl acetate as solvent.

Lane No.	No. of compounds	Rf for hexane: ethyl acetate		Florescence colour	Wavelength	Fraction No.
		50:50	60:40			
A1	1	Nd	7.1	Blue	Long	Fraction 1
A2	2	Nd	7.1	Blue	Long	Fraction 2
			6.5	Blue	Short	
A3 – A4	3	Nd	7.1	Blue	Long	Fraction 3
			6.5	Blue	Short	
			6.2	Green	Long	
A5	2	Nd	7.1	Blue	Long	Fraction 4
			6.2	Green	Long	
A6	3	7.0	7.1	Blue	Long	Fraction 5
		6.6	6.4	Green	Long	
		5.4	4.8	Blue	Long	
A7	1	5.4	4.8	Blue	Long	Fraction 6
A8	2	5.4	4.8	Blue	Long	Fraction 7
		Nd	4.0	Light blue	Long	
A9 – A10	3	5.4	5.0	Blue	Long	Fraction 8
		4.9	4.3	Blue	Long	
		4.1	2.9	Blue	Short	
B1	2	5.1	3.9	Blue	Long	Fraction 9
		4.1	2.8	Light blue	Short	
B2	3	5.1	3.9	Blue	Long	Fraction 10
		4.6	3.3	Blue	Long	
		4.1	2.8	Light blue	Short	
B3 – B4	2	5.1	3.9	Blue	Long	Fraction 11
		4.6	3.3	Blue	Long	
B5 – B8	2	4.6	3.3	Blue	Long	Fraction 12
		3.3	2.0	Blue	Long	
B9 – C10	1	3.3	2.0	Blue	Long	Fraction 13
D1 – D10	1	3.0	Nd	Blue	Long	Fraction 14
E1 – E5	1	2.7	Nd	Blue	Long	Fraction 15
E6 – F3	1	2.7	Nd	Blue	Long	Fraction 16
F4 – F10	1	2.7	Nd	Blue	Long	Fraction 17
G1 – G10	4	3.2	Nd	Blue	Long	Fraction 18
		2.7	Nd	Blue	Long	
		2.4	Nd	Blue	Long	
		1.2	Nd	Yellow	Short	
H1 – H10	1	1.2	Nd	Yellow	Long	Fraction 19
I1 – L4	1	1.2	Nd	Yellow	Long	Fraction 20

*Nd = Not Detected **Short wavelength – 245nm ; Long wavelength – 365nm

Figure 3.12 Visual separation of *Aquilaria* samples by thin layer chromatography using hexane:ethyl acetate as solvent to determine the compounds present in each fraction.

From the LC-ESI-MŚ/MŚ results, 7 chromones were identified (Table 3.13). An example of the fragmentation pattern of 2-(2-phenylethyl)-chromone is shown in Figure 3.13. The fragmentation pattern of each identified chromone is illustrated in the Appendix B.

Table 3.13 2-(2-Phenylethyl)-chromones detected in *A. malaccensis* wood chip extract.

Fraction No.	No. of compounds detected	MW of molecule	Daughter ion detected	Proposed molecule	CAS RN
A8	1	250.9	90.9 ; 150.9	2-(2-phenylethyl)- chromone	61828-53-3
B2-B4	2	281.1	91 ; 190	6-methoxy-2-(2-phenylethyl)-chromones	84294-89-3
		311.1	121 ; 189.9	6-methoxy-2[2-(3-methoxyphenyl)ethyl]-chromone	111286-05-6
B9-B10	3	297.0	120.9 ; 176.4	5-hydroxy-6methoxy-2-(2-phenyethyl)- chromone	125092-36-6
		311.1	90.7 ; 219.9	6,7-dimethoxy-2-[2-phenyl) ethyl] chromones	84294-87-1
		327.1	190.8 ; 136.9	6-methoxy-2-[2-(3-methoxy-4-hydrophenyl) ethyl] chromones	84294-88-2
D1-D10	2	311.1	90.8 ; 220	6,7-dimethoxy-2-[2-phenyl) ethyl] chromones	84294-87-1
		327.1	136 ; 190.8	6-methoxy-2-[2-(3-methoxy-4-hydropheyl) ethyl] chromones	84294-88-2
E1-E10	3	311.2	137 ; 190.8	6-methoxy-2-[2-(3-methoxy-4-hydroxyphenyl)ethyl]chromone	449728-40-9
		327.1	190.7; 137.0	6-methoxy-2-[2-(3-methoxy-4-hydrophenyl) ethyl] chromones	84294-88-2
		341.1	220 ; 120.8	6,7-dimethoxy-2-[2-(4methoxyphenyl)ethyl]-chromones	117596-92-6

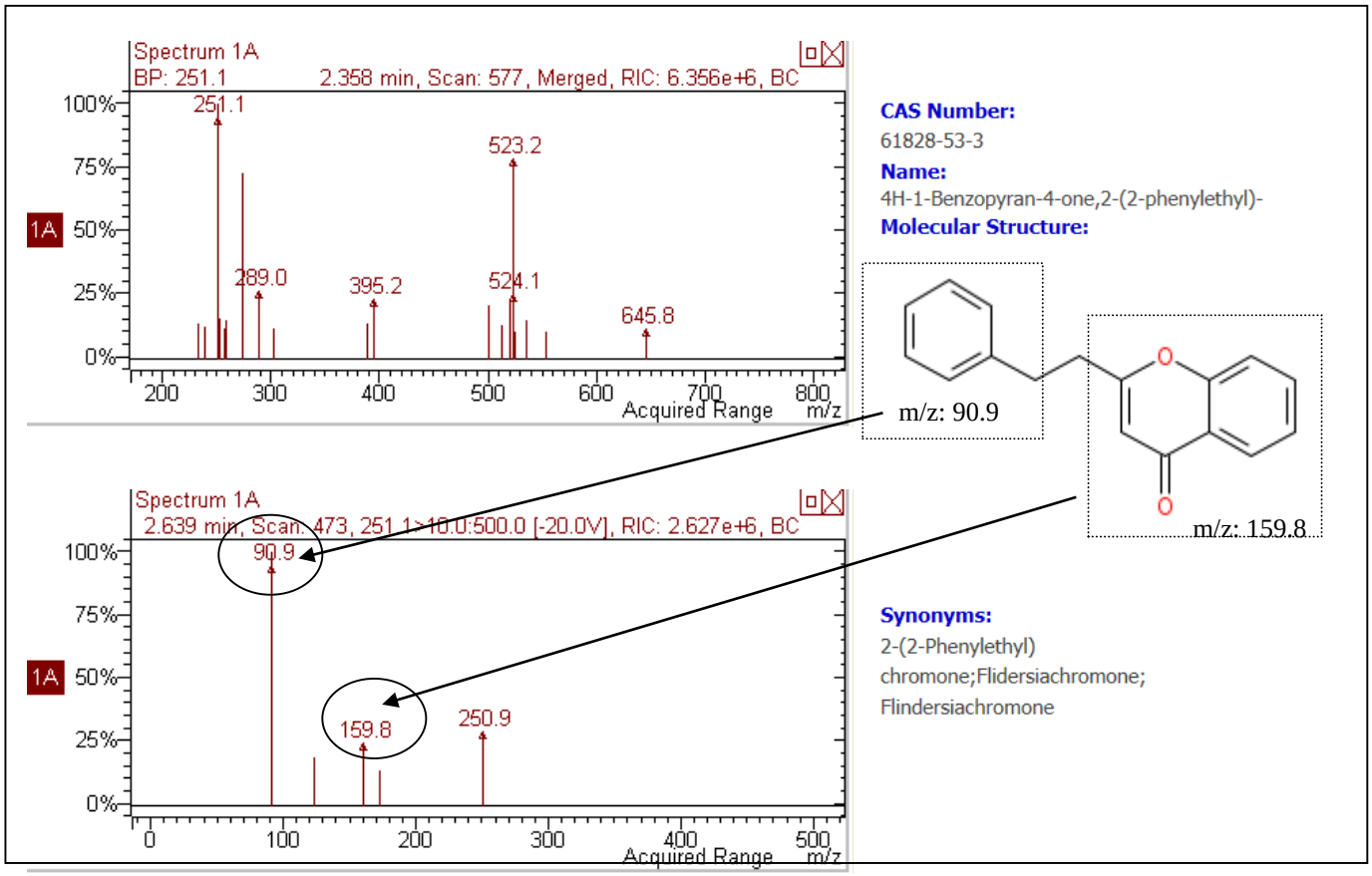


Figure 3.13 LC-ESI-MŚ/MŚ spectra of 2-(2-phenylethyl)-chromone in fraction A8.

3.2.2.1. Detection and analysis of chromones in *Aquilaria* cell suspensions using solvent extraction techniques.

The sample preparation method described in Section 3.2.2.2 resulted in the failure to detect any chromones in the methanol extract indicating that the method might not be suitable for the analysis of trace analytes such as chromones.

3.2.3.2. Detection and analysis of chromones in *Aquilaria* cell suspension using LC-SPME-LC-ESI-MŚ/MŚ technique

The sample preparation method described in Section 3.2.2.2 resulted into the detection of 7 chromones in which 4 were positively identified (Table 3.14). Out of the 4 chromones identifies only 2 were similar to the chromones identified in the oil of *A. malaccensis* earlier in Table 3.13. It was important to note that out of 5 samples analysed, chromones were only detected in 3 samples and among these samples the type of chromones produced varied from one sample to another.

Table 3.14 Fragmentation pattern of chromones present in cell suspension of *A. malaccensis*.

Sample No.	**No. of compounds detected**	**MW of molecule**	**Daughter ion detected**	**Proposed molecule**	**CAS RN**
R1	3	281.1	90.8 ; 190.4	6-methoxy-2-(2-phenylethyl)-chromones	84294-89-3
		310.9	182.7 ; 137.1	5,8-dihydroxy-2-(2-(4-methoxyphenyl)ethyl)chromone	128922-70-3
		331.4	281.8; 51.9	Unknown	Unknown
R2	2	281.1	205.8 ; 84.9	7-hydroxy-2-(2-phenylethyl)chromone	449728-44-3
		311.1	191; 121	6-methoxy-2-[2-(3-methoxy-4-hydroxyphenyl)ethyl]chromone	449728-40-9
R3	2	331.4	281.8 ; 51.9	6-hydroxy-2-[2-(4-hydroxyphenyl)ethyl]chromone OR 6-hydroxy-2-[2-(2-hydroxyphenyl)ethyl]chromone	449728-42-1 OR 449728-43-2
		331.4	281.8 ; 51.9	Unknown	unknown

3.3 Discussion

3.3.1 Sesquiterpenes

Due to the varying conditions used for GC-MŚ and SPME sampling, standard terpenes were used to assess the suitability of the HS-SPME technique. The repeatability of the SPME technique was found to be quite high (Section 3.3.1.1) as the average CV value generated was 0.05%, which was considered reasonably low reflecting the low spread of the data (Forkman, 2009). Therefore it was concluded that the SPME technique enabled the detection of terpenes.

Subsequent experiments were then focused on developing the HS-SPME technique for the detection of sesquiterpenes and volatile compounds in *Aquilaria malaccensis* extracts and subsequently in cell suspensions. Three chemical compounds, specifically alpha humulene, delta guaiene and alpha guaiene, were selected as 'chemical markers' because they have been regularly detected in *Aquilaria* extracts and detected in 'artificially stressed' cell cultures of *A. sinensis* (Kumeta & Ito, 2010). In addition, they are known to play an important role in the sesquiterpene pathway and would act as intermediate molecules in the formation of more complex sesquiterpenes, such as agrospirol, jinkoh-eremol, kusonol and agarofuran (Ito *et al*., 2005). To avoid confusion, the term 'chemical marker' has been used throughout this thesis to refer to these three compounds.

Traditionally, the analysis of sesquiterpenes and volatile compounds has been done by direct injection of diluted samples into the GC-MŚ, however one of the major disadvantages of this method is that 'dominant' compounds within the mixture have a tendency to suppress the signal of trace compounds present (Camara *et al*., 2006). Fractionation of the extract through liquid – liquid extraction or chromatography can reduce the complexity of the mixture but then requires the analysis of each fraction which is both time consuming and requires a

whole range of chemicals during the sample preparation procedures (Augusto *et al.*, 2003; Tschiggerl & Bucar, 2012). Sample preparation by head space - solid phase micro extraction (HS-SPME) enabled the detection by GC-MŚ of 11 compounds in diluted *A. malaccensis* oil as compared with only 4 compounds by direct injection (Figure 3.7)

Alpha humulene was the only chemical marker found in the oil sample tested. Although the mean peak area (%) following SPME was slightly lower as compared to the direct injection method the difference was not significant. This indicated that the HS-SPME method is more efficient than the direct injection method due to its capability of detecting a higher number of analytes (high resolution) while still maintaining comparable signal strength.

In order to analyse sesquiterpenes and volatile compounds in cell cultures, identification of the most suitable solvent should permit the detection of analytes of interest from the crude sample with minimal sample preparation (Pawliszyn, 1995). This is because the more complex the sample preparation step, the higher the possibility of 'losing' analytes (Steffen & Pawliszyn, 1996). This is particularly critical when processing cell suspension samples which possess a low concentration of analytes due to the limited mass available. In addition to using methanol, pentane and ethyl acetate to dilute *A. malaccensis* oil prior to SPME sampling, water was tested as any alternative medium/solvent to explore the possibility of sampling cell cultures directly without the need to extract the analytes to a more volatile solvent prior to SPME sampling or direct injection.

Prior to the introduction of SPME techniques, the use of water-based samples had been impossible in GC-MŚ analysis due to water's high boiling point which makes it extremely difficult to vaporise. Also, the introduction of water into a GC-MŚ system would cause irreversible damage to the filament and column making analysis practically impossible,

therefore analysis of water based samples has been limited to liquid chromatography (Khun , 2002 ; Agilent, 2005). The introduction of HS-SPME in sample preparation allows water-based samples to be analysed via GC-MŚ because, during the sampling process, analytes are vaporised into the air space and subsequently transferred to the SPME fiber which then releases the analytes into the GC-MŚ via desorption. In this process, water molecules are not introduced into the GC-MŚ system (Zhang & Pawliszyn, 1993).

It was interesting to find that water-based samples yielded the highest number of analytes. The 22 compounds detected represented a total of 17.88% of the mean peak area identified in the essential oil of *A. malaccensis.* Pentane-based samples yielded a total of 21 compounds which represented a mean peak area of 24.24%. The difference between the water and pentane systems was mainly attributed to the mean peak area of gamma eudesmol which was significantly higher in the pentane sample. This was most likely due to the lack of interactions between molecules (e.g. dipole forces and hydrogen bonding) between the solvent (pentane) and the gamma eudesmol which enabled the molecule to vaporise more easily as compared to the other solvents (water, ethyl acetate and methanol) which contain oxygen molecules.

Due to the complexity of the mixture, the focus of the analysis was on the efficiency of the solvents to yield/detect the selected 'chemical markers' (Table 3.7). Only two (water and methanol) out of the four solvents were found to be capable of detecting the presence of all three 'chemical markers'. The mean peak area of all three analytes (Tables 3.7) was found to be significantly higher in water and methanol when compared to pentane and ethyl acetate. Based on these results, the effect of solvent on the analysis should be investigated further and verified using tissue culture samples. Ethyl acetate was deemed to be unsuitable for the

detection of sesquiterpenes and volatile compounds in the oil of *A. malaccensis*, consequently the solvents used in subsequent experiments were limited to pentane, methanol and water.

