AQUILARIA MALACCENSIS POLYPLOIDS AS IMPROVED PLANTING MATERIALS

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SITI SUHAILA AR, NORIHAN MS, NORWATI M, NOR AZAH MA, MAHANI MC, PARAMESWARI N, KODI IK, MAILINA J, AZRINA A, NOR HASNIDA H, HALIZA I, NAZIRAH A & MUHD FUAD Y. 2015. *Aquilaria malaccensis* polyploids as improved planting materials. *Aquilaria malaccensis* is an agarwood-producing timber species used in many traditional remedies and modern therapeutic treatments and perfume industries. In this study, we aimed to enhance *A. malaccensis* phytochemical content through in-vitro polyploidisation. Shoot tip and nodal segment from 8-week-old in-vitro *A. malaccensis* plantlets were treated with different concentrations of colchicine and trifluralin at various exposure times to obtain polyploids. Tetraploid plantlets (10%) was obtained using nodal segment explants treated with 0.1 mM trifluralin at 120 hours. Chemical profiling of diploid and tetraploid samples (leaf, stem and root) was evaluated separately using headspace–solid phase microextraction (HS–SPME) combined with gas chromatograph mass spectrometry (GCMS). Phytochemical content increased in tetraploid, particularly in stem whereby the total phytochemical contents were 43.19% in tetraploid compared with 5.87% in diploid. The HS–SPME–GCMS analyses showed that tetraploid stem contained high levels of sesquiterpenoids found in agarwood oil such as α-eudesmol (18.3%), α-gurjunene (8.61%) and γ-gurjunene (6.22%). On the other hand, aromadendrene (2.49%) and α-humulene (3.38%) were detected in diploid samples. Tetraploid leaf samples were observed to contain α-humulene (3.79%) while diploid only contained (2E) tridecenol (19%). There were no significant differences between diploid and tetraploid in terms of total phytochemical content in root samples. Nevertheless, high sesquiterpenoid content, γ-gurjunene (14.0%), was detected in tetraploid sample while γ-muurolene (2.96%), in diploid. α-Guaiene content was higher in root samples of diploid (6.49%) than tetraploid (1.09%). These results demonstrated that tetraploid plantlets led to higher yield of total phytochemical content and might facilitate production of high quality *A. malaccensis* clones.

Keywords: Polyploidisation, colchicine, trifluralin, phytochemical content

INTRODUCTION

Aquilaria malaccensis is an agarwood-producing timber species which belongs to the family Thymeleaeceae. It is distributed mainly in Asia such as Malaysia, Indonesia, India, Thailand and Cambodia (Norwati 2000, Kassan 2013). The species produces secondary metabolites known as agarwood as defence mechanism against pathogens in wounded bark (Hashim et al. 2010, Taha 2010, Kassan 2013). Agarwood has had a very long history of use in traditional medicine as incense as well as aromatic and therapeutic oils. The quality of agarwood is based on the amount of resin impregnated in cells (Huda et al. 2009, Tajuddin & Yusoff 2010). The resin consists of sesquiterpenoid groups and phenyl ethyl chromone derivatives. In the highest quality agarwood, the resin contains various oxygenated sesquiterpenes (sesquiterpenoids)

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and chromone derivatives which contribute to the aroma of agarwood (Yagura et al. 2003, Tajuddin & Yusoff 2010). The prices of agarwood oil and woodchip depend on factors such as colour, viscosity and long-lasting aroma (Nor Azah et al. 2008, Tajuddin & Yusoff 2010, Nor Azah et al. 2013). It has become a lucrative and highly valued forest product especially to tourists from the Middle East who purchase agarwood blocks and oil as souvenirs (Hashim et al. 2010). The agarwood oil is also incorporated into making high-end fragrances and cosmetics (Yagura et al. 2003). It was reported by Malaysia Timber Industry Board that between 2005 and 2009, the agarwood market exceeded USD508 million in Malaysia (Hashim et al. 2010). Due to overwhelming illegal harvesting of *Aquilaria* species in the wild, the species is listed under the Convention on International Trade in Endangered Species of Wild Flora and Fauna in Appendix II (IUCN 2007).

