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Full Length Research Paper

Enhancement of phytochemical production through *in vitro* polyploidization of agarwood-producing species, *Aquilaria malaccensis*

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***Aquilaria malaccensis* is an endangered agarwood-producing plant found in most Asian countries and has a very long history of use in traditional medicine as incense, aromatic and therapeutic oil. Polyploidization is an established method to enhance the desired horticultural characteristics in many ornamental plants. In some medicinal plants, it proves to have positive effects on the production of secondary metabolites and/or bioactive compounds. Thus, the aims of this study are to induce polyploidization in *A. malaccensis* and investigate the phytochemical constituents using Headspace-Solid Phase Microextraction (HS-SPME), coupled with Gas Chromatography-Mass Spectrometry (GCMS) analyses. The present study showed that the highest percentage of polyploidization in *A. malaccensis* samples was at 120 h of exposure time using 1 mM colchicine and 0.1 mM trifluralin on shoot tip and nodal segment, respectively. Through HS-SPME/GCMS, the phytochemical constituents in polyploid doubled the amount than in diploid samples. The phytochemical constituents increased 4-folds in stem and twice in roots of polyploid samples as compared to diploid. The seedling however, had higher number of phytochemical constituents due to age differences against the *in vitro* samples. Enhancement of phytochemical production through polyploidization in *A. malaccensis* will provide new breeds for commercial plantation program and support the effort to reduce over-collection of the species in the wild.**

Key words: *Aquilaria malaccensis*, diploid, polyploid, colchicine, trifluralin, Head Space-Solid Phase Microextraction (HS-SPME), Gas Chromatography-Mass Spectrometry (GC-MS).

INTRODUCTION

It has been well documented that ploidy level changes occur naturally and/or induced artificially in plants resulting in more vigorous growth rates, and in some plants, enhanced secondary metabolites due to higher number of genes and enzyme activities involved in a cell (Tate et al., 2005). Polyploidy usually produces bigger vegetative and reproductive parts of the plants due to cell enlargements in order to contain more than two sets of chromosomes (Dhooghe et al., 2011). Artificial induction of polyploidization has been successfully applied in plants

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especially in ornamentals and food crops. Other workers reported increase in secondary metabolites and bioactive compounds in some medicinal plants (Marriot et al., 2001; Gonzalez and Wheathers, 2003; Sanwal et al., 2010). Based on these studies, an increase of interest in applying the technique to timber trees had occurred and it has shown encouraging results by some timber trees such as enhancement of growth rates, diameter increments and higher biomass yield for biofuel purposes (Blakesley et al., 2002; Gamage et al., 2007; 2011).

Flow cytometer has been applied in the studies of polyploidization to confirm the ploidy level changes and genome sizes due to its fast and accurate methods (Galbraith et al., 1983; Dolezel et al., 2005, 2007). In the case of *Aquilaria malaccensis* (locally known as Karas), its fragrant resin called agarwood had been used in traditional medicine as anti-asthma, antitoxic, antioxidant, hypertension (anti-stress), hepatitis, cirrhosis, diuretic, painkiller, and many other diseases (Nor Azah et al., 2008). Today, it is incorporated in modern therapeutic and