UNIVERSITI PUTRA MALAYSIA

ENHANCEMENT OF KEY CHEMICAL CONSTITUENTS IN Aquilaria malaccensis LAMARCK (KARAS) THROUGH In Vitro POLYPLOIDIZATION

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Doctor of Philosophy

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Chairman : Associate Professor Norihan binti Mohd. Saleh, PhD
Faculty : Biotechnology and Biomolecular Sciences

Aquilaria malaccensis is a highly valued timber species in Malaysia for its ability to produce fragrant resin known as agarwood. The agarwood consists of volatile chemical (sesquiterpenoid groups and phenyl ethyl chromones derivatives), reported to be triggered as a defence mechanism due to attacks by microorganisms at the wounded areas. In the natural forest, only up to 10% of the wild *A. malaccensis* were found to be able to produce agarwood. Malaysia is currently facing threats of *A. malaccensis* extinction due to illegal harvesting of the species which have caused this species to be listed in CITES (Appendix II). In view of this situation, there is a need to develop *A. malaccensis* clones with enhanced chemical constituents for commercial plantation. The *in vitro* polyploidization has been proven to improve desired characteristics in plant such as fast growing, increased in bioactive compounds and secondary metabolites. Therefore, the objectives of this study were to develop *in vitro* polyploidization protocol in *A. malaccensis*; to determine the morphological characteristics difference between the polyploids to its diploid counterparts and; to evaluate and compare the chemical constituents in the different ploidy levels of *A. malaccensis*. The *A. malaccensis in vitro* polyploidization was carried out using antimitotic agents (colchicine and trifluralin) at different concentrations and exposure times on two types of explants (shoot tip and nodal segment). The ploidy levels of the *in vitro* induced polyploid plantlets were determined using flow cytometer, chromosome count and stomata size measurement. Diploid plantlets were used as reference. The plant height, leaf length and leaf width were also measured in tetraploids and diploids plantlets. The chemical profiling of the *in vitro* induced polyploids and diploids plantlets were evaluated using the Headspace-Solid Phase Microextraction (HS-SPME) and hydro distillation, HD (for essential oil extraction, if any);both coupled with gas chromatography-mass spectrometry (GC/MS).The experiment showed that *A. malaccensis* polyploids can be induced with tetraploids obtained using nodal segments treated with 0.1 mM trifluralin for 120 hours. However,

other treatments were only able to induce mixoploids in shoot tip (treated with 1 mM colchicine at 24 and 120 hours exposure time; 2 mM colchicine at 48 hours exposure time; while 0.05 mM and 0.1 mM trifluralin both at 120 hours exposure time) and nodal segment (0.05 mM trifluralin at 120 hours exposure time). The DNA content and genome sizes were quantified as 1.84 pg $2C^{-1}$ and 899 Mbp in the diploid and, 3.86 pg $2C^{-1}$ and 1887 Mbp in the tetraploids. *A. malaccensis* chromosome number was determined to be *2x*=14 (diploids), and *4x*=28 (tetraploids). The tetraploids showed larger stomata guard cell sizes (33.3±0.6 µm) in leaves as compared to diploids (23.1±0.5µm). Plant height of 24 months old tetraploids were 49±0.05 cm, more than double the height of diploids, stem diameter of tetraploids were 0.7±0.02 cm compared to 0.5±0.02 cm in diploids, the leaf area also doubled in size compared to diploids, with 24.07±0.04 cm^2 in tetraploids leaf sample. Chemical profiling of four different sources were compared: seedling grown under normal conditions, seedling grown *in vitro*, *in vitro* diploids and *in vitro* tetraploids. Through HS-SPME/GCMS, highest amount of important sesquiterpenes (volatile chemical constituents in agarwood oil from mature trees) such as α-eudesmol and α-guaiene, was found in stem and root samples of *in vitro* tetraploids. The HD-GC/MS method showed all samples did not contain essential oil. However, 60% of the hydrosol water from leaves and 49.1% of the residual water from root samples of *in vitro* tetraploids consists of important sesquiterpenes. Further evaluation in *A. malaccensis* diploid and tetraploid plants at 24 months old showed fewer amounts of chemical constituents than the 4 months old in all plant parts. The tetraploids root however, contains important sesquiterpene, α-guaiene (2.92%), which was not detected in diploids. These results demonstrated that *A. malaccensis* polyploid plantlets can be induced for plant improvement. The *A. malaccensis* tetraploids contained higher chemical constituents which can be promoted as high quality *A. malaccensis* clones for commercial plantation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENINGKATAN KONSTITUEN KIMIA MELALUI PENGHASILAN POLIPLOIDI *Aquilaria malaccensis* Lamarck (KARAS) SECARA *In Vitro*