In-vitro polyploidisation using antimitotic agents such as colchicine, oryzalin and trifluralin is a means to increase agronomic properties in many ornamental, medicinal and woody plants (Doležel & Bartos 2005, Shapiro 2005, Doležel et al. 2007, Loureiro et al. 2007). Each antimitotic agent acted and/or resulted differently in terms of chromosome doubling success. Even though there are reports suggesting that colchicine gives higher chromosome doubling compared with others, this is not necessarily true as other factors such as concentrations used, plant species and explant susceptibility also play important roles in the success of polyploidisation (Kermani et al. 2003, Petersen et al. 2003, Allum et al. 2007, Dhooghe et al. 2011). Based on these studies, interest in applying the technique to timber trees has occurred to assist current problems in forest plantations such as long life cycle, small diameter timber and low biomass (Gamage et al. 2007, 2011). *Aquilaria malaccensis* is a natural diploid with chromosome number 2x = 14 (Siti Suhaila et al. 2013). In-vitro polyploidisation may be the method to investigate secondary metabolite production in other ploidy levels (Dhawan & Lavania 1996, Asif et al. 2001). Studies have shown changes and increasing secondary metabolites and bioactive compounds in medicinal plants such as *Vertiveria zizanioides* (Lavania 1988), *Artemisia annua* (Gonzalez & Wheathers 2003) and *Zingiber officinale* (Sanwal et al. 2010) through in-vitro polyploidisation. The development of flow cytometer application in plant researches has facilitated faster and more reliable methods to differentiate ploidy levels in plants along with established chromosome counting (Doležel et al. 2007). Further identification and chemical profiling were done using phytochemistry analyses. The analyses of agarwood oil contents via gas chromatograph mass spectrometry (GCMS) is favourable because it is an easy, fast and proven method in chemical profiling of phytochemical constituents (Ismail et al. 2013). The technique is a combination of gas chromatography to separate the mixture of components and mass spectroscopy to characterise each constituent. The chemical constituents of agarwood oil are identified by matching their retention indices with the spectra available at the National Institute of Standards Technology library (www.sisweb.com/manuals/nist05manual.pdf). The additional method to identify agarwood oil chemical profiles is headspace–solid phase microextraction (HS–SPME) combined with GCMS (HS–SPME–GCMS) which is based on the odour-active volatile components released in samples. This paper reports results of the use of colchicine and trifluralin to induce polyploidy and changes to phytochemical constituents using HS–SPME–GCMS.

MATERIALS AND METHODS

Establishment of in-vitro plantlets and polyploidy induction

The in-vitro clone (pK17) of *A. malaccensis* plantlets were propagated in MS medium (Murashige & Skoog 1962) supplemented with 0.1 mg L^{-1} benzylaminopurine. Cultures were maintained in a room at 22 ± 2 °C under 2000 lux [16 hours light (L)/8 hours dark (D)] light regime (Kandasamy 2004). To induce polyploidy, 10 shoot tips and nodal segments were used as explants for each experiment with three replications. After pre-treatment in MS hormone-free medium for 8–9 days, the explants were immersed in antimitotic agents, colchicine and trifluralin. The concentrations used were: [colchicine (0, 0.5, 1.0 and 2.0 mM) and trifluralin (0, 0.01, 0.05 and 0.1 mM)] at various exposure durations (0, 6, 12, 24, 48 and

120 hours). Each treatment combination was coupled with 1% dimethyl sulfoxide (DMSO) to facilitate penetration of antimitotic agents into plant cells. The explants were shaken at 90 rpm at 22 ± 2 °C under dark treatment. Following this treatment, the explants were rinsed three times with sterile distilled water and transferred to fresh MS medium and incubated under 2000 lux (16L/8D) light regime at 22 ± 2 °C for 8 weeks.