For the analysis of '*in vitro*' propagated plantlets, water was also found to be the best medium to yield and detect the presence of all three 'chemical markers' in 'actively growing' and 'senescing' shoot tips. It was also interesting to detect the presence of an additional sesquiterpenes namely beta caryophyllene which was consistently detected in plant sample, but found to be absent in oil extracts. The absence of beta caryophyllene in oil sample may attributed to the nature of beta caryophyllene which is volatile (usually produced by plant to deter herbivory - Bernacosni *et al.*, 1998; Turlings & Ton, 2006) and maybe have evaporated over time. Overall it was found that, a higher concentration of the 'chemical markers' in 'senescing' shoots was expected since sesquiterpenes are considered to be secondary metabolites produced usually in relation to stress (Ito *et al*., 2005; Siegler, 1998). Alpha humulene was the only marker detected in the pentane-based samples and only in the live shoots. The absence of all chemical markers in the methanol-based samples and of two in the pentane–based samples may have been due to the broad solvent peak and the increased baseline (Figure 3.9). Broad solvent peaks in GC-MŚ chromatograms are mainly due to saturation of the volatile solvent at the start of the run leading to an increase in the baseline and a consequent suppression of the detection of analytes (Decker, n.d.). Based on these results, it was decided that the analysis of cell suspensions would be done without the introduction of a solvent and the number of chemical markers would be expanded to include beta caryophyllene.

Ito *et al*. (2005) reported that 'chemical markers' such as alpha humulene were only expressed in *A. sinensis* cell suspensions after being subjected to artificial stress, specifically methyl jasmonate (MEJA). However in the present study, the presence of all four chemical markers in tissue cultured shoots in the absence of any 'artificial stress' being applied to the

culture system may have been due to the tissues being cultured in 'multiplication' medium which accelerates cell division. This medium often forces the cells to undergo organogenesis, thereby indirectly causing the cells to be stressed (Gaspar *et al*., 2002). When studying metabolite production in cell suspensions, it is important to understand how and where the compounds of interest are produced. If the metabolite remains intracellular, extraction procedures such as cell lysis or permeabilisation would need to be performed, however this often results in loss of viable biomass. Conversely, if the metabolite is secreted out of the cell (extracellular production) then metabolites could be harvested without destroying viable cells (Rao & Ravishankar, 2002; Bourgaud *et al*., 2001; Kieran *et al*., 1997).

The only 'proven' extraction method used in the study of *Aquilaria* cell suspensions has involved the extraction of metabolites from cells using solvent systems combined with crushing/agitation (Ito *et al*., 2005; Kumeta & Ito, 2010). This method is based on the assumption that sesquiterpenes remain intracellular after their production. In our study, sesquiterpenes were only detected in samples which contain plant cells and were subject to agitation (Table 3.11). This finding supports Ito *et al.,* (2005) assumption of which sesquiterpenes are not mobilized out of the cell after synthesis as they were not detected in the culture medium of cell suspensions (Table 3.11). Based on these results it was concluded that solvents can be excluded from the analysis, and that agitation is necessary to ensure sesquiterpenes are released from the cells.

The limited biomass from tissue cultured material meant that optimization of parameters such as vial size and total sample volume was also important to be able to detect trace amounts of metabolites in extremely small samples. In preliminary studies, the total sample volume used was 50% of the total vial volume which ranged between 5 and 10ml in which two vial size namely 10ml and 20ml vials (Figure 3.10). When analysing the samples,

the 20ml vials showed good separation of analytes regardless on sample volume. The results obtained from the 10ml vials was found unsatisfactory as the baseline was significantly elevated leading to poor detection of analytes (Figure 3.11). The difference of behaviour/ results mainly contributed the smaller headspace in the 10ml vials which lead to the increase of vapour pressure in the headspace of the vial. With increased vapour pressure the minute amount of water would be deposited on the PDMS fiber leading to unintentional introduction into the GC system resulting in the presence of an elevated baseline (Zhang & Pawliszyn, 1993). Further optimisation on sample size indicated that reducing the sample volume by half from 10ml to 5ml had no effect on mean peak size (%) of targeted analytes from all three sample analysed (Figure 3.11) therefore it was concluded that the optimal sample volume was 5ml using 20ml headspace vials.

3.3.2 Chromones (phenyl–ethyl chromones)

The establishment of standards and knowledge of the nature of the compounds of interest plays an important role in successful identification of specific compounds. Use of pure standards allows validation of putative compounds by comparing retention time and fragmentation patterns. It was not possible to obtain any standards of agarwood-based chromones as, to date, all chromones which have been successfully identified were isolated and purified by chemists in different parts of the world and they are not available commercially. Unlike GC-MŚ systems, LC-MŚ systems do not have a built-in database as retention times differ between different LC-MŚ brands, detectors, columns and solvent systems. To overcome this limitation, a standard was established by extracting 2-(2-phenylethyl)-chromones from locally sourced wood chips (*A. malaccensis*) based on the method described by Shimada *et al*. (1982). Extracts from the wood chips were subjected to basic chromatography separation techniques to reduce the complexity of the samples because dirty or complex samples would damage/contaminate the

LC-MŚ system. Unlike the procedure used by Shimada, individual compounds were not purified. Once the oil had been fractionated and characterised based on TLC properties (Table 3.12 & Figure 3.12), each fraction was then analysed using LC-ESI-MŚ/MŚ. Out of a total of 7 putative compounds, 2 chromones were successfully identified. The accuracy of this result is considered to be quite high due to the unique fragmentation patterns of chromones. Fragmentation of the parent ion usually takes place when it is subjected to high amount of collision energy (during tandem MŚ) in which the weakest bond of the parent ion (as shown in Figure 3.13) would break producing daughter ions (Hoffmann & Stroobant, 2007). Based on the results obtained, it was concluded that tandem MŚ could be used to positively identify basic chromones, such as 2-(2-phenylethyl)-chromones. Compared to the conventional method of isolating, purifying and identification via NMR, the detection of chromones using LC-ESI-MŚ/MŚ overcomes the limitation of a lack of standard and also reduces sample preparation time.

The MŚ spectrum generated by each sample was screened manually for chromones. When chromones were detected (based on mass (m/z) value) the compound was then subjected to tandem MŚ. Collision energy of the tandem MŚ was checked in the range of 10-30V and 20V was found to give the best fragmentation patterns.

Using the sample preparation method developed by Qi *et al*. (2005) and Okudera & Ito (2009), the LC-ESI-MŚ/MŚ failed to detect the presence of any chromones in cell suspension sample extract indicating that either chromones were not present in the cell suspension or the sample preparation technique used was not suitable for trace element analysis (Section 3.3.3.1). Based on this observation, we subsequently move on to explore the possibility of using C18 LC SPME fibers to extract analytes from the sample which resulted in the detection of 7 chromone like-elements in which 4 were successfully identified (Table 3.14). The utilization

of SPME as a sample preparative step was found to be effective in detecting chromones in unstressed cell suspensions of *A. malaccensis*. In general, the fragmentation patterns of the chromones detected (Appendix B - Table 3.16) were similar to the chromones identified in Section 3.2.1 (Appendix B – Table 3.15), however only two (6-methoxy-2-[2-(3-methoxy-4-hydroxyphenyl)ethyl]chromone and 6-methoxy-2-(2-phenylethyl)-chromones) out of the four chromones identified in the cell suspensions were found to present in the oil extract.

It was interesting to note that all the chromones identified in the cell suspension were similar to those identified in the resinous wood of *A. malaccensis* by Konishi *et al*. (2002). Konishi reported that the production of chromones was limited to the resinous tissue of *Aquilaria* species and, to date, only two 2-(2-phenylethyl) chromones have been isolated from non-resinous tissue, i.e. roots of *A. sinensis* (Yang *et al*., 1989). The results from the present study indicate that either chromones are produced in trace amounts which were not detectable using conventional extraction methods or the cell suspensions were under stress at the time of analysis causing the cells to produce chromones. The latter is probable because, although cell suspensions are assumed to be growing under 'ideal/optimal' conditions, the accelerated growth rate of cell suspensions would indirectly cause stress to the cells. In addition, other uncontrollable factors such as changes in humidity, temperature throughout the experiment or analytical procedures may indirectly contribute to the stress.

Although the experiment yield positive result, it was ultimately decided that chromone analysis would be excluded from the study due absence of a consistent production of specific analytes which could act as chemical markers. Without the presence of specific chemical markers it would not be practical to manually screen each sample for a total of 39 different molecular masses (39 types of chromones).

3.4 Conclusions

The application of solid phase micro extraction fibers (SPME) was found to be extremely effective in detecting sesquiterpenes and chromones in cell suspensions of *A. malaccensis*. Overall four sesquiterpenes (alpha humulene, delta guaiene, beta caryophyllene and alpha guaiene) and four chromones (6-methoxy-2-(2-phenylethyl)- chromone, 5,8-dihydroxy-2-(2-(4-methoxyphenylethyl)-chromone,7-hydroxy-2-(2-phenylethyl]-chromone and 6-methoxy-2-[2-(3-methoxyphenyl)ethyl] chromones) were found to be produced in unstressed *Aquilaria malaccensis* cell suspensions

The following procedures are recommended for the analysis of sesquiterpenes through HS-SPME GC-MŚ and chromones through SPME-LC-MŚ/MŚ:

Analysis of sesquiterpenes through HS-SPME GC-MŚ

Sample 5ml of cell suspension into 20ml headspace vials using a CTC auto-sampler. Insert a 100µm polydimethylsiloxane (PDMS) fiber into the headspace of the sampling vial and maintain at 80ºC with agitation at 300 rpm for 40 minutes. Inject the fiber (containing the analytes) into the injector port of the GC-MŚ. Run the GC-MŚ program with the following parameters: injector temperature set at 270ºC, start the column oven program at 60ºC and hold for 2 minutes before progressively increasing to 180ºC at 30ºC per minute. Then increase the temperature by 1.5ºC per minute until it reaches 200 ºC. Then increase to 280ºC at a rate of 20ºC per minute and hold for 15 minutes.

Analysis of chromones through SPME LC-ESI-MŚ/MŚ

Introduce samples into an Agilent® Pursuit XRs 3C18 column (100 x 0.2mm) with the mobile phase of methanol:water (80:20, v/v) at a flow rate of 200µl/min with the column set at room temperature and injection volume of 15µl. Set the electrospray capillary potential at 60V. Maintain the drying gas pressure at 35psi/400ºC and the nebulizing gas pressure at 50psi. Collect spectra over the mass range of *m/z* 200-800. Identify analytes using a Varian-320 LC-ESI-MŚ/MŚ equipped with a Prostar 410 auto-sampler, two Prostar 210 pumps and a 1200L electrospray source tandem mass spectrometer. Screen the compounds manually to identify chromone molecules. Reanalyse targeted molecules using conditions described above after subjecting to 20V of collision energy to fragment the molecules into ions.

CHAPTER 4

Establishment of molecular techniques to study synthase expression in *Aquilaria malaccensis*

4.0 Introduction

Manipulation of terpene metabolism in plants has long been a target of interest in plant biotechnology. Examples of plants which are highly sought after are *Artemisia annua* for the production of the anti-malarial drug artemisinin (Cai *et al*., 2002), *Zingiber* species for the production of zerumbone (Picaud *et al*., 2006; Yu *et al*., 2008) and *Taxus* species for the production of the anti-tumor compound paclitaxel (Tabata, 2004). However, to exploit fully the potential of plants, it is imperative to understand the complete biosynthetic pathway of the metabolite of interest. With reference to *Aquilaria* species, sesquiterpenes and phenyl ethyl chromones represent the main constituents of the highly prized agarwood. The lack of understanding about the biosynthesis of fragrance components in agarwood has meant that it is difficult to establish the molecular basis for its production in *Aquilaria* species.

All terpenoids are derived from isopentyl diphosphate which serves as a central precursor that is synthesised in the cytosol and plastids (Trapp & Croteau, 2001). Sesquiterpenes, which are the focus of this study, are synthesised in the cytosol through the acetate/mevalonate pathway (Figure 4.1). They are also known as phytoalexins and play an important role in the defensive system of plants whereby, when damage is inflicted on a plant, sesquiterpenes are released to deter herbivory and protect the plant from pathogenic infections, thus minimising further damage. To date, more than 40 synthases originating from 48 species have been identified (Degenhardt *et al.*, 2009; Wang *et al*., 2009b; Kumeta & Ito, 2011). Terpene synthase genes are usually up-regulated either in highly specialised cells such as glandular trichomes in mint, during specific developmental stages or during short periods of transient defence reactions (Bohlmann *et al*., 1998). The current study will focus on the third criterion in which artificial stress, i.e. methyl jasmonate and salicylic acid, will be used to activate the plant's defence mechanism in order to analyse terpene synthase (Tps) activity.

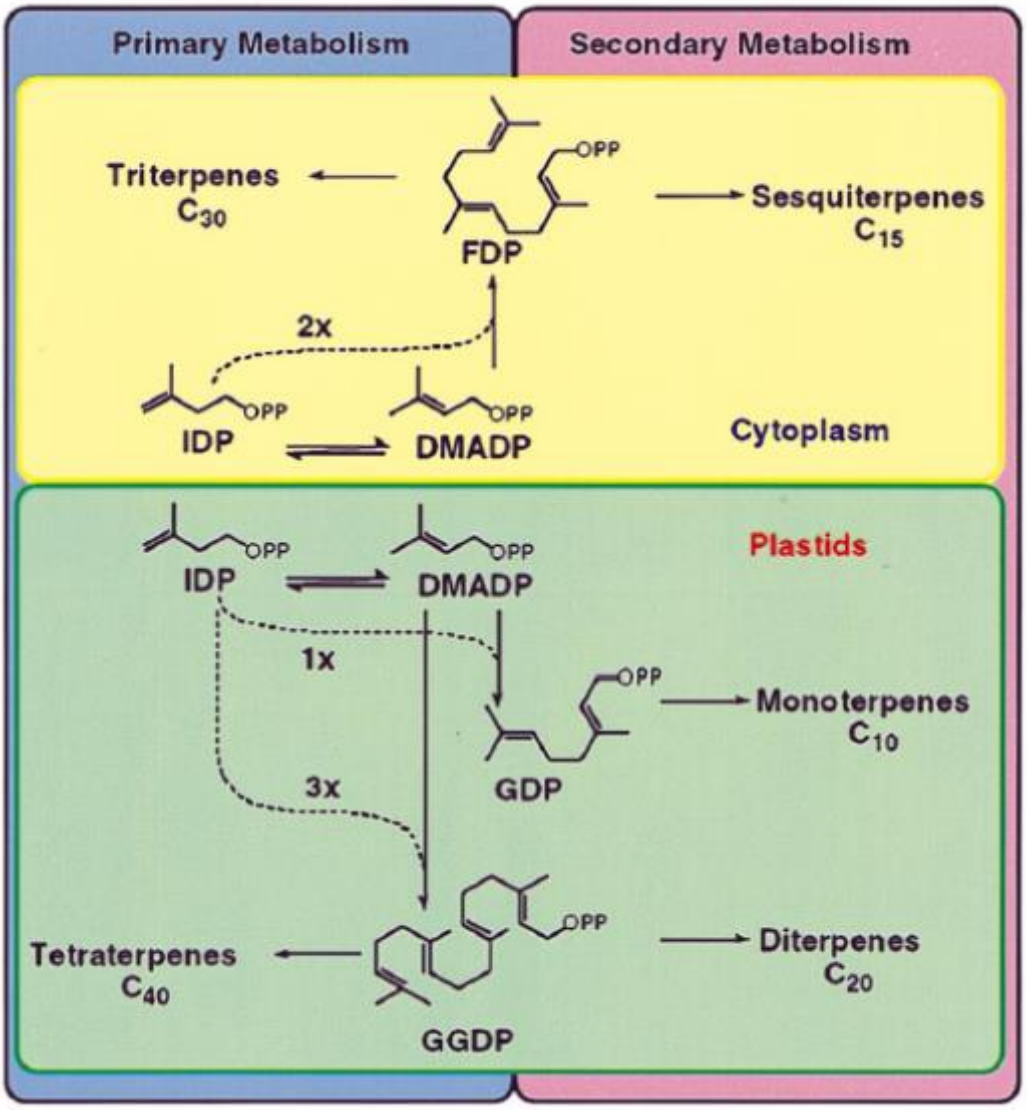


Figure 4.1 Organisation of terpene biosynthesis in plants (Bohlmann *et al.*, 1998)

Gene expression studies were used to identify and understand gene expression patterns when the plant defence system and metabolites such sesquiterpenes are produced; for sesquiterpene, most molecular studies done were performed by enriching plant material (subjecting plants to artificially induced conditions or stress) prior to mRNA isolation. Sesquiterpenes synthases such as α-humulene synthase isolated from *Zingiber zerumbet* (Yu *et al*., 2008) , δ-guaiene from *Aquilaria crassna* (Kumeta & Ito , 2011) and β- caryophyllene

from *Artemisia annua* (Cai *et al*., 2002) are examples of synthases involved in phytoalexin biosynthesis which have been successfully isolated and identified through artificially stressing cell or callus cultures with the application of chemical stress/elicitor such as methyl jasmonate or through the inoculation of fungal extracts.