Oleh

SITI SUHAILA BINTI A. RAHMAN

Mac 2017

Pengerusi : Profesor Madya Norihan binti Mohd. Saleh, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Aquilaria malaccensis adalah spesies kayu bernilai tinggi di Malaysia kerana kebolehupayaannya menghasilkan resin wangi dikenali sebagai gaharu, yang digunakan secara tradisional dan industri terapeutik/aromatik moden. Gaharu ini terdiri daripada bahan kimia meruap (kumpulan sesquiterpenoid dan olahan kromon phenyl etil), dilaporkan akan terhasil sebagai mekanisme pertahanan terhadap serangan oleh mikroorganisma di kawasan cedera pada batang pokok. Di dalam hutan semula jadi, hanya lebih kurang 10% daripada *A. malaccensis* liar mampu menghasilkan gaharu. Malaysia kini menghadapi ancaman kepupusan *A. malaccensis* ekoran daripada pengambilan gaharu secara haram yang telah mengakibatkan spesies ini disenaraikan dalam CITES (Lampiran II). Keadaan ini telah mewujudkan keperluan untuk menghasilkan klon *A. malaccensis* dengan konstituen kimia yang dipertingkatkan untuk tujuan perladangan komersial. Poliploidi telah terbukti mampu meningkatkan ciri-ciri yang dikehendaki dalam tumbuhan seperti tumbesaran yang lebih cepat, peningkatan sebatian bioaktif dan metabolit sekunder. Oleh itu, objektif kajian ini adalah untuk membangunkan protokol menghasilkan *A. malaccensis* poliploidi secara *in vitro*; untuk menentukan perbezaan ciri-ciri morfologi antara anak pokok poliploid dengan diploid dan; untuk menilai dan membandingkan konstituen kimia dari peringkat ploidi *A. malaccensis* yang berbeza. Protokol poliploidi secara *in vitro* telah dibangunkan menggunakan ejen antimitotik (colchicine dan trifluralin) pada kepekatan dan masa pendedahan yang berbeza ke atas dua jenis eksplan (pucuk dan segmen nod). Tahap ploidi anak pokok poliploid *in vitro* ditentukan dengan menggunakan kaedah aliran sitometri, bilangan kromosom dan pengukuran saiz stomata. Anak pokok diploid digunakan sebagai rujukan. Ketinggian anak pokok, panjang daun dan lebar daun diukur dalam anak pokok tetraploid dan diploid. Pemprofilan kimia terhadap anak pokok tetraploid dan diploid dinilai menggunakan kaedah *Headspace-Solid Phase Microextraction (HS-SPME)* dan hidro penyulingan, HD (untuk minyak gaharu, jika ada); kedua-duanya dinilai dengan kaedah gas kromatografi-spektrometri jisim (GC/MS). Eksperimen menunjukkan bahawa *A. malaccensis* poliploidi boleh