Analysis of ploidy level by flow cytometry

The ploidy analysis was performed with flow cytometer. The position of a known ploidy, diploid *Raphanus sativus* cv. Saxa (2C = 1.11 pg DNA) (Doležel et al. 1992) peak on a histogram needed to be confirmed through flow cytometry analysis. Once the position had been confirmed, the unknown 2C DNA nuclei samples (the DNA content value of diploid *A. malaccensis*) were analysed together with the standard. After 8 weeks of in-vitro incubation, three leaf samples were aseptically excised for evaluation and each was repeated three times for confirmation purposes. Samples were analysed with flow cytometer using settings listed in Table 1. The flow cytometer was equipped with an air-cooled argon-ion laser tuned to 15 MW and operating at 488 nm. Fluorescence was collected through a 645 nm dichroic long-pass filter in reflecting mode and a 620 nm band-pass filter. Nuclei suspension samples were prepared using general purpose buffer (0.5 mM spermine.4HCl, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate, 80 mM KCl, 20 mM NaCl, 0.5% (v/v) Triton X-100 (pH 7.0) (Loureiro et al. 2007)), prepared using ultra-pure water and stored as 150 mL aliquots stored in the dark at 4 °C. *Aquilaria malaccensis* and *R. sativus* cv. Saxa leaf samples (1 cm^2) were chopped together in Petri dish containing ice-cold general purpose buffer using razor blade. The resulting homogenates were removed with 10 mL disposable syringes and filtered through 50-µm nylon disposable syringe filters into 3.5 mL sample tubes containing ice-cold 50 µL propidium iodide (1 mg mL^{-1}) and ice-cold 50 µL RNAse (1 mg mL^{-1}). The blade was rinsed and blotted with paper towel between each sample and replaced after every 30 samples. Samples were analysed immediately and data were collected until each sample and reference standard histogram peaks reached 10,000 counts. The various 2C peaks mean positions were determined by manual gating to ensure that the highest point of the histogram fell in the middle of each gate. The coefficient of variation (CV) of *A. malaccensis* sample peaks must be lower than 5% throughout the study (computed by flow cytometer). The DNA content of the specimen was calculated from the ratio of peak positions of the *A. malaccensis* sample and standard reference whereby the absolute DNA amount of a sample was calculated based on the values of the peak means.

Preparation of chromosomes for ploidy level determination

True tetraploid and diploid samples from flow cytometer analyses were subjected to root induction medium (MS medium supplemented with 1 mg L^{-1} indol-3-butyric acid) for 4 weeks of root development. After 4 weeks, three root tips from each plant were excised and pre-treated with 0.5 mg L^{-1} colchicine for 180 min and fixed overnight with 3:1 (v/v) absolute EtOH:glacial acetic acid at 4 °C. These root tips were rinsed and hydrolysed for 7 min in 1N HCl at 60 °C in waterbath, rinsed with sterile distilled water and stained with Schiff's reagent for 2 hours. Stained root tips were then rinsed and treated with 200 µL of 2% pectinase (w/v) and 200 µL of 20% cellulase (w/v) for 15 min at room temperature in the dark. After rinsing, the meristem of the

Table 1 Summary of flow cytometer instrument settings used

Parameter	Gain	Log scale	Lower limit	Upper limit
FS	125	Log_3	50.0	999.9
SS	225	Log_3	10.0	999.9
FL2	470	Linear	70.0	999.9

FS = forward scatter correlates with cell size, SS = side scatter depends on the density of particle/cell, FL2 = propidium iodide fluorescence

root tips (about 2 mm long) were removed and placed on a clean slide. A drop of aceto-orcein solution was added and the slide covered. The samples were observed under optical microscope 1000× magnification with four cells selected for each sample for chromosome count. These true tetraploid plantlets were maintained under in-vitro conditions and the ploidy level evaluated using flow cytometer.

Preparation of stomata guard cell analysis for ploidy level determination

The method by Huang et al. (2010) was used for stomata guard cell analysis. Ten in-vitro *A. malaccensis* diploid and tetraploid leaves (from 8-week-old plantlets) were randomly selected. Three leaves from each plantlet were excised and immediately a clear tape was placed on the abaxial side of the leaf. After 15 min, the tape was gently stripped off so that only the leaf cuticle was attached to it and the guard cell shape was imprinted. The tape was placed on a microscope slide. Four separate areas were selected on each leaf sample for stomata observations under 400× magnification. Statistical analysis using analysis of variance and Duncan's multiple range test were used to determine significant difference ($p < 0.05$) in (a) number of stomata per area (38 mm × 25 mm) and (b) guard cell sizes between *A. malaccensis* diploid and polyploid.