Although the establishment of cDNA libraries is ideal for studying gene expression patterns within plants, the scope of this study could not include the establishment of a cDNA library due to time limitations. As an alternative, the study was based on information made available from published cDNA studies which have been successful in identifying sesquiterpene synthases, specifically for α-humulene, β-caryophyllene, δ-guaiene and α–guaiene. Primers designed to detect specific sesquiterpene synthases present in other species were used to identify gene sequences which may correlate to the biosynthesis of sesquiterpenes.

The sesquiterpenes α-humulene , α-guaiene, δ-guaiene and β-caryophyllene belongs to the Tps-a subfamily of the Tps gene family. A minimum of 40% of the sesquiterpene synthase sequences of each member are similiar to other members (Bohlmann *et al*., 1998). It is important to note that, due to this high similarity among synthases within the subfamily, the mode of action of these synthases is not limited to the regulation of a single sesquiterpene but it is most likely involved in generating multiple products. For example, the γ-humulene synthase in *Abies grandis* is known to generate 52 sesquiterpenes (Steele *et al*., 1998), while TPS4 synthase of *Zea mays* has been found to regulate 2 sesquiterpenes and 12 olefins in minor amounts (Köllner *et al*., 2004). Therefore, based on this knowledge, the selection of primers was done by screening literature for relevant synthases (Table 4.1).

Table 4.1 Synthases and primers involved in sesquiterpene biosynthesis.

Gene Bank accession no.	Main products	Name of primer	Species	References
GU083700	δ-guaiene	ACL 154	*Aquilaria crassna*	Kumeta & Ito (2010)
AB786708	β-caryophyllene	QHS 1	*Artemisia annua*	Cai *et al*. (2002)
AB263736	α-humulene	ZSS 1	*Zingiber zerumbet*	Yu *et al*. (2008)
DQ872158	β-caryophyllene	OsTPS3	*Oryza sativa*	Cheng *et al*. (2007)
DQ872159		OsTPS13		
FJ767894	β-caryophyllene	MmCS	*Mikania micrantha ()*	*Wang et al*. (2009b)

This molecular based approach was included in this study to complement the chemical analyses. Given that a lot of work has been done on illustrating and understanding the basic metabolic pathway of sesquiterpenes, the use of a semi-quantitative PCR approach will complement the biochemical results presented in chapter 5 and would provide further insight into the molecular mechanisms which are involved in the onset and production of agarwood in *Aquilaria* species.

The aims of this part of the study were to

(i) Develop and establish molecular techniques required to test the applicability of primers targeting gene sequences related to the synthesis of sesquiterpenes, and

(ii) Establish a semi quantitative PCR screening method which would enable the study of gene expression levels in cell suspensions in response to chemical stress or stimulation.

4.1 Materials and Method

4.1.1 Plant Material

Cells of *Aquilaria malaccensis* established and maintained through cell suspension culture, as described in Chapter 2, were used.

4.1.2 Extraction of Genomic DNA

The CTAB method developed by Phil Jones (personal communications) with some modifications was used. Approximately 3g of cell powder (cells obtained from cell suspension were frozen using liquid nitrogen and grounded into powder using mortar and pestle) was transferred to a 50ml centrifuge tube containing 10ml modified CTAB buffer (2% w/v CTAB, 0.02M EDTA pH 8, 1.4M NaCl, 0.1M Tris-Cl pH 8.0). Other components added fresh were 20µl β-mercaptoethanol and 70µg RNase A. The mixture was incubated at 65°C for 30 minutes. After incubation, it was mixed thoroughly with an equal volume of 24:1 chloroform:isoamyl alcohol, followed by centrifugation at 13,000 x *g* for 15 minutes. The upper aqueous phase was transferred to a new tube, where the DNA was precipitated with a 0.6 volume of cold isopropanol at -20°C for 2 hours. DNA was then collected by centrifuging at 13,000 x *g* at 4°C for 15 minutes and the pellet was washed with 5ml 70% ethanol before being dissolved in 100µl TE buffer (10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0). The quantity of extracted DNA was assessed using the absorbance reading (NanoDrop™ 1000 Spectrophotometer, Thermo Scientific) and its purity determined from the A_{260}/A_{280} ratio. The integrity of the DNA was also checked by viewing the samples after running on a 0.6% TAE agarose gel stained with SYBR® Safe (Cat. No. S33102, Invitrogen) as described in Section 4.1.3.

4.1.3 Agarose Gel Electrophoresis

Agarose gel was prepared by mixing 1x TAE buffer, 0.6% agarose powder and 1x SYBR® Safe (Cat. No. S33102, Invitrogen). The gel was then cast in varying sizes of moulds, depending on the number of samples to be runs. Gels were run at 10V/cm at room temperature using 1x TAE as the running buffer and samples were mixed with 6x loading dye in a ratio of 5 to 1 prior to the run. Fragment separation was visualised and photographed using a FluoroChem HD2 MultiImage ® II (Alpha Innotech).

4.1.4 Primer Selection and PCR Optimisation

Six primers (Table 4.2), adopted from existing primers available from the literature, were used to detect the presence of selected sesquiterpene genes (β-caryophyllene, α-humulene, δ-guaiene) in the DNA extracted from *A. malaccensis* cells.

Amplification of DNA was done using i-Taq™ polymerase (Cat no. 25021, Intron Biotechnology) using 20µl reaction mixture comprised of 1x Buffer, 2.5U i-Taq™ polymerase, 0.2mM dNTPs, 0.5µM primers, ultrapure water and 1µg template. PCR was done using a GS2 Thermal Cycler (G-Storm) with initial denaturation at 95ºC for 5 minutes, 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and final extension at 72ºC for 135 seconds. The resulting products were then viewed using 0.6% SYBR® Safe (Cat. No. S33102, Invitrogen) stained TAE agarose gel (Section 4.1.3).

Table 4.2 Sequences of primers used.

Name of primer	Sequence of primer	Size of gene sequence	Plant used	Targeted analyte
ACL 154	Forward 5'-ATGTCTTCGGCAAAACTAGGTTCTGCCTCC-3' Reverse 5'-GATTTCAATAGCATGACGCAACAAGGCAGC-3'	1641bp	*Aquilaria crassna* (Kumeta & Ito, 2011)	δ-guaiene
QHS 1	Forward 5'-ATGGTGATGACTGGAAAGGC-3' Reverse 5'-TGGAAACTTCGCTAAGCTCC-3'	1902 bp	*Artemisia annua* (Cai *et al*., 2002)	β-caryophyllene
ZSS 1	Forward 5'-ATGGAGAGGCAGTCGATGGCCCTTG-3' Reverse 5'-AATAAGAAGGATTCAACAAATATGAGAG-3'	1644bp	*Zingiber zerumbet* (Yu *et al*., 2008)	α-humulene
OsTPS3	Forward 5'-CGGGATCCATGGCAACCTCTGTTCCG -3' Reverse CCGCTCGAGTTAAACAGAGAGGATGTA-3'	1731bp	*Oryza sativa* (Cheng *et al*., 2007)	β-caryophyllene
OsTPS13	Forward 5'-CGGGATCCATGGCGCCGGCTTTC-3' Reverse 5'-CGAGCTCTTAATCGTAATCTATATGATTAAGC-3'	1623bp	*Oryza sativa* (Cheng *et al*., 2007)	
MmCS	Forward 5'-TAAGAAGGAGCAAGAAAGAGTGC -3' Reverse 5'-CTCTTTGATGTCTTCTTCCACTTC-3'	1898bp	*Mikania micrantha (Wang et al*., 2009)	β-caryophyllene

PCR optimisation

Optimisation of PCR was carried out using selected primers and to ensure that high quality (pure) PCR products were produced for sequencing, factors such as the annealing temperature, inclusion of $MgCl_2$ and DMSO and different buffer/PCR kits were also assessed.

a) PCR kit optimisation

   Besides i-Taq polymerase, other PCR reagents such as Vivantis Taq polymerase (Cat No. PL1202, Vivantis) was used.

   i) PCR amplification using Vivantis Taq polymerase: Amplification of DNA was done using 25µl reaction mixture comprising of 1 X Buffer S, 2U Vivantis Taq polymerase, 0.2mM dNTPs, 0.5µM primers, ultrapure water and 1µg template, as described above.

b) Optimisation of PCR condition by varying annealing temperature – ACL 154 & ZSS1

   Gradient PCR was conducted for ACL 154 and ZSS1 fragments using Vivantis Taq polymerase and i-Taq™ polymerase respectively, so that an optimum annealing temperature could be determined. The PCR conditions were as described in Section 4.1.4 except the annealing temperature was set at a range of 50 to 60°C.

c) Optimisation of PCR conditions with the addition of magnesium chloride ($MgCl_2$)

   Magnesium chloride at 1.5mM, 2.5mM, 3.5mM and 4.5mM was mixed with Vivantis ® Taq polymerase (as described in Section 4.1.4). PCR amplification was done using ACL 154 using the conditions described above.

4.1.5 DNA Purification, Cloning and Sequencing

After amplification, PCR products were mixed with 6x EZ-Vision®DNA Dye (Cat. No. N133, Ameresco ®) in the ratio 1:1 prior to being loaded onto 1% TAE agarose gels. Gels were run at 90V for 45 minutes using 1X TAE as the running buffer at room temperature. Fragment separation was visualised using a UV trans-illuminator, and the band of interest was sliced out using a scalpel, transferred to a 1.5ml centrifuge tube and stored at 4ºC. The PCR products were purified from agarose gels using MEGAquick-spin™ gel purification kit (Cat.No. 17282, Intron Biotechnology). The purified products were then ligated into pCR™4-TOPO® vector and transformed into competent TOP10 *E. coli* following manufacturer's instruction (Invitrogen, Life Technologies). Positive colonies were selected by PCR, prior to extracting plasmid (Exprep™ Plasmid SV, GeneAll) after an overnight incubation. The extracted plasmid were sent for Sanger sequencing using M13 forward and reverse primers. Results were then analysed using Bioedit v 7.2.5 and run through BLAST.

4.1.6 RNA Extraction & Quantification

RNA was extracted using the method described in the Trizol ® reagent product manual. Cells sampled from cell suspensions were frozen in liquid nitrogen and ground to a fine powder. Approximately 0.5g of the cell powder was transferred to a 2ml centrifuge tube containing 1.5ml of Trizol® reagent. The mixture was inverted several times before centrifuging at 12,000 x *g* at 4°C for 10 minutes. The supernatant was transferred to a new tube and 0.3ml of chloroform added. The mixture was agitated vigorously for 15 seconds, incubated at room temperature for 3 minutes and then centrifuged at 12, 000 x *g* at 4°C for 15 minutes. The upper aqueous phase was transferred to a new tube, where the RNA was precipitated with 0.8ml cold isopropanol. The RNA was then collected by centrifuging at 12,000 x g at 4^0C for 10 minutes.

The RNA pellet was washed with 200µl 75% ethanol before being dissolved in nuclease free water. The quantity of extracted RNA was assessed via NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) while the quality of the RNA was checked by viewing the samples after running on a 0.3% of SYBR® Safe (Cat No. S33102, Invitrogen) stained TAE agarose gel (Section 4.1.3).

If the sample was found to contain multiple bands, it was treated with DNase to remove any contaminating DNA by adding 2 µl DNase I (Cat No. 1709, Vivantis) and incubating at 37^{0}C for 2 hours. After incubation, 2 µl 0.5M EDTA pH 8.0 was added to the mixture and incubated at 65^{0}C for 10 minutes. An equal volume of 24:23:1 phenol:chloroform:isoamyl alcohol was then added to the mixture, followed by centrifugation at 13,000 x *g* for 10 minutes. The upper aqueous phase was transferred to a new tube, where the RNA was precipitated with 0.6 volume of cold isopropanol at -20^{0}C for 2 hours. RNA was then collected by centrifuging at 13,000 x g at 4^{0}C for 15 minutes. The RNA pellet was washed with 200µl of 75% ethanol before being dissolved in nuclease-free water. Initial quantification of RNA was assessed via Nano Drop while more accurate quantification was done using a Quant-it ™ RiboGreen ® RNA quantification kit (Cat No. R11491, Invitrogen) (Section 4.1.5). The quality of the RNA was checked by viewing the samples after running on a 0.3% SYBR® Safe (Cat No. S33102, Invitrogen) stained TAE agarose gel (Section 4.1.3).

RNA Quantification

RNA quantification was performed using a Quant-iT™ RiboGreen® RNA Quantification Kit with the high range assay protocol recommended by the manufacturer. A standard curve was generated for RNA concentrations ranging from 20ng to 1µg. For each analysis, 0.5µl of sample was mixed with 99.5µl 1X TE buffer and 100µl Quant-iT™ RiboGreen RNA working solution (diluted 200 fold using 1x TE buffer) in 96 well micro-plates. Sample fluorescence was then measured at standard fluorescein wavelengths (excitation ~ 480nm; emission ~ 520 nm) using a Varioskan™ Flask Spectral Reader (Thermoscientific) in clear round-bottom micro-plates (Corning). Each sample was prepared in duplicate and the average flourometric value was used to calculate RNA concentration based on the equation generated using the standard curve.