diaruhkan dengan terhasilnya tetraploid melalui rawatan segmen nod dengan 0.1 mM trifluralin selama 120 jam. Beberapa rawatan yang lainnya juga menghasilkan poliploidi pada peringkat mixoploidi pada eksplan pucuk (dirawat dengan 1 mM colchicine pada 24 and 120 jam masa pendedahan; 2 mM colchicine pada 48 hours pendedahan masa; 0.05 mM dan 0.1 mM trifluralin kedua-duanya pada 120 masa pendedahan) dan segmen nod (0.05 mM trifluralin pada 120 jam masa pendedahan). Kandungan DNA dan saiz genom adalah 1.84 pg $2C^{-1}$ dan 899 Mbp dalam diploid dan, 3.86 pg $2C^{-1}$ dan 1887 Mbp dalam tetraploid. *A. malaccensis* mempunyai bilangan kromosom *2x* = 14 (diploid), dan *4x* = 28 (tetraploid). Daun tetraploid menunjukkan saiz pengawal sel stomata lebih besar (33.3 ± 0.6 µm) berbanding diploid (23.1 ± 0.5 µm). Ketinggian pokok tetraploid berusia 24 bulan adalah 49.0 ± 0.05 cm, lebih daripada dua kali ganda ketinggian pokok diploid, diameter batang tetraploid adalah 0.7 ± 0.02 cm, berbanding 0.5 ± 0.02 cm diploid. Keluasan daun juga adalah dua kali ganda berbanding saiz pada diploid, di mana sampel daun tetraploid adalah 24.07 ± 0.04 cm^2. Perbandingan profil kimia dilaksanakan terhadap empat sumber yang berbeza: anak benih ditanam di bawah keadaan biasa, anak benih ditanam secara *in vitro*, diploid *in vitro* dan tetraploid *in vitro*. Melalui kaedah *HS-SPME/GCMS*, jumlah sesquiterpene penting ditemui paling tinggi di dalam batang dan akar sampel tetraploid *in vitro*. Kaedah *HD-GC/MS* menunjukkan semua sampel tidak mengandungi minyak pati. Walau bagaimanapun, 60% daripada air hydrosol dari daun dan 49.1% daripada air sisa daripada sampel akar dalam tetraploid *in vitro* terdiri daripada sesquiterpene penting. Penilaian lanjut ke atas *A. malaccensis* diploid dan tetraploid pada umur 24 bulan menunjukkan jumlah konstituen kimia yang lebih sedikit daripada pada umur 4 bulan di semua bahagian pokok. Walau bagaimanapun, akar tetraploid mengandungi sesquiterpene penting, α-guaiene (2.92%), yang tidak dikesan dari akar diploid *in vitro*.Hasil kajian ini menunjukkan bahawa anak pokok *A. malaccensis* polyploid boleh diaruh untuk penambahbaikan kandungan konstituen kimia yang lebih tinggi yang boleh dipromosikan sebagai klon *A. malaccensis* berkualiti tinggi untuk perladangan komersial.

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I certify that a Thesis Examination Committee has met on 24 March 2017 to conduct the final examination of Siti Suhaila bt. A. Rahman on her thesis entitled "Enhancement of Key Chemical Constituents in *Aquilaria malaccensis* Lamarck (Karas) through *In Vitro* Polyploidization" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

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

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℃	temperature in degree Celsius
µl	microliter
BAP	6-benzylaminopurine
CITES	Conference on International Trades of Endangered Species
cm	centimeter
cm^2	centimeter square
DNA	dinucleic acid
g/L	gram per liter
GC/MS	Gas Chromatography-Mass Spectrometry
GPB	general purpose buffer
HD	hydro distillation
HS-SPME/GCMS	Headspace-Solid Phase Microextraction
IBA	indo-3-butyric acid
kg	kilogram(s)
kPa	kilo Pascal pressure
m	meter
mg	milli gram(s)
mg/ml	milligram per milliliter
mm	millimeter
mM	milli Molar
MTIB	Malaysian Timber Industry Board
N^{ns}	Normal seed grown under natural condition
pg $2C^{-1}$	DNA content in picogram per pair of chromosome
PGR	plant growth regulator
PI	propidium iodide
RM	Malaysian currency (Ringgits)
RNAse	ribonuclease enzyme
UAE	United Arab Emeritus
US	United States of America
V^{ns}	Normal seeds grown under *in vitro* condition

CHAPTER 1

INTRODUCTION

Aquilaria malaccensis is one of the agarwood-producing species which belongs to the Thymealaeceae family, distributed throughout Peninsular Malaysia, Indonesia, India, Vietnam and Thailand (Md. Salleh, 2010; Chang et al., 2002). *Aquilaria* species is best known for its distinguished and highly-valued fragrant resin (agarwood) used in incense making, in traditional medicines and perfumeries for hundreds of years, throughout the world (Nor Azah et al., 2013; Taha et al., 2010). The agarwood however, is not naturally produced. Its formation must be induced by wounding and/or infection by microbial attack (Dahlan, 2010; Lok and Yahya, 2010). Only up to10% of the trees are able to produce agarwood in the wild, either through natural (wounded by nature and infected) or mechanical (artificial inoculation) methods (Chang et al., 2002; Ng et al., 1997). The agarwood contain mainly volatile sesquiterpenes and methyl ethyl groups (Tajuddin and Yusoff, 2010; Nor Azah et al., 2008).