Determination of phytochemical content by HS–SPME coupled with GCMS

Four-month-old in-vitro *A. malaccensis* diploid and tetraploid plantlets were selected and freshly chopped, placed into vials and analysed separately (each plant part sample weighing 20 g for the experiment). Chemical analyses of fresh samples were undertaken by GCMS. Gas chromatograph coupled to a mass selective detector system equipped with capillary column (30 m × 0.25 mm internal diameter, film thickness 0.25 µm) was utilised. The electron ionisation system was set with ionisation energy of 70eV. Helium was the carrier gas, set at flow rate of 1 mL min^{-1}. Injector and ion source temperatures were both set at 250 °C. Injection volume was 1 µL (split ratio 50:1). Polydimethylsiloxane fibre was used to absorb the volatile gases released by all samples during analyses with incubation temperature of 80 °C. The temperature programme was set initially at 60 °C for 10 min then to 180 °C for 1 min and then at 3 °C min^{-1}. The components were identified by comparing their mass spectrum with that of mass spectral library (HPCH 2205.L; Wiley7 NiST05.L; NIST0.5a-L) along with retention indices. Only compounds with > 90% matching from the NIST05 library were listed.

RESULTS

Effects of colchicine and trifluralin on polyploidy induction

The shoot tip and nodal segment explants showed decreased survival with increasing exposure time towards antimitotic agents (Tables 2 and 3). From the flow cytometer analyses using *R. sativus* cv. Saxa (internal standard) together with *A. malaccensis* leaf samples (diploid and tetraploid), the internal standard showed its expected position and was calculated to be 1.1 pg C^{-1} and the diploid *A. malaccensis* appeared to have DNA nuclei of 1.86 pg C^{-1} (Figure 1a). When tetraploid samples were tested, they showed a single peak further on the x-axis than diploid, with DNA nuclei value of 3.86 pg C^{-1} (Figure 1b). Compared with the diploid peak, *A. malaccensis* tetraploid cells exhibited a peak twice the size of the channel position, thereby confirming chromosome doubling as a result of antimitotic agent application.

The highest percentage for ploidy changes (13.3% of mixoploids) in shoot tip was by treatment of 1 mM colchicine at 24 hours exposure time (Table 2) but no tetraploids was obtained from shoot tip explants. However, in nodal segments, both mixoploids and tetraploids were induced with 0.05 and 0.10 mM of trifluralin at 120 hours with 6.7 and 10% respectively (Table 3). The mixoploids were observed to occur at higher frequencies in shoot tip than nodal segment explants. Nevertheless, the results showed that shoot tip explants were more responsive towards the type and concentration of antimitotic agents used. Even though most of the explants survived the treatments, the concentration and exposure time tested were not sufficient to induce polyploidy at high numbers. It was reported that *A. malaccensis* had very small genome size (900–918 Mbp);

Table 2 Percentage of ploidy level changes in *Aquilaria malaccensis* shoot tip explants as treated by colchicine and trifluralin at various exposure times

Colchicine (mM)	Exposure (hours)	Sample (n)	2n + 4n (Mean% ± SD)	4n (Mean% ± SD)	Trifluralin (mM)	Exposure (hours)	Sample (n)	2n + 4n (Mean% ± SD)	4n (Mean% ± SD)
0	6	30	0	0	0	6	30	0	0
	12	30	0	0		12	30	0	0
	24	30	0	0		24	30	0	0
	48	30	0	0		48	30	0	0
	120	30	0	0		120	30	0	0
0.5	6	30	0	0	0.01	6	30	0	0
	12	30	0	0		12	30	0	0
	24	30	0	0		24	30	0	0
	48	30	0	0		48	30	0	0
	120	30	0	0		120	30	0	0
1.0	6	30	0	0	0.05	6	30	0	0
	12	30	0	0		12	30	0	0
	24	30	13.3 ± 0.58	0		24	30	0	0
	48	30	0	0		48	30	0	0
	120	30	0.67 ± 0.58	0		120	30	6.7 ± 0.58	0
2.0	6	30	0	0	0.10	6	30	0	0
	12	30	0	0		12	30	0	0
	24	30	0	0		24	30	0	0
	48	30	3.3 ± 0.58	0		48	30	0	0
	120	30	0	0		120	30	6.7 ± 0.58	0

SD = standard deviation, 2n + 4n = mixoploids, 4n = tetraploids

Table 3 Percentage of ploidy level changes in *Aquilaria malaccensis* nodal segment explants as treated by colchicine and trifluralin at various exposure times