4.1.7 Reverse Transcriptase and cDNA verification

RNA was amplified using an Omniscript® Reverse Transcriptase (RT) Kit (Cat No.205111, Qiagen®). RT-PCR was performed in a final volume of 20 µl containing 2µg RNA (sample), 1x buffer RT, 0.5µM each dNTP, 10U Omniscript® reverse transcriptase and 1 µM Oligo $(dT)_{18}$ Primer Mix. The mixture was incubated at 37ºC for 60 minutes, then stored at -20ºC. Selective amplification of cDNA was done using ACL154 primer. Amplification of cDNA was performed as previously described in Section 4.1.4. PCR products were analysed using 0.3% agarose gel as described in Section 4.1.3.

4.2 Results

4.2.1 PCR Optimisation

Preliminary screening using DNA

In the evaluation of the 6 primers for the detection of the gene sequence of Tps synthases within the *Aquilaria* genome, only two samples, QHS 1 and ACL 154, showed encouraging results in which one band was observed while other primers had multiple bands (Figure 4.2). Further optimization of PCR conditions was therefore focused on QHS 1, ACL 154 and ZSS1 due to the presence of one band (ACL 154, QHS 1) or one dominant band (ZSS1). The other primers were excluded from the study.

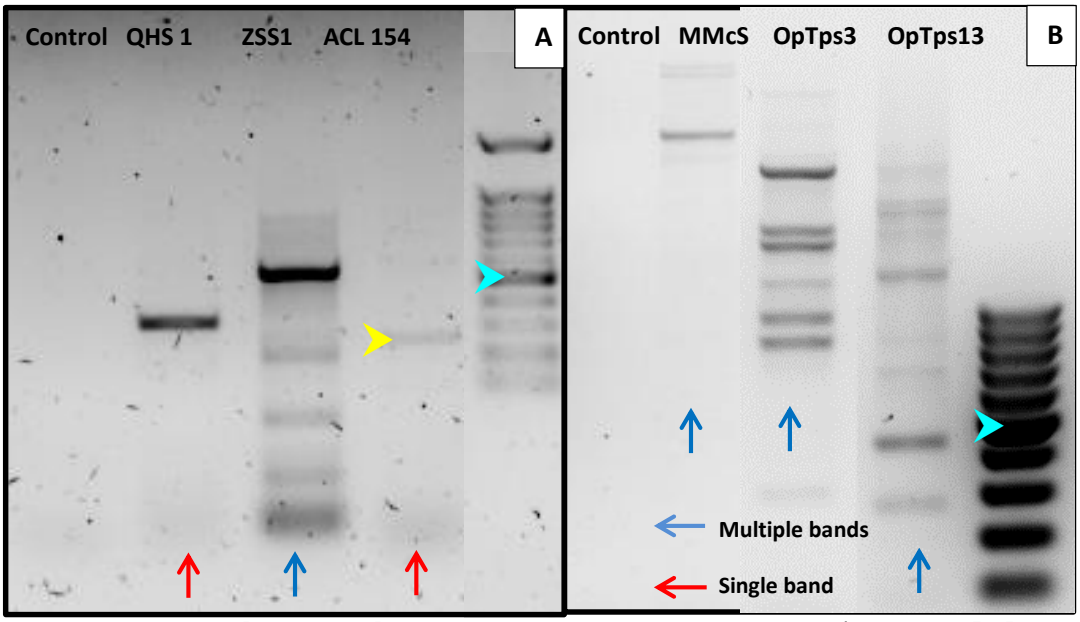


Figure 4.2 Primer selection and PCR optimisation using i-Taq™ polymerase. [A] Negative Control , QHS1, ZSS1 & ACL154. [B] Negative Control , MMcS, OpTps3 & OpTps13. (➤) : 500bp ; (➤) :300bp.

Further optimisation (DNA amplification)

Due to the presence of a prominent single band using QHS 1, further optimisation was deemed unnecessary, and the sample was subsequently amplified and sent for sequencing. Using ACL 154 and ZSS1 resulted in multiple bands with one dominant band, so further optimisation of the PCR conditions was pursued.

a) PCR reagents

The specificity of the primer ACL154 improved greatly with the use of Vivantis Taq polymerase (Figure 4.3). For the primer ZSS1, although Vivantis Taq polymerase was not able to reduce unspecific bands, the intensity of the dominant band was increased.

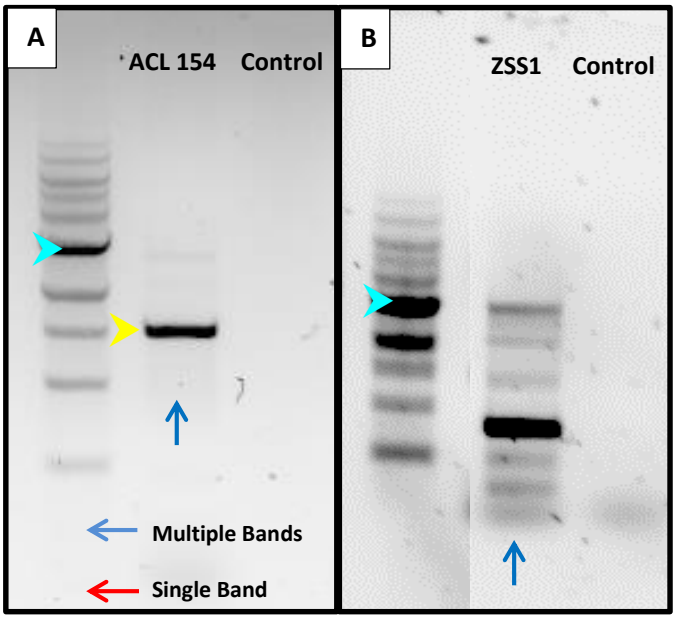


Figure 4.3 PCR optimisation using Vivantis DNA polymerase. [A] ACL I54 + DNA; Negative control. [B] ZSS1 + DNA; Negative control. . (➤) : 500bp ; (➤) :300bp.

b) PCR annealing temperature

Vivantis Taq polymerase was found to be more suitable for amplification of ACL 154 and ZSS1 amplicons, however due to the presence of multiple non-specific bands, further optimisation of PCR conditions was done by subjecting samples to higher annealing temperature varying (50 – 60 ^{o}C). Varying the annealing temperature did not improve the amplification of the ACL 154 or the ZSS1 amplicons (Figure 4.4). For ZSS 1 there was a reduction of the non-specific bands (multiple bands) but this was at the expense of the dominant band (~ 1000bp). At 59.7^{o}C only the dominant band remained but its intensity was greatly reduced. Based on these results, it was decided to continue only to optimize ACL 154 primer conditions, aimed mainly at further improvement of primer specificity, band intensity and minimisation of smearing.

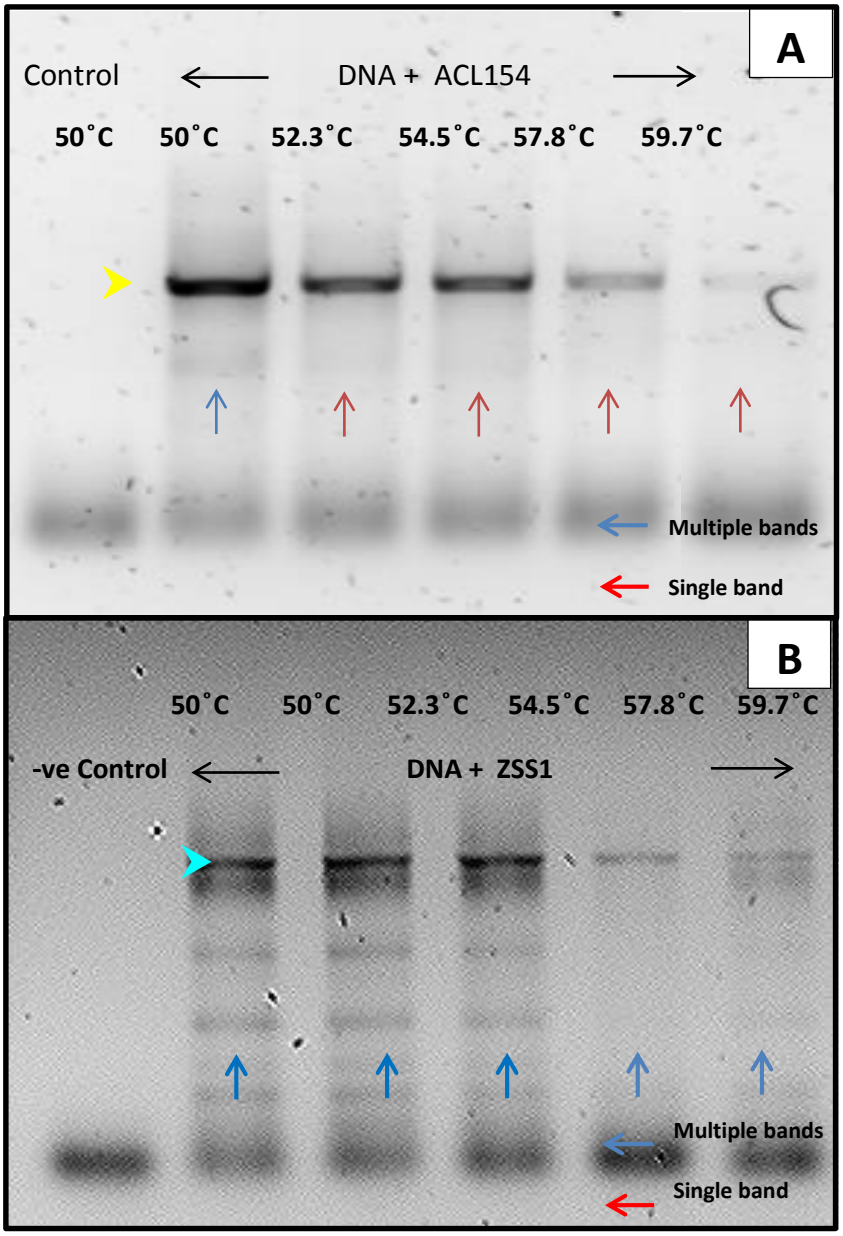


Figure 4.4 PCR amplification with [A] ACL 154 and [B] ZSS1 at varying annealing temperatures. (➤) : 500bp ; (➤) :300bp.

c) Magnesium chloride ($MgCl_2$)

The addition of $MgCl_2$ did not enhance specificity or band intensity (Figure 4.5). Therefore it was decided to proceed with scaling up amplification of the ACL 154 amplicon followed by separation and recovery of the single band via gel electrophoresis and DNA purification before proceeding to DNA sequencing (sent out for sequencing).

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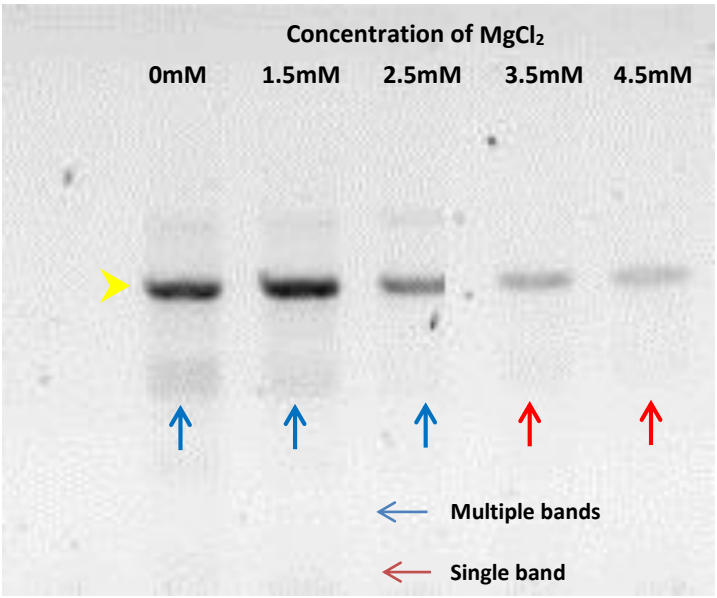

Figure 4.5 Effect of $MgCl_2$ concentration on PCR amplification with ACL 154. (➤) :300bp.

4.2.2 DNA Sequencing

Sequencing was done to verify if the amplified amplicon represented to the gene of interest. Upon DNA sequencing, the QHS 1 amplicon was not able to yield any results as multiple signals were detected, indicating the presence of two or more DNA templates in the sample. This was further verified by running the sample in a denser gel (2%). A thicker band was observed indicating there were overlapping bands of similar size (Figure 4.6 – Appendix C). Despite efforts to further purify and separate the bands, this was not achievable, so QHS 1 primer was deemed unsuitable for the study. Notwithstanding the presence of multiple bands,

it was decided to amplify the ZSS1 amplicon in the hope that the dominant band would contain an amplicon homologous to the synthase. However, despite successful attempts to purify the amplicon (Figure 4.3 & 4.4), sequencing the amplicon failed (unable to obtain good sequencing information). For the ACL 154 amplicon, numerous attempts to sequence it failed. It was then decided to excise the band of interest (~500kb) directly from the electrophoresis gels prior to purification (Figure 4.7) and, from this, a short 151bp amplicon was successfully sequenced. The nucleotide sequence was then analysed by screening for similar sequences within the GenBank database using BLAST.

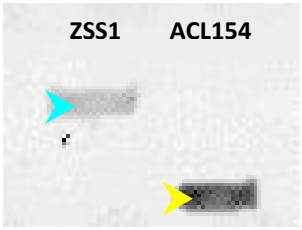

Figure 4.7 Gel electrophoresis image of purified samples of ZSS1 and ACL 154 amplicon using 0.8% SYBR® stained agarose gel. (➤) : 500bp ; (➤) :300bp.

The ACL 154 fragment (151bp) was found to resemble δ-guaiene synthases found in both *Aquilaria crassna* and *A. sinensis,* indicating that the ACL 154 primer was suitable for analysing gene expression patterns related to δ-guaiene and similar sesquiterpenes (Table 4.3). The ACL 154 amplicon was observed to have a high level of homology to delta synthases originating from *A. sinensis* and *A. crassna* whereby the area of coverage achieved was 99% identical (Table 4.3; Figure 4.8).