Among the *Aquilaria* genus, *A. malaccensis* received significant attention in the past few decades and is favored by consumers especially from the Middle East and European countries, where it is worth more than 5 million US dollars (Mohd. Yusoff, 2014). However, the increase in demand had led to diminishing supplies and uncontrollable market pricing which are driving some of these species into extinction, if proper regulations are not enforced (Hashim et al., 2010). Malaysia and other agarwood-producing countries such as India, Indonesia, Vietnam and Thailand are seriously considering agarwood plantation programs (Lok and Yahya, 2010). Commercial plantation of *A. malaccensis* seems to be a practical alternative towards increasing the volume of agarwood production. At the same time, illegal harvesting can be inhibited through these plantation programmes where more manageable operations are in place, providing sustainable supply of agarwood. A sustainable commercial plantation practices coupled with proven inoculation formulation and technique seems to work in reducing harvesting of *A. malaccensis* trees from the wild. However, the metabolisms and pathways involved in the formation of agarwood remains a challenge for researchers as they discover agarwood is not a uniform product as in other commodity crops. For example, the chemical constituents were found to be highly variable between species and intraspecies, even when treated with the same inoculants. The understanding of the chemical constituents synthesized at genomic level is still at preliminary stage and genes that are involved in the agarwood regulation are still unknown. Several attempts were made to synthetically manufacture the agarwood essential oil chemical structures and/or induce the chemical compounds of agarwood using elicitors (Okudera and Ito, 2009). Although there are some initial successes, the operations and scale-up may not be economically feasible for industrial scale, at this stage. Therefore, improving the planting materials and selection of elite clones for commercial plantation programs need to be addressed to fulfill the growing demand of the agarwood and agarwood-based products.

Polyploidization (whole-genome duplication) has played a pervasive role in the evolution of eukaryotic cells (Adams and Wendel, 2005; Abbot and Lowe, 2004). Plants as well as fungi and animals undergo polyploidization as part of the natural phenomenon of speciation and evolution processes (Tate et al., 2005; Soltis, 2005). It is an important mechanism with a profound impact on biodiversity dynamics, ecosystem functioning, as well as health and sosio-economic aspects (Adams and Wendel, 2005). Polyploids are frequently superior compared to their parent plants which over a long period of evolutionary time, increased the morphological complexity in plants and able to reduce the risk of species extinction (Soltis et al., 2004). The induction of artificial polyploidy has been proven to be useful, not only to enhance the morphological aspects (such as plant size, color intensity on flower petals and biomass) but also increasing the production of secondary metabolites and bioactive compounds, in important medicinal plants (Lavania et al., 2012; Gonzalez and Wheathers; 2003; Lavania, 1988). Even though it has been proven to be effective in producing superior polyploid lines in some cases, the increase in chromosome number through artificial polyploidization could inhibit or slow down the initial plant growth and development, but later showed better growth performances in comparison to the diploids (Oiyama and Okudai, 1986). There are many factors that play a role in polyploid induction, for example woody plant groups are harder to induce than herbaceous plants (Gamage et al., 2011), the response of explants towards antimitotic agents and concentrations used (Dhooghe et al., 2011; Allum et al., 2007). Therefore, factors such as plant groups, explants types, types and concentrations of the antimitotic agents, and exposure time, needed to be carefully assessed to determine the degree of success in a polyploidization induction study (Dhooghe et al., 2011).

In this study, the use of *in vitro* polyploidization for *A. malaccensis* for the improvement in morphological characteristics as well as enhancement of the amount of secondary metabolites produced are studied. The challenges with *A. malaccensis* being a woody plant, indicates considerably difficult to induce, and coupled with lack of information on the morphological and cytological aspects of *A. malaccensis*. Following the above considerations, this study has four major objectives; i.e. to:

1. Develop an *in vitro* polyploidization protocol for *A. malaccensis*
2. Compare the morphological characteristics in polyploids to their diploid counterparts
3. Conduct cytological study on *A. malaccensis* diploids and polyploids
4. Evaluate the key chemical constituents in *A. malaccensis* polyploids compared to the diploid counterparts

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