Colchicine (mM)	Exposure (hours)	Sample (n)	2n + 4n (Mean% ± SD)	4n (Mean% ± SD)	Trifluralin (mM)	Exposure (hours)	Sample (n)	2n + 4n (Mean% ± SD)	4n (Mean% ± SD)
0	6	30	0	0	0	6	30	0	0
	12	30	0	0		12	30	0	0
	24	30	0	0		24	30	0	0
	48	30	0	0		48	30	0	0
	120	30	0	0		120	30	0	0
0.5	6	30	0	0	0.01	6	30	0	0
	12	30	0	0		12	30	0	0
	24	30	0	0		24	30	0	0
	48	30	0	0		48	30	0	0
	120	30	0	0		120	30	0	0
1.0	6	30	0	0	0.05	6	30	0	0
	12	30	0	0		12	30	0	0
	24	30	0	0		24	30	0	0
	48	30	0	0		48	30	0	0
	120	30	0	0		120	30	6.7 ± 0.58	0
2.0	6	30	0	0	0.10	6	30	0	0
	12	30	0	0		12	30	0	0
	24	30	0	0		24	30	0	0
	48	30	0	0		48	30	0	0
	120	30	0	0		120	30	6.7 ± 0.58	10 ± 0.00

SD = standard deviation, 2n + 4n = mixoploids, 4n = tetraploids

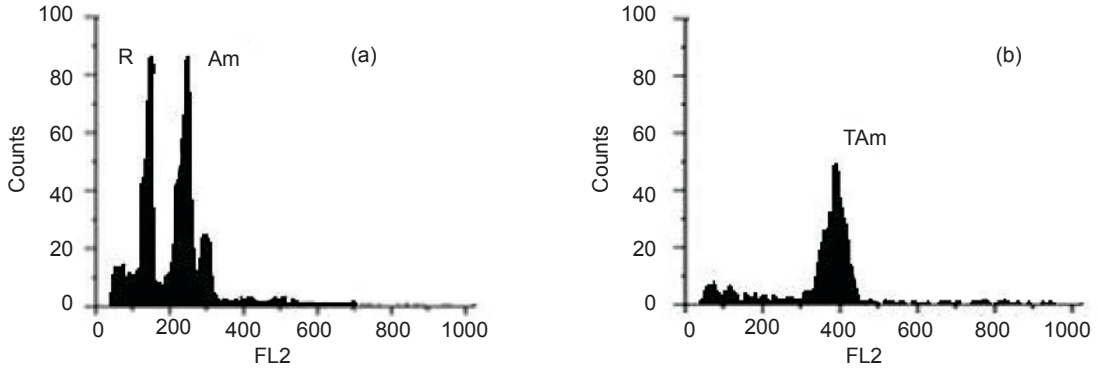

Figure 1 Histograms showing the number of nuclei per channel as a function of relative fluorescence intensity resulting from flow cytometric analysis of nuclei stained with propidium iodide (a) internal reference *Raphanus sativus* cv. Saxa—R, 1.1 pg C^{-1} and diploid *Aquilaria malaccensis*—Am, 1.86 ± 0.02 pg C^{-1} and (b) tetraploid—TAm, 3.86 ± 0.03 pg C^{-1}

therefore chromosome count might be difficult to attempt (Siti Suhaila et al. 2013). In this study, the root squashed method was able to obtain chromosome number of tetraploid samples, 4x = 28 (Figure 2b), double the amount of diploid chromosome number, 2x = 14 (Figure 2a), as reported by Siti Suhaila et al. (2013). It was observed that the densities of stomata guard cells were higher in diploid (33.3 ± 0.6 µm; Figure 3a) compared with tetraploid (23.1 ± 0.5 µm; Figure 3b). However, the average values of stomata guard cell sizes in diploid were smaller then tetraploid (Figure 4).