Table 4.3 Summary of the blastN search results obtained from the ACL 154 fragment

Description	**Organism**	**Blast score**	**Query cover**	**E value**	**Identity**	**Accssion no.**
n6 δ-guaiene synthase 2 gene	*Aquilaria crassna*	470	100%	$4\ e^{-134}$	97%	JF289166.1
n6 δ-guaiene synthase 3 gene	*Aquilaria crassna*	457	100%	$2\ e^{-130}$	96%	JF289267.1
n6 δ-guaiene synthase 1 pseudogene	*Aquilaria crassna*	426	97%	$4\ e^{-121}$	94%	JF289265.1
δ-guaiene synthase gene	*Aquilaria sinensis*	412	97%	$9\ e^{-117}$	93%	KC149961.1
n14 δ-guaiene synthase 5 gene	*Aquilaria crassna*	412	97%	$9\ e^{-117}$	93%	JF289269.1
n6 δ-guaiene synthase 4 gene	*Aquilaria crassna*	408	97%	$1\ e^{-115}$	93%	JF289268.1

```
AM-ACL154    1  ATGTCTTCGGCAAAACTAGGTTCTGCCTCCGAAGATATTAGCCGCCGAGATGCCAATTAC  60
KC149961.1   1  ATGTCTTCGGCAAAACTAGGTTCTGCCTCCGAAGATGTTAGCCGCCGAGATGCCAATTAC  60
JF289266.1   1  ATGTCTTCGGCAAAACTAGGTTCTGCCTCCGAAGATGTTAGCCGCCGAGATGCCAATTAC  60
                ********************************** ************************

AM-ACL154   61  CATCCCACTGTTTGGGGGGACTTTTTCCTCACTCATTCTTCCAACTTCTTGGTAACTTTT  120
KC149961.1  61  CATCCCACCGTTTGGGGGGACTTTTTCCTCACTCATTCTTCCAACTTCTTGGTAACTTTT  120
JF289266.1  61  CATCCCACTGTTTGGGGGGACTTTTTCCTCACTCATTCTTCCAACTTCTTGGTAACTTTT  120
                ******** ************************************************

AM-ACL154  121  TTGATCATTCAACTTCTACCTTAGCTCATCATTGTTTTTTTAGTTAGAGCTAAGATGTGA  180
KC149961.1 121  TT-ATCATTGAACTTCTATCTCAGCTCACCACTGTTTTCTTAATTAGAGCGAAGATGTGA  180
JF289266.1 121  TTTATCATTGAACTTCTACCTTAGCTCATCACTTTTTTCTTAGTTAGAGCTAAGATGTGA  180
                ** ****** ******** ** ****** ** * **** *** ******* *********

AM-ACL154  181  AACTAGCATTTTATCGCTCTTTTTGGTTTTGAATGGTGGTTTCACGTATCTTAGTTCCAA  240
KC149961.1 181  AACTAGCATTTTATCACTCTTTTTGGTTTTGAATGGTGGTTGCATGTATTTTAGTTCCAA  240
JF289266.1 181  AACTAGCATTTTATCACTCTTCTTGG-TTTGAATGGTGGTTTCACGTATCTTAGTTCCAA  240
                *************** ***** **** ************* ** **** **********

AM-ACL154  241  TATTTTGGCGGCTCAGATTGCGGAGCTTCTGCTAAAATCTAAGTC---------------  300
KC149961.1 241  TATTTTGGTGGCTCAGATTGTGGAACTTCTGCTGAAATTTGATTCAGTACTTCTCTTTAT  300
JF289266.1 241  TATTTTGGCGGCTCAGATTGCGGAGCTTCTGCTAAAATCTAAGTCAGAACTTCTCTTTAT  300
                ******** *********** *** ******** **** * * **
```

Figure 4.8 Comparison of nucleotide sequences between A. *malaccensis* ACL 154 amplicon sequence (AM-ACL154) with other sesquiterpene synthases of other *Aquilaria* species. KC149961.1 - δ-guaiene synthase gene of A. *sinensis* ; JF289266.1 - n6 δ-guaiene synthase 2 gene of A. *crassna*. In each column, nucleotides that are highlighted on a grey background when they are identical to one other nucleotide sequence of another *Aquilaria* species or on a black background when different from both nucleotide sequences.

4.2.3 RNA Extraction and Quantification

For RNA quantification, a standard curve (Figure 4.9) was plotted for each 96 well plate and analysed to ensure consistency among samples. In addition, the quality of the RNA was also verified through imaging (gel electrophoresis) to ensure that minimal RNA degradation had occurred (Figure 4.11 – Appendix C).

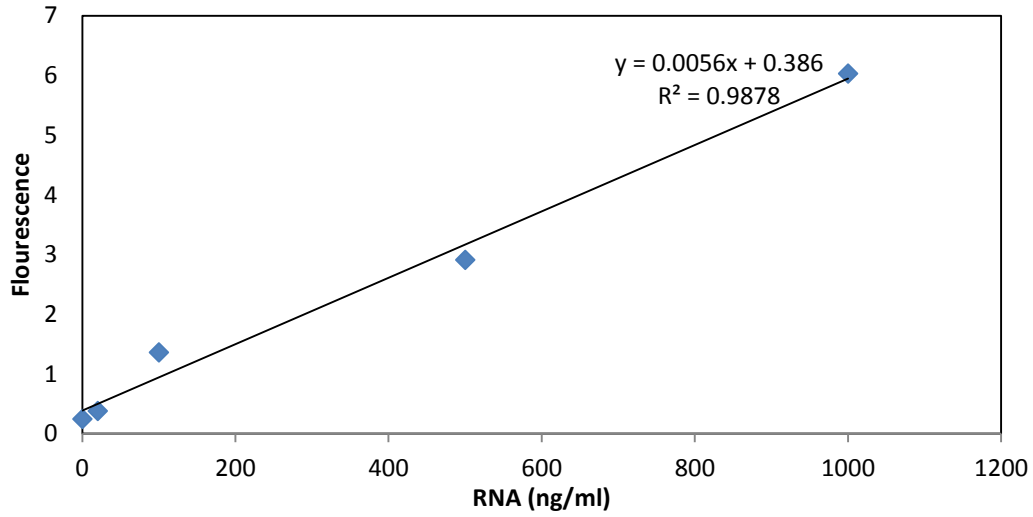

Figure 4.9 Example of standard curve generated for the high-range assay of Quant iT™ Ribogreen® reagent. Samples were excited at 485nm and measured at 530nm using a fluorescence microplate reader. **Using the standard curve equation, the concentration of RNA per sample was tabulated.

4.2.4 Reverse Transcription and cDNA Verification

Based on the known RNA concentration of each sample, reverse transcription was done by standardising the RNA at 2µg per reaction. Upon generating cDNA transcripts, PCR amplification using ACL 154 primer was carried out. The sample used was from the control (not stressed cell suspensions) which had been screened by GCMŚ for the presence of selected analytes, namely α-humulene, δ-guaiene, α-guaiene and β-caryophyllene. A low level of gene expression was detected as a faint band which is approximately 150bp in size (Figure 4.10). This is consistent with the GCMŚ results (results not shown) whereby traces of all four analytes were detected.

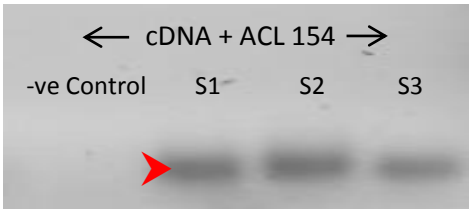

Figure 4.10 Gel electrophoresis of cDNA sample subjected to PCR amplification using ACL 154 primer and viewed using 0.8% SYBR® stained agarose gel. (➤) :150bp. **S1, S2 & S3 represent different cell suspension lines which were also analysed as illustrated in chapter 3 (Figure 3.11).

4.3 Discussion

Progress towards understanding agarwood production has benefitted from the recent identification of the gene for δ-guaiene synthase which is believed to play an important role during the onset of agarwood production (Kumeta & Ito, 2010, 2011; Okudera & Ito , 2009; Xu *et al.*, 2013). However, more biochemical and molecular investigations are needed to understand fully the various aspects of the pathway. This chapter represents the first study to investigate and identify a gene sequence related to the biosynthesis of selected sesquiterpenes in *Aquilaria malaccensis*. Out of the 6 primers used to screen for correlating gene sequences related to sesquiterpene synthases, only ACL 154 amplicon was successfully sequenced after optimising the PCR amplification conditions (Section 4.2.1) and subsequently cloning amplicon prior to sequencing. Primers designed for other species were deemed unsuitable as multiple bands were observed, suggesting a low level of homology between sesquiterpene synthases (of different species). A low level of homology between synthases of *Aquilaria* species when compared to other species was also observed by Kumeta and Ito (2010) who reported that the homology of the sesquiterpene synthase isolated from *A. crassna* shared only 40% identity in amino acids with other sesquiterpene synthases.

The ACL 154 primer was designed by Kumeta & Ito (2010) and subsequently aided the identification of five δ-guaiene synthases (AcC1, AcC2, AcC3, AcC4 and ACL 154) cloned from cell cultures of *A. crassna*. In the present study, the 280bp amplicon amplified via the ACL 154 primer correlated to a portion of the δ-guaiene synthases which have previously been isolated and identified. The 280bp product showed 96 – 97% similarity towards the front section (1-280) of δ-guaiene synthase of *A. sinensis* and *A. crassna* (Figure 4.8). Guaiene-type sesquiterpenes, such as δ-guaiene, have unique five- and seven-membered ring systems which are synthesised via two cyclisation reactions. The first involves C1 to C10 cyclisation, yielding

a germacrene line intermediate and the second cyclisation occurs between C2 and C6, generating the guaiene product (Kumeta & Ito, 2010). Although guaiene-type sesquiterpenes are common in nature (Naef, 2011), to date very few synthases related to their synthesis have been successfully identified (Xu *et al*., 2013; Kumeta & Ito, 2011; Deguerry *et al*., 2006).

Previous phylogenetic analyses done by Kumeta & Ito (2010) showed that δ-guaiene synthase of *A. crassna* belongs to the Tps-a subfamily which consists of sesquiterpenes and diterpenes related to angiosperm secondary metabolism. Based on the analysis done (Figure 4.8), the results obtained suggests that δ-guaiene synthases identified from the *Aquilaria* genus share a high degree of similarity among closely related species suggesting that they all belong to the Tps-a subfamily. The results also reflects that the DNA sequence of the ACL 154 amplicon is part of a highly conserved region within the δ-guaiene synthases of the *Aquilaria* genus, making it an ideal primer for the study of the regulation of sesquiterpene related synthases in response to induced stress (Chapter 5).

Once the primer had been tested, preliminary screening of cell suspensions focused on developing a semi-quantitative PCR protocol to study the gene expression levels. RNA extraction using Trizol® was found sufficient (Section 4.2.3). Low levels of transcripts were detectable (Section 4.2.4) and were consistent with the GCMŚ results (results not shown-similar to results obtained in Chapter 3) which indicated low levels (low signal strength) of analytes (δ-guaiene, α- humulene, α-guaiene and β-caryophyllene). It is important to note that, despite the low band intensity and small band size (approximately 150bp), the band should not be confused as primer dimer since control samples (without cDNA) did not show the formation of such bands. However, due to the low expression levels, it was not possible to detect any correlation between gene expression and the amount of analyte (GCMŚ-Total peak area) present in the sample. Consequently this study continued by investigating the gene expression

levels of sesquiterpene synthases in cell suspensions of *A. malaccensis* subjected to administered chemical stress (Chapter 5).

4.5 Conclusion

By developing and optimising molecular protocols, ACL 154 can be used to study gene expression patterns related to the synthesis of sesquiterpenes using a semi-quantitative approach.

CHAPTER 6

Summary and Concluding Remarks

The work reported in this thesis focused on the development and application of plant cell culture techniques to increase our understanding on the production of agarwood specifically in A. *malaccensis*. The establishment of cell cultures using shoot and leaf segments was done with the intention of establishing a model system which would enable us to study the chemical profile in cells, with a focus on the production of fragrance associate molecules namely sesquiterpenes and chromones. Also molecular studies were conducted to explore the possibility of establishing a linkage between gene expression patterns with the chemical profiles obtained.

In the last decade, several *Aquilaria* species namely *Aquilaria sinensis* (Qi *et al*., 2005) and *Aquilaria crassna* (Ito *et al*., 2005) have been introduced into culture with intentions to understand the fundamentals of agarwood production. To date it is generally acknowledged that the production of agarwood in a mature tree is initiated by physiological disturbance which is associated with wounding or insect attacks (Ng *et al*., 1997), which is followed by colonisation of the exposed tissues by naturally occurring microbes (bacteria & fungi). As a response to the stress of wounding and microbial infection, *Aquilaria* trees would activate their defence system triggering the production of a unique type of resin which is high in volatile organic compounds that aid in suppressing and localising the infected area (Nobuchi & Siripatanadilok, 1991). However to date, the detailed mechanism and factors which influence agarwood production remain unclear resulting in the absence of an alternative agarwood source (besides natural population).

In this study, cell suspension lines were introduced using *in vitro* grown *Aquilaria malaccensis* plantlets. Leaves and shoots were cultured on semi-solid media supplemented with selected hormones with desired levels. It was found that it was necessary to continuously subculture the callus (every 4 weeks) and to remove the older brown callus at every subculture interval in order to maintain continuous growth. A number of cell suspension lines were

developed from both leaf- and shoot-derived callus. But upon further optimisation, it was found that the leaf-derived callus (CS11) resulted in the establishment of a more homogenous cell suspension culture which, was deemed as a critical criteria when establishing cell suspension lines. Optimisation of culture conditions at early stage of cell suspension culture establishment indicated that agitation of 100 rpm (or higher) was not suitable, as this caused sheering of cells. Therefore the agitation speed was reduced to 75rpm. Cultures were maintained by sub culturing at an interval of 6 weeks using an optimum of 5ml of cells (settled cell volume) per 50 ml of liquid medium. The use of 2.3µM 2,4-D and 2.2µM BAP was found necessary to sustain active cell division in these lines.

Besides the establishment of stable cell suspension lines, the development and optimisation of analytical techniques was deemed to be an important element in this study. The use of a relatively new technology known as solid phase micro extraction technique in plant culture systems created new opportunity to detect and study the presence of fragrance molecules specifically sesquiterpenes and chromones despite being present in low concentration. As chromones and sesquiterpenes belong to different classes of molecules the development of two different analytical methods namely a SPME-LC-MŚ system for chromones and HS-SPME-GC-MŚ system for sesquiterpenes was deemed necessary.

Optimisation of both analytical systems were vital for the extraction and detection of the fragrance compounds. The biochemical profile of the cell culture was found to be different from the profiles reported for cell cultures of *Aquilaria crassna* (Kumeta & Ito, 2009) and *Aquilaria sinensis* (Qi *et al*., 2005). Six sesquiterpenes (alpha humulene, delta guaiene, beta caryophyllene, alpha guaiene, alpha elemene and beta selinene) and four chromones (6-methoxy-2-(2-phenylethyl)- chromone, 5,8-dihydroxy-2-(2-(4-methoxyphenylethyl) chromones,7-hydroxy-2-(2-phenylethyl]-chromone and 6-methoxy-2-

[2-(3-methoxyphenyl) ethyl] chromones) was found in cell suspension. All the fragrance molecules were found in low concentrations in unstressed cell cultures.

Although the effect of stress on chromones was not studied, the presence of MEJA, SA and ethanol was found to increase the production of sesquiterpenes. The correlation between the increases in sesquiterpenes production in relation to sesquiterpenes synthase expression was also explored in a preliminary study done using the ACL 154 primer where, the increase in alpha humulene production was found to correlate with an increase in delta guaiene synthase activity, suggesting that delta guaiene synthase maybe responsible for alpha humulene production in *Aquilaria malaccensis*.

Moving forward, the techniques developed in this study would enable further analysis on the effect of different stress factors on the production of fragrance compounds in agarwood. As agarwood price increases and wild population of *Aquilaria* continues to decline, there is an urgent need for the understanding of biochemical pathway changes which contribute to the establishment of agarwood in *Aquilaria* species.

In conclusion, the results of the current study advance the use of plant cell and tissue culture techniques together with existing analytical techniques applicable to *Aquilaria* species as part of the efforts to understand the mechanism behind the induction and production of agarwood.

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Appendices

Appendix A - Plant Cell and Tissue culture

Table 2.3. Analysis of variance for the effect of varying phytohormone concentration on the formation of callus on shoot tips.