Phytochemical content in diploid and tetraploid leaf, stem and root

The study showed some similarities and differences in the presence of the phytochemical content using HS–SPME coupled with GCMS from different plant parts of *A. malaccensis*. The total phytochemical content in tetraploid was higher than diploid, particularly from the stem part (43.19%) compared with diploid 5.87% (Table 4). Interestingly, tetraploid showed a number of important phytochemical constituents (known as sesquiterpenoids in agarwood oil) which was not detected and/or at lower level in diploid samples. For example, tetraploid stem samples contained high levels of α-eudesmol (18.3%), α-gurjunene (8.61%) and γ-gurjunene (6.22%), which were not present in diploid samples. However, there were two sesquiterpenoids, i.e. aromadendrene (2.49%) and α-humulene (3.38%) detected at low levels in diploid stem samples. Even though aromadendrene was not present in the tetraploid stem samples, a higher level of α-humulene (3.79%) was found in tetraploid samples. However, it was not detected in stem as diploid samples did. Instead it was in the tetraploid leaf samples. In leaf samples, both diploid and tetraploid had only one but different compound detected. In diploid it was (2E)-tridecenol (19.4%) while in tetraploid, α-humulene was detected.

There were almost similar amounts in terms of total phytochemical content identified in roots between diploid (30.68%) and tetraploid (30.25%). However, the sesquiterpenoids detected were different. For example, diploid root samples had higher contents of α-guaiene (6.49%) and α-bulnesene (17.60%) than tetraploid, i.e. 1.09% and 3.05% respectively. Diploid roots also contained γ-muurolene (2.96%) and δ-cadinene (3.63%) which were not detected in tetraploid. However, tetraploid were able to synthesise higher sesquiterpenoids in its roots such as γ-gurjunene (14.00%), β-patchoulene (2.00%), allo-aromadendrene (1.24%), β-cedrene (1.19%) and aristolone (2.37%) that were not present in diploid samples.

DISCUSSION

It has been reported that colchicine is the most common and most effective antimitotic agent for chromosome doubling such as in *Zea mays*

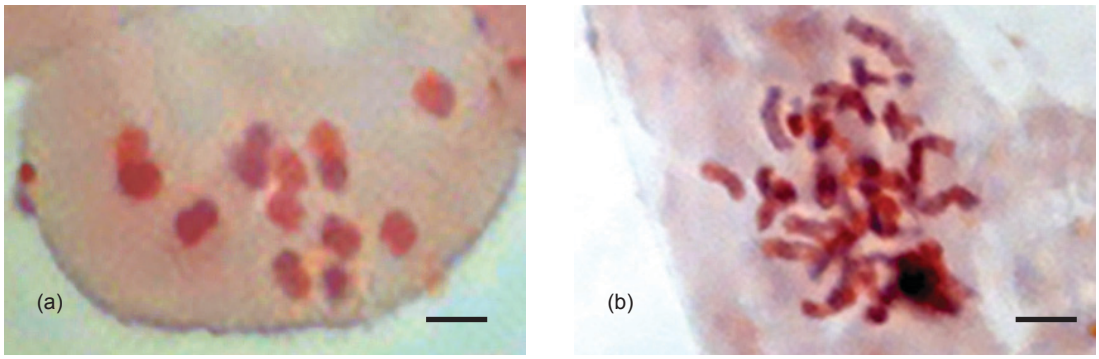

Figure 2 Chromosome numbers were determined using optimised root tip squashing method from (a) diploid *Aquilaria malaccensis*, 2x = 14 and (b) tetraploid, 4x = 28; bar = 5 µm

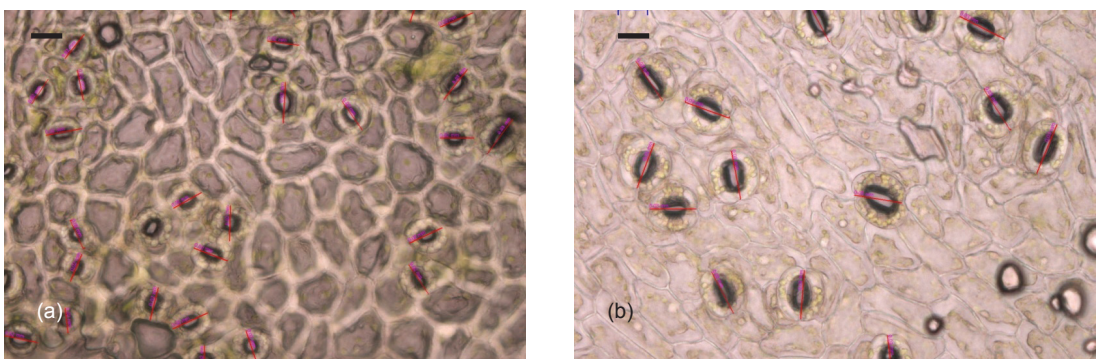

Figure 3 The density of stomata in *Aquilaria malaccensis* (a) diploid (23.1 ± 0.1) and (b) tetraploid (13.3 ± 0.2) samples; bar = 20 µm

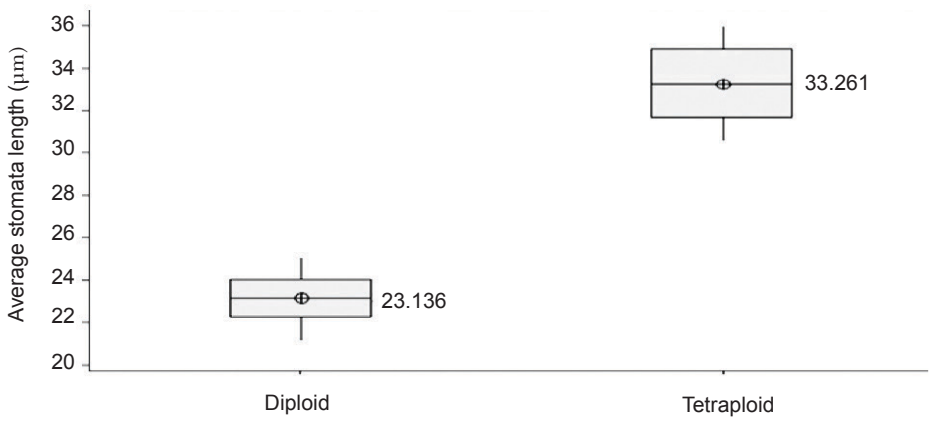

Figure 4 Stomata length of *Aquilaria malaccensis* (a) diploid (23.1 ± 0.5 µm) and (b) tetraploid (33.3 ± 0.6 µm) samples

Table 4 Volatile constituents identified using HS–SPM–GCMS method from different sources of *Aquilaria malaccensis*

Chemical constituent	RI	Diploid (%)			Tetraploid (%)			Confirmation method
		L	S	R	L	S	R	
β-Patchoulene	1379	–	–	–	–	–	2.00	MS
β-Elemene	1389	–	–	–	–	1.33	–	RI, MS
Longifolene	1407	–	–	–	–	–	1.04	MS
β-Cedrene	1419	–	–	–	–	–	1.19	MS
Didehydro-cycloisolongifolene	1423	–	–	–	–	–	2.10	MS
α-Gurjunene	1431	–	–	–	–	8.61	–	MS
α-Guaiene	1437	–	–	6.49	–	–	1.09	RI, MS
Aromadendrene	1439	–	2.49	–	–	–	–	MS
α-Humulene	1452	–	3.38	–	3.79	–	–	RI, MS
Allo-aromadendrene	1458	–	–	–	–	–	1.24	MS
4,5-di-epi-Aristolochene	1471	–	–	–	–	3.07	2.17	RI, MS
γ-Gurjunene	1475	–	–	–	–	6.22	14.00	RI, MS
Valencene	1496	–	–	–	–	4.06	–	MS
Pseudowiddrene	1498	–	–	–	–	1.60	–	MS
α-Bulnesene	1506	–	–	17.6	–	–	3.05	RI, MS
δ-Cadinene	1523	–	–	3.63	–	–	–	RI, MS
γ-Muurolene	1525	–	–	2.96	–	–	–	MS
(2E)-Tridecenol	1570	19.40	–	–	–	–	–	MS
α-Eudesmol	1652	–	–	–	–	18.3	–	MS
Aristolone	1762	–	–	–	–	–	2.37	MS

L = leaf, S = stem, R = root, MS = mass spectrometry; HS–SPME–GCMS = headspace–solid phase microextraction/gas chromatograph mass spectrometry

(Barnabás et al. 1999), *Brassica napus* (Zaki & Dickinson 1995), *Triticum aestivum* (Barnabás et al. 1991) and *Nicotiana tabacum* (Takashima et al. 1995). However, chromosome doubling also depends on explants used (Kermani et al. 2003, Petersen et al. 2003) and susceptibility and permeability of the meristem and/or plant tissue towards the antimitotic agents (Allum et al. 2007). In spite of the effectiveness of colchicine, alternatives to colchicine are encouraged as it has side effects such as sterility, abnormal growth, chromosome loss, chromosome rearrangement and gene mutation (Luckett 1989, Dhooghe et al. 2011) as well as high toxicity to humans (Morejohn et al. 1984). Moreover, the solvent in which the antimitotic agents are dissolved increased the toxicity on explants, especially during exposure period (Stanys et al. 2006, Allum et al. 2007, Zhang et al. 2007, Greplova et al. 2009). Therefore, a general protocol for in-vitro polyploidisation is not available due to the complexity of the process (Dhooghe et al. 2011).