Treatment	Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
2,4 D + BAP (Treatment A)	Overall Treatment	295.568	24	12.315	78.944	.000**
	2,4D	173.168	4	43.292	277.513	.000**
	BAP	105.488	4	26.372	169.051	.000**
	2,4D x BAP	16.912	16	1.057	6.776	.000**
NAA + Kinetin (Treatment B)	Overall Treatment	295.568	24	12.315	78.944	.000**
	NAA	173.168	4	43.292	277.513	.000**
	Kinetin	105.488	4	26.372	169.051	.000**
	NAA x Kinetin	16.912	16	1.057	6.776	.000**

** Significant to 1% level ; *significant at 5% level ; ^{ns} not significant

Table 2.4. Analysis of variance for the effect of varying phytohormone concentration on the formation of callus on leaves.

Treatment	Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
2,4 D + BAP	Overall Treatment	295.568	24	12.315	78.944	.000**
	2,4D	173.168	4	43.292	277.513	.000**
	BAP	105.488	4	26.372	169.051	.000**
	2,4D x BAP	16.912	16	1.057	6.776	.000**
NAA + Kinetin	Overall Treatment	86.400	24	3.6	18.578	.000**
	NAA	44.32	4	11.08	57.179	.000**
	Kinetin	27.68	4	6.92	35.711	.000**
	NAA x Kinetin	14.4	16	0.9	4.644	.000**

** Significant to 1% level ; *significant at 5% level ; ^{ns} not significant

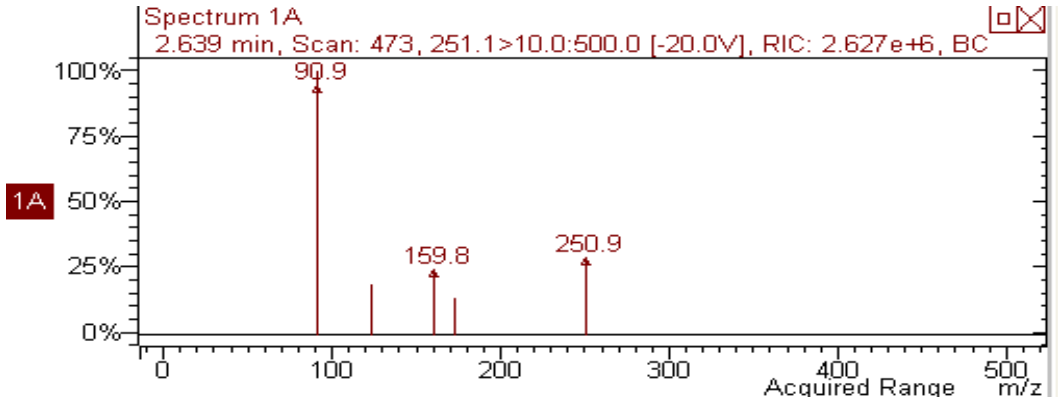
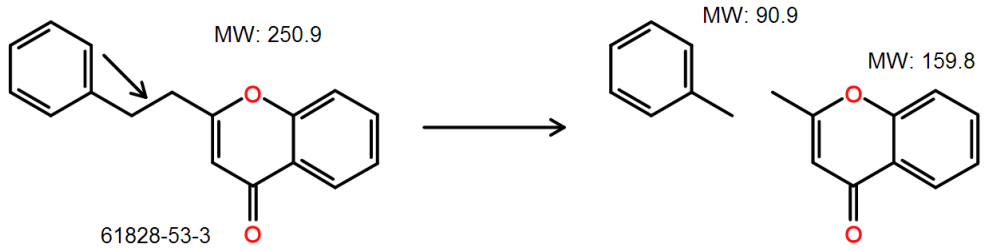
Table 2.5. Analysis of variance on the effect of varying phytohormone concentration on callus proliferation.

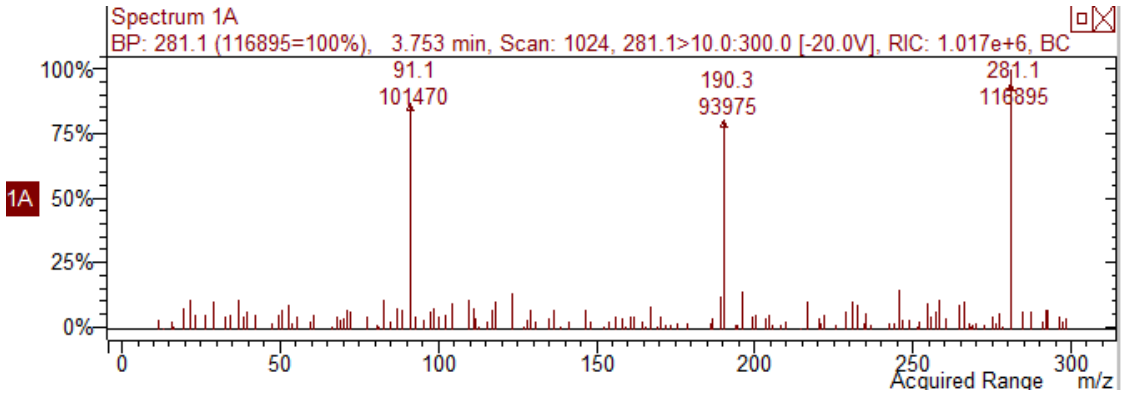
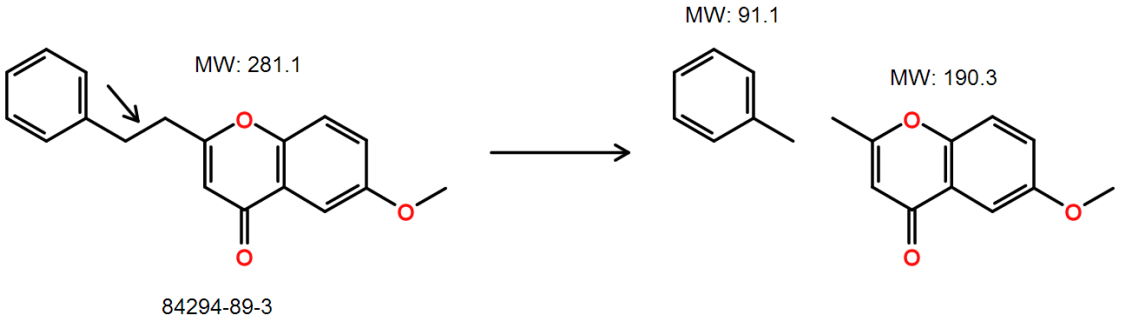
Dependant Variable	Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
First Subculture	Overall Treatment	24.018	15	1.601	2.418	.018**
Second Subculture	Overall Treatment	8.836	8	1.104	1.543	.211ns
Third Subculture	Overall Treatment	41.032	8	5.129	7.668	.000**

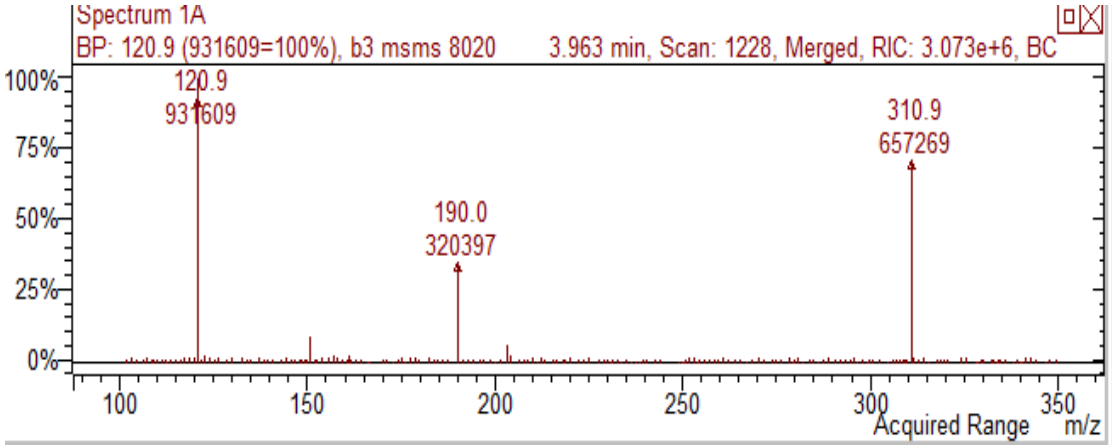
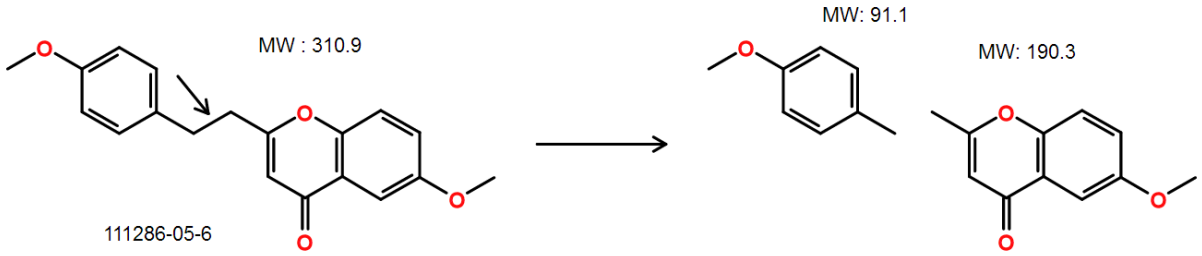
**significant at 1% level *significant at 5% level ns not significant

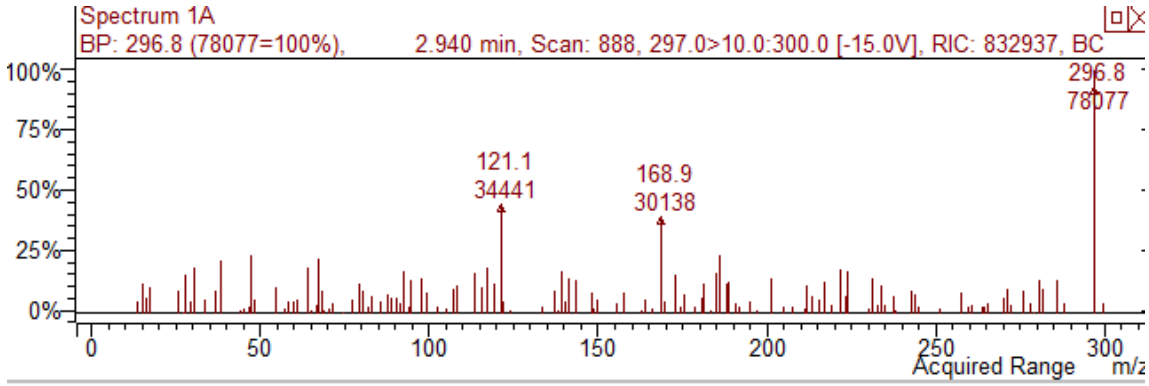
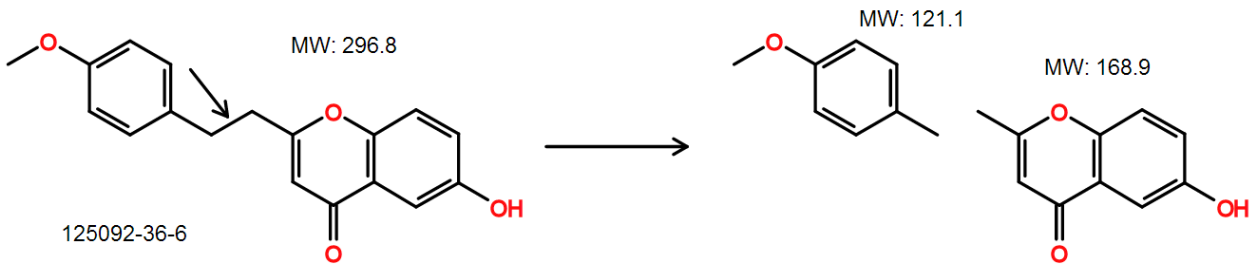
Appendix B – Chemical Analysis

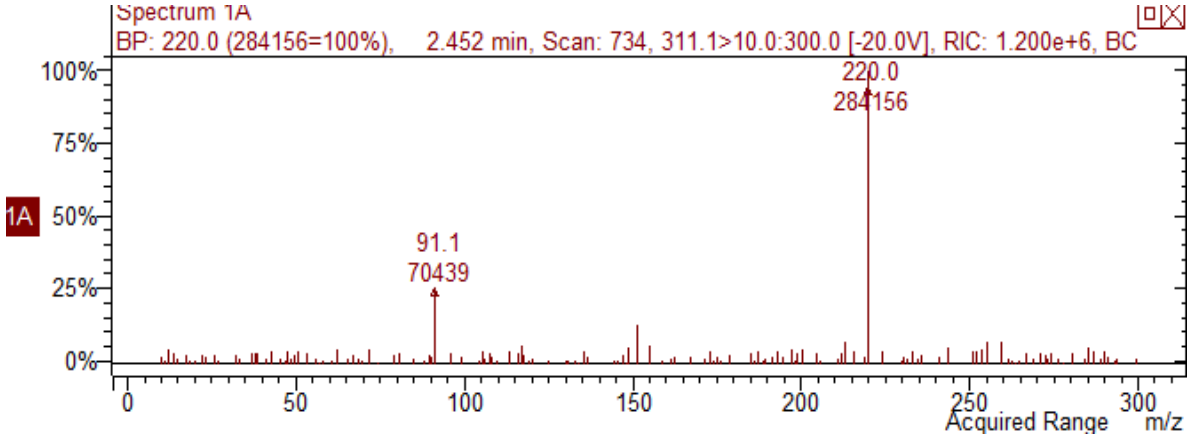
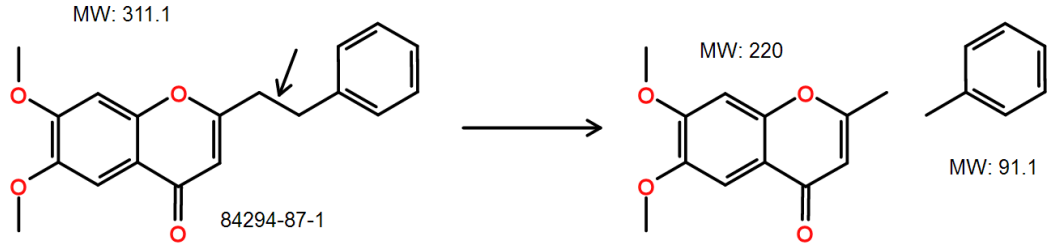
Table 3.15 LC-ESI-MŚ/MŚ chromatogram and fragmentation pattern of chromones present in resinous tissue of *Aquilaria malaccensis*

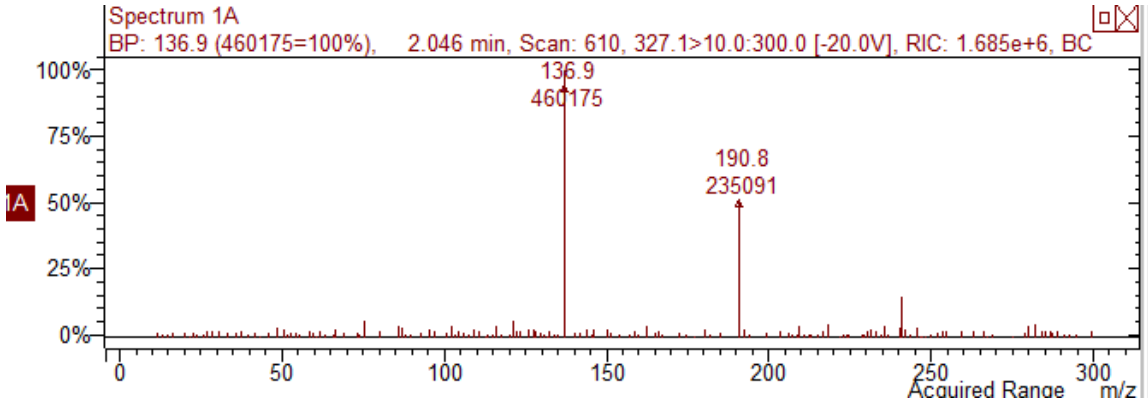
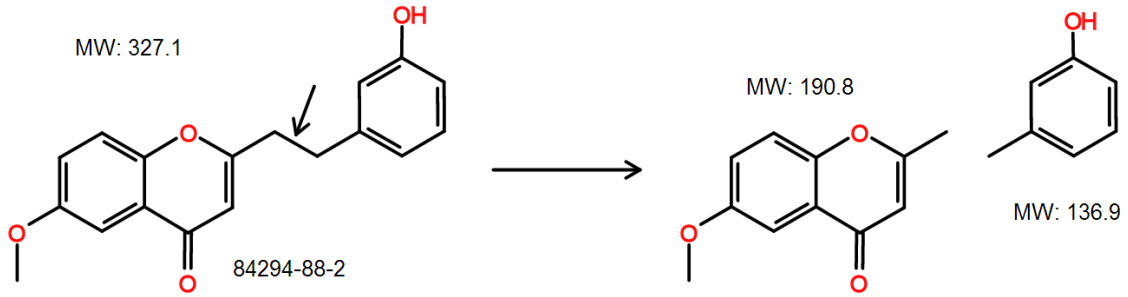
1.	Fraction ID : A8 CAS Number : 61828-53-3 Name : 2-(2-phenylethyl)- chromone MW : 250.9 Daughter Ions Detected : 90.9 ; 159.8	 

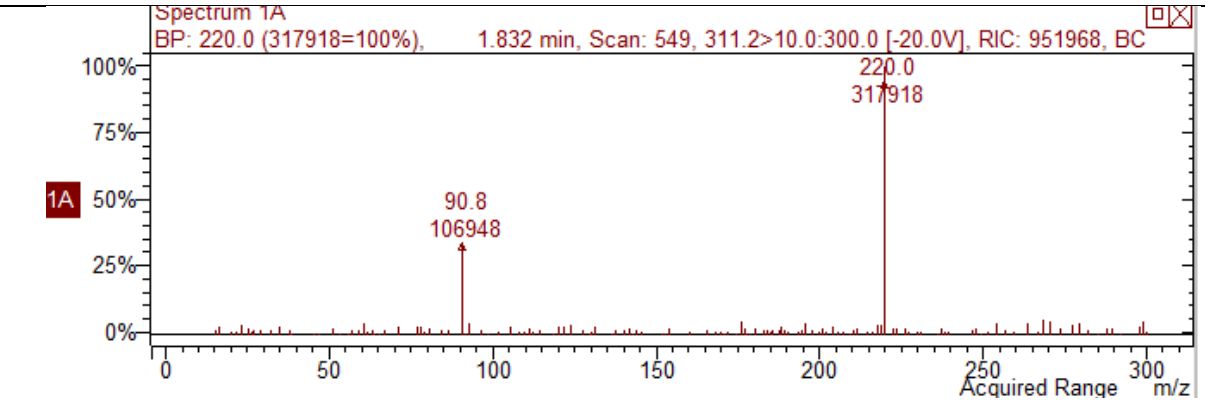
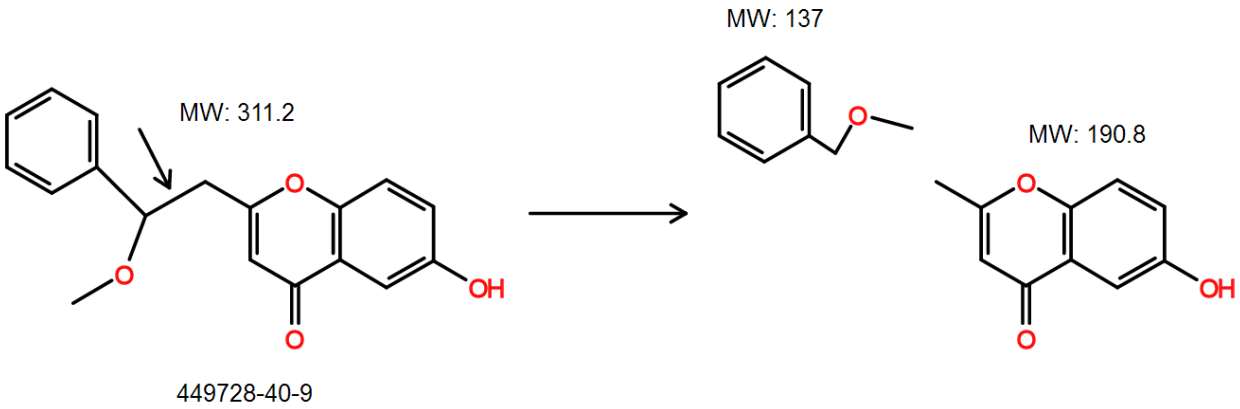
2.	Fraction ID : B2 CAS Number : 84294-89-3 Name : MW : 280.9 Daughter Ions Detected : 94.7 & 189.4	 

3.	Fraction ID : B3 CAS Number : 111286-05-6 Name : 6-methoxy-2[2-(3-methoxyphenyl)ethyl]-chromone MW : 310.9 Daughter Ions Detected : 94.7 & 189.4	 

4.	Fraction ID : B9-10 CAS Number : 125092-36-6 Name : 5-Hydroxy-6methoxy-2-(2-phenyethyl)- chromone MW : 296.8 Daughter Ions Detected : 121.1 ; 168.9	 

5.	Fraction ID : B9-10 CAS Number : 84294-87-1 Name : 6,7-dimethoxy-2-[2-phenyl) ethyl] chromones MW : 311.1 Daughter Ions Detected : 91.1 ; 220	 

6.	Fraction ID : B9-10 CAS Number : 84294-88-2 Name : 6-methoxy-2-[2-(3-methoxy-4-hydrophenyl) ethyl] chromones MW : 327.1 Daughter Ions Detected : 190.8 ; 136.9	 

7.	Fraction ID : E1-E10 CAS Number : 449728-40-9 Name : 6-methoxy-2-[2-(3-methoxy-4-hydroxyphenyl)ethyl]chromone MW : 311.2 Daughter Ions Detected : 220 ; 90.8	  449728-40-9

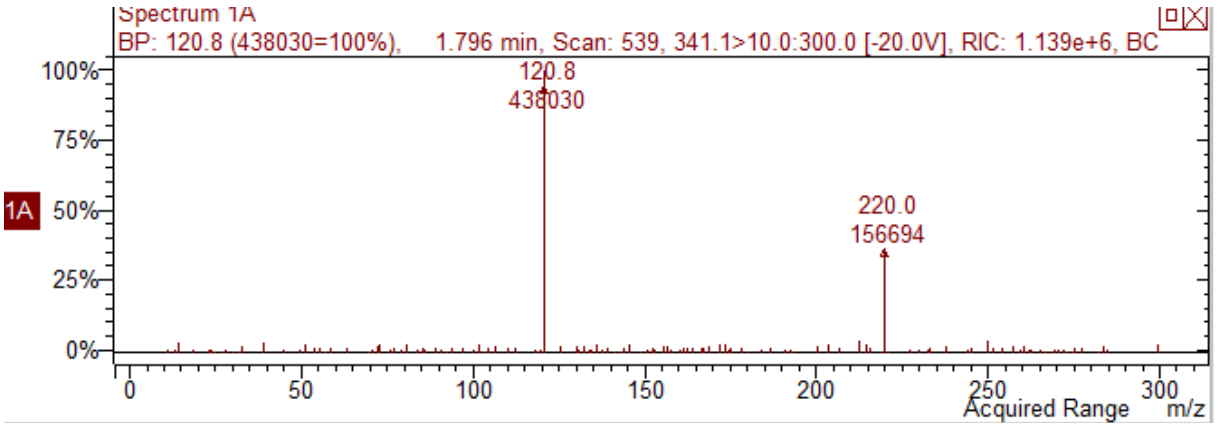
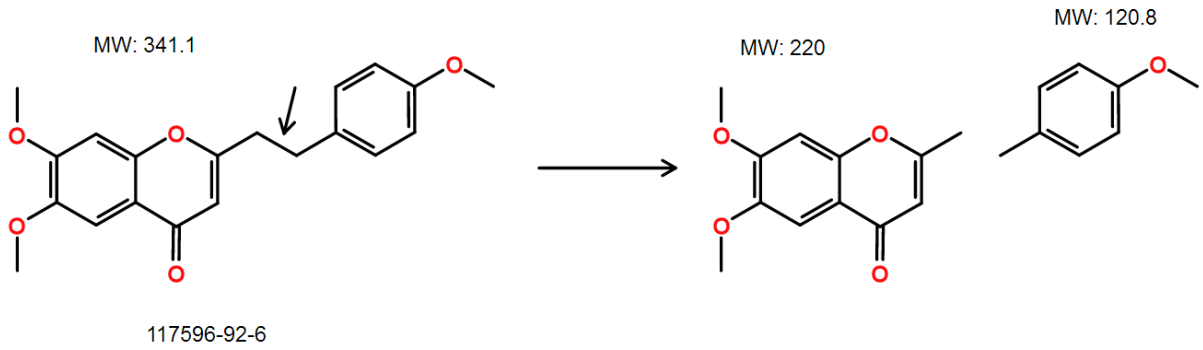
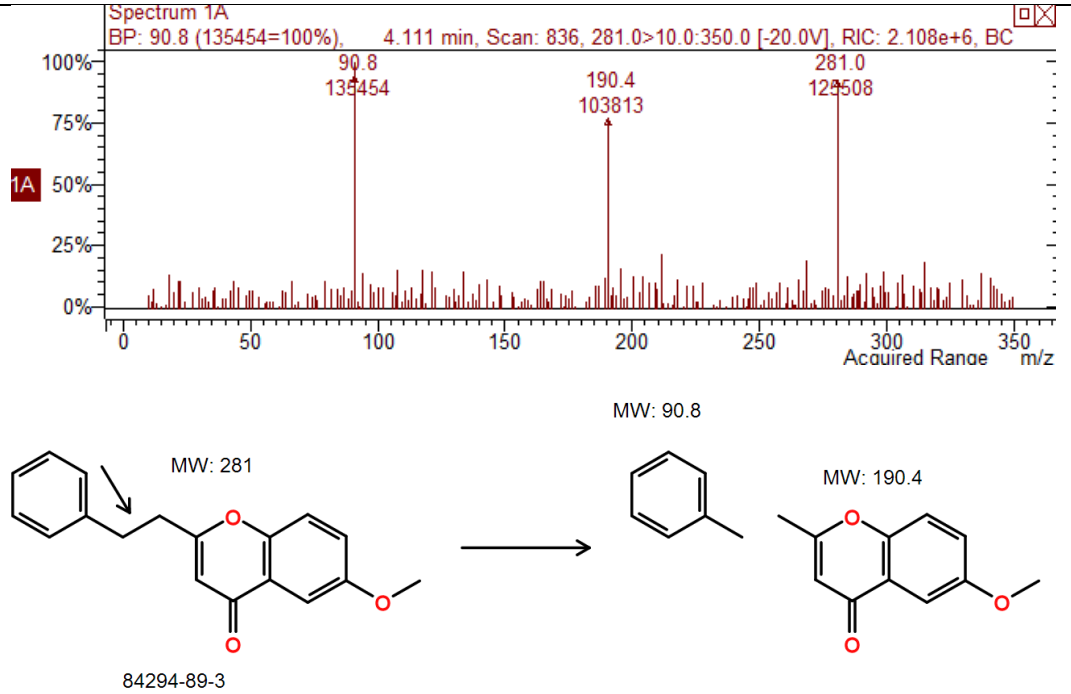
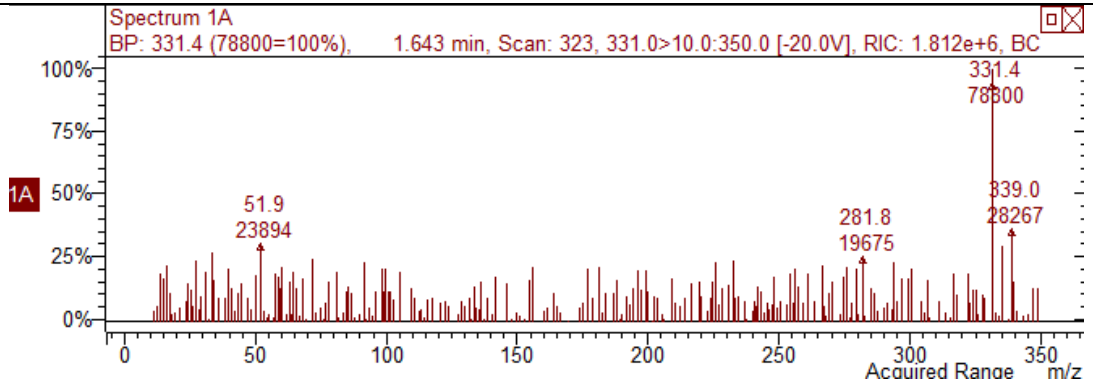
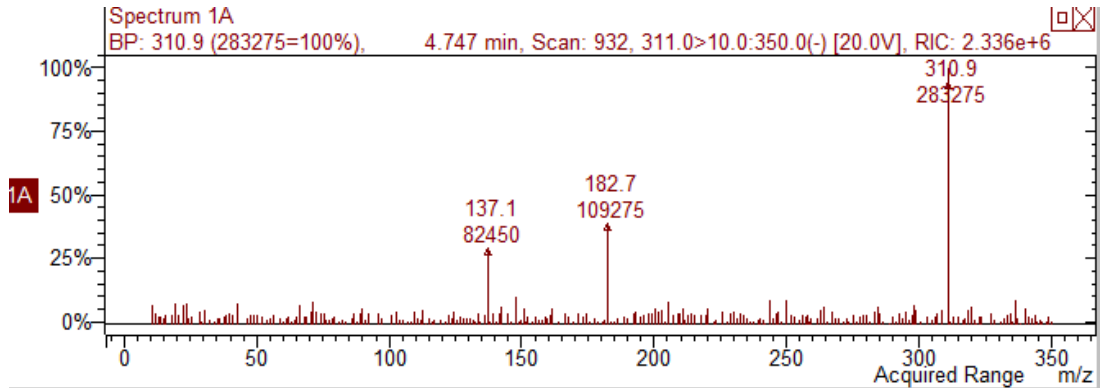
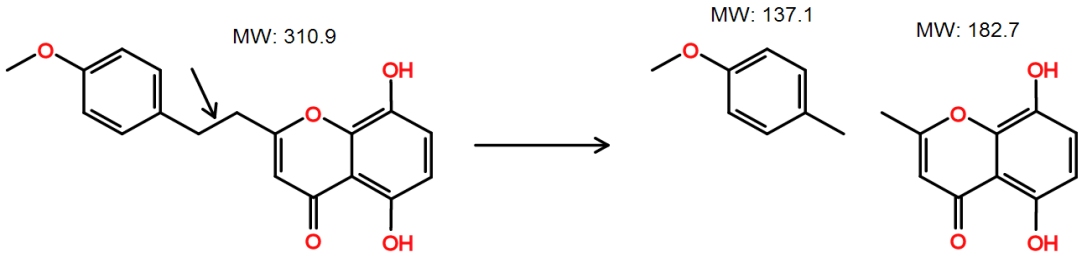
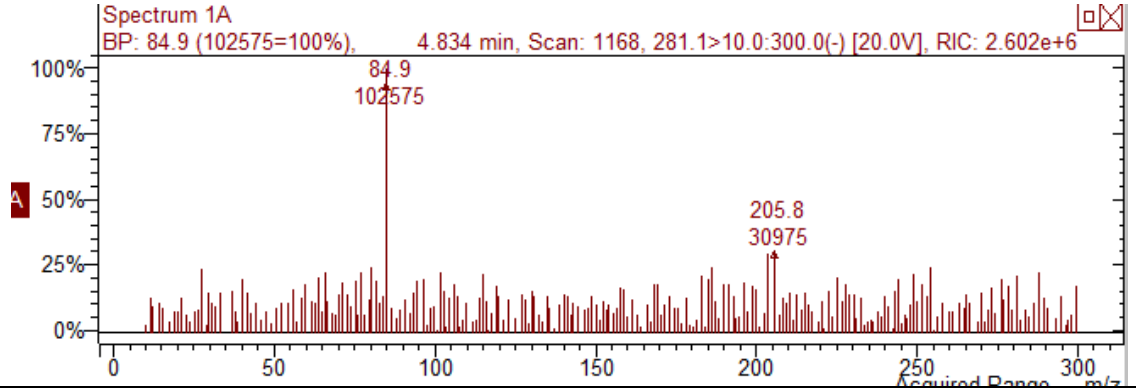
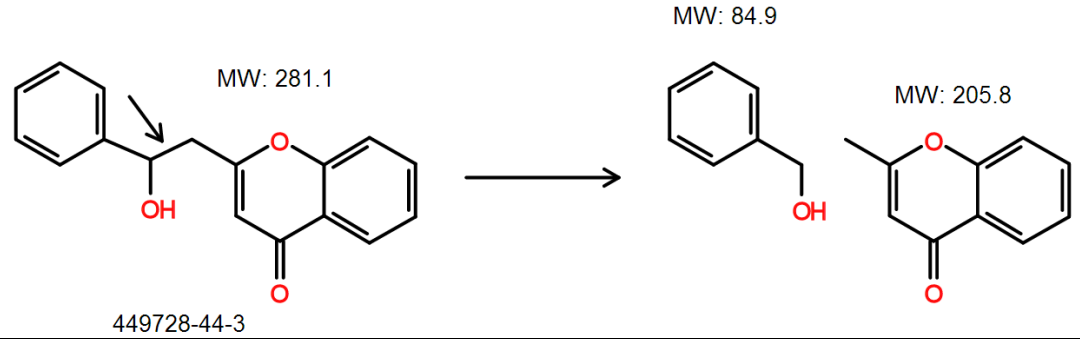
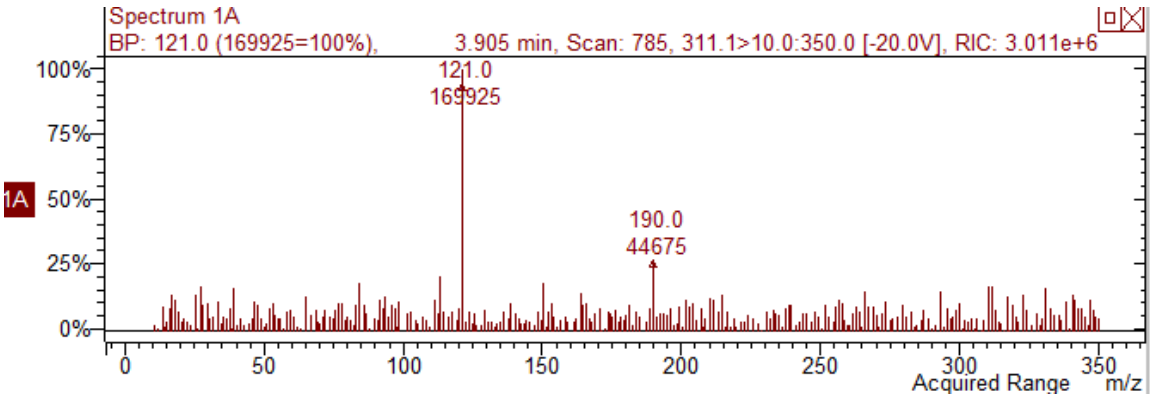
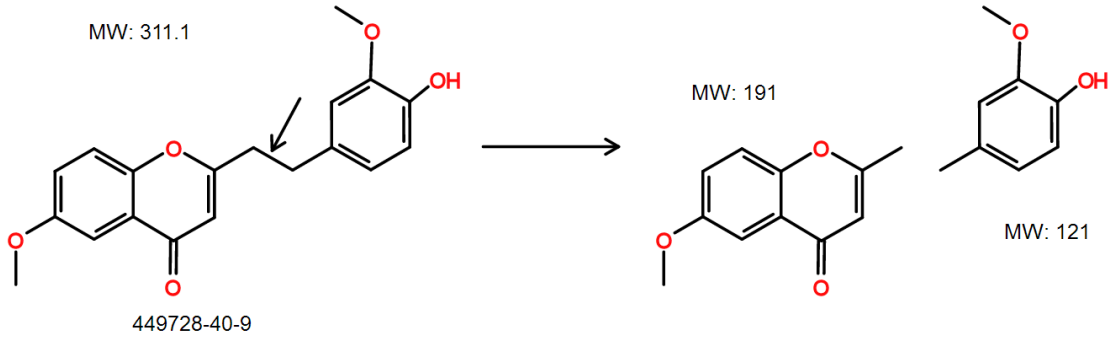
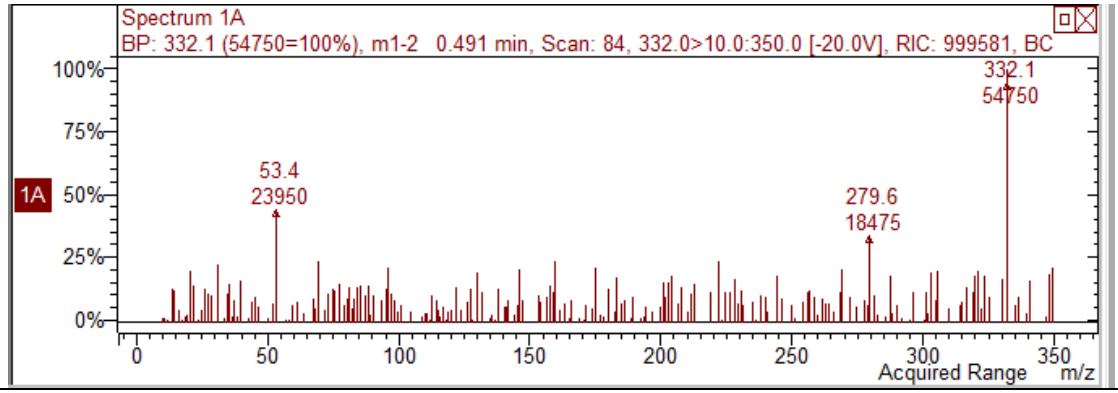
8.	Fraction ID : E1-E10 CAS Number : 117596-92-6 Name : 6,7-dimethoxy-2-[2-(4methoxyphenyl)ethyl]-chromones MW : 341.1 Daughter Ions Detected : 220 ; 120.8	 

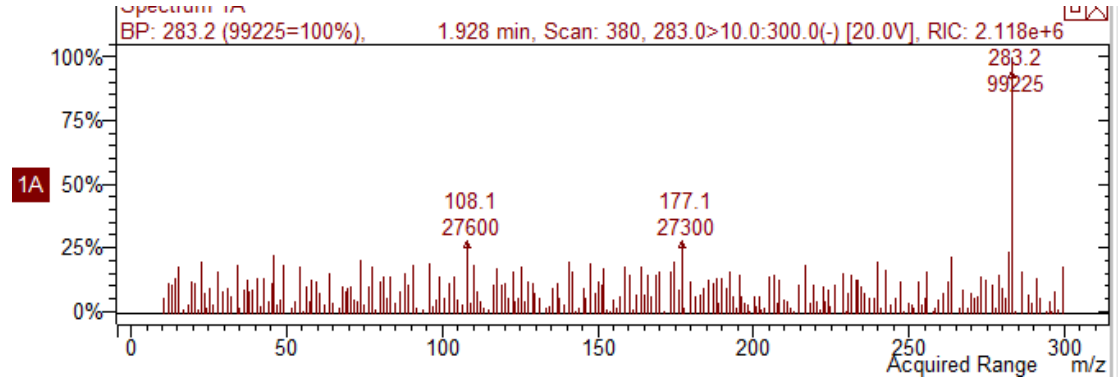
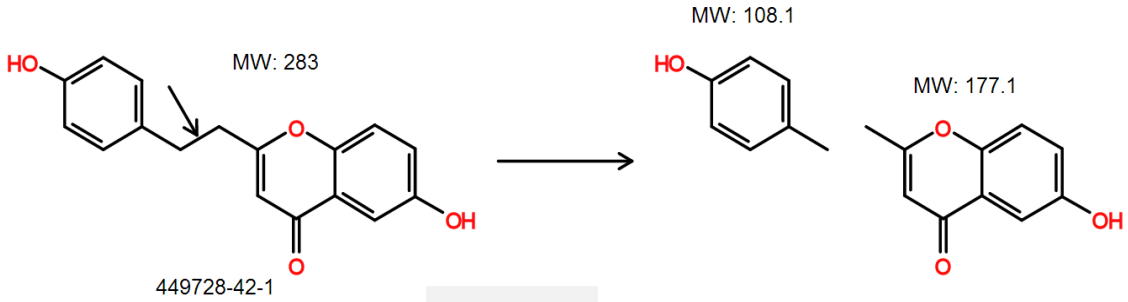
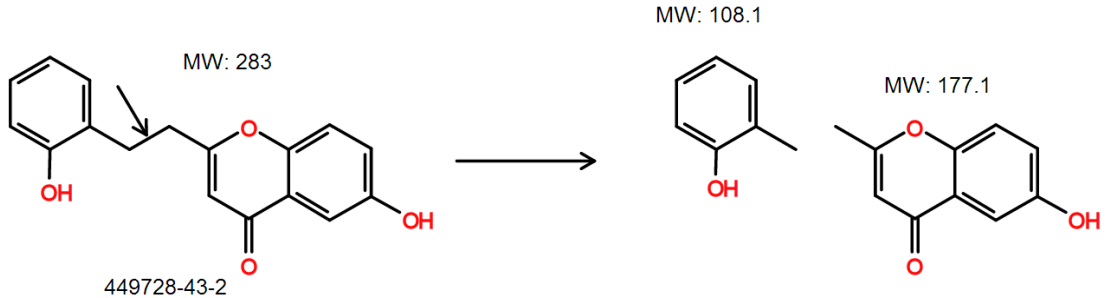
Table 3.16 LC-ESI-MŚ/MŚ Chromatogram and Fragmentation pattern of chromones present in cell suspensions of *Aquilaria malaccensis*.

1.	Sample ID : Control R1 CAS Number : 84294-89-3 Name : 6-methoxy-2-(2-phenylethyl)chromone MW : 281.0 Daughter Ions Detected : 190.4 ; 90.8	
2.	Sample ID : Control R1 CAS : **Unknown** Name : **Unknown** MW : 331.4 Daughter Ions Detected : 281.8 ; 51.9	

3.	Sample ID : Control R1 CAS : 128922-70-3 Name : 5,8-Dihydroxy-2-(2-(4-methoxyphenyl)ethyl)chromone MW : 310.9 Daughter Ions Detected : 182.7 ; 137.1	  128922-70-3
4.	Sample ID : Control R2 CAS : 449728-44-3 Name : 7-hydroxy-2-(2-phenylethyl)chromone MW : 281.1 Daughter Ions Detected : 205.8 ; 84.9	

		
5.	Sample ID : Control R2 CAS : 449728-40-9 Name : 6-methoxy-2-[2-(3-methoxy-4-hydroxyphenyl)ethyl]chromone MW : 311.1 Daughter Ions Detected : 191; 121	

		
6.	Sample ID : Control R3 CAS : **Unknown** Name : **Unknown** MW : 331.4 Daughter Ions Detected : 281.8 ; 51.9	

7.	Sample ID : Control R3 MW : 331.4 Daughter Ions Detected : 281.8 ; 51.9 CAS : 449728-42-1 Name : 7-hydroxy-2-(2-phenylethyl)chromones **OR** CAS : 449728-43-2 Name : 7-hydroxy-2-(2-phenylethyl)chromone	  **OR** 

Appendices C – Molecular analysis

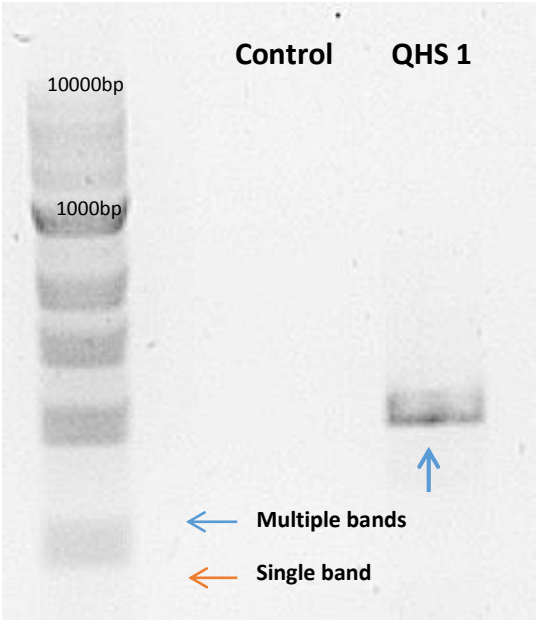


Figure 4.6 Gel electrophoresis image of QHS 1 amplicon using 2% SYBR® stained agarose gel.

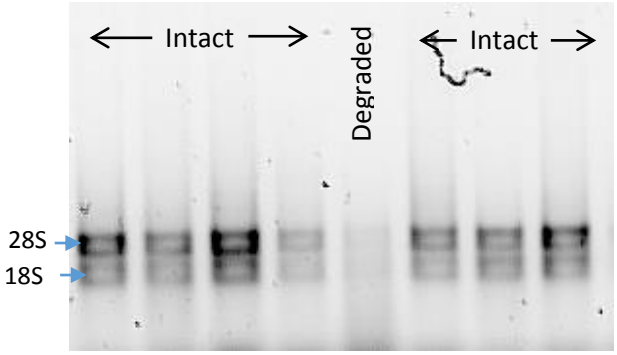


Figure 4.11 Example of a gel electrophoresis image of RNA extracted from multiple samples prior to RNA quantification using 0.8% SYBR® stained agarose gel.