In this study, polyploidisation in *A. malaccensis* has successfully been induced artificially. However, it was at lower percentages (< 13.3%) compared with other studies (15–33%) using antimitotic agents. The responses of the explants towards antimitotic agents were different probably due to the kinetics of mitotic division in culture (Möllers et al. 1994, Zaki & Dickinson 1995). This study revealed that the shoot tip and nodal segments of *A. malaccensis* reacted differently under the same concentration of antimitotic agent and exposure time. This is in accordance to earlier researches which reported that explant type, concentration of antimitotic agent and exposure time were important parameters for successful polyploidisation (Allum et al. 2007, Dhooghe et al. 2011). Other works have shown

that longer exposure time than that needed by cells during mitosis often resulted in higher than desired ploidy levels (Allum et al. 2007). In this study, the longest exposure time (120 hours) in both explants increased the potential of obtaining higher chromosome doubling in *A. malaccensis*. Incorporation of DMSO (2 or 4%) facilitates absorption of chemicals into cells but at the same time may decrease survival rates (Hamill et al. 1992). Therefore, this study had used lower concentration of DMSO (1%) than that of Hamill et al's to ensure high survival rates and to obtain polyploids. Flow cytometry proves to be an advantage as it allows rapid analyses of large numbers of plant samples (Doležel et al. 2007), which can be done at a much earlier stage of plant development in order to save time and space (Väinölä 2000). The analysis was more accurate than morphological and anatomical observations to determine ploidy levels in plants (Brummer et al. 1999, Zlesak et al. 2005). Compared with chromosome counting, flow cytometer can further analyse other heterogeneous population of cells (for example mixoploid cells) in plant samples (Doležel et al. 2007).

Aquilaria malaccensis polyploidy clones (in this case, tetraploid) were responsible for higher phytochemical content as observed in species such as *V. zizanioides* (Lavania 1988). The GCMS was a reliable method for chemical profiling and probing of phytochemical constituents in *A. malaccensis* as reported by other researchers (Tajuddin & Yusoff 2010, Nor Azah et al. 2013). Polyploidy plantlets enhanced the production of total phytochemical content in *A. malaccensis*. However, there were a few exceptions whereby the diploid contained higher percentage of certain phytochemical constituents than tetraploid. However, chemical profiling showed that many of them were not considered as important phytochemical constituents in determining agarwood oil quality but as first line defence mechanism in plants. Different ploidy levels and different plant parts of the same clone and age of plants produced different phytochemical constituents (Achakzai et al. 2009, Dhooghe et al. 2011), as discovered in this study. Higher ploidy level (such as tetraploid) cells may be involved in other synthesised pathways (due to chromosome doubling), producing different enzymes and/or complex enzymes combinations which results in higher number of phytochemical being formed and contributes to the total phytochemical content, as proven by other workers (Lavania 1988, Gonzalez & Weathers 2003, Sanwal et al. 2010). The results also confirmed that in-vitro leaves did not function effectively compared with leaves from the field. There were minimal phytochemical constituents detected from in-vitro leaf samples due to light source from fluorescent tubes in laboratory versus light intensities from the sun (Kandasamy et al. 2004). Tetraploid leaf samples showed higher phytochemical constituent compared with diploid which may in turn be a non-destructive method to extract and obtain valuable phytochemicals through leaf harvesting. This study also revealed that in-vitro plantlets were able to produce sesquiterpenoids at a young age in in-vitro plantlets of *A. malaccensis*. Induction of polyploidy, followed by screening of phytochemical contents could be beneficial for the production of high quality *A. malaccensis* clones. These clones of polyploidy stock plants can in turn be crossed with diploid plants to produce triploid progeny with novel characteristics. Alternatively, polyploidy can also be used as parental stock in hybridisation programme.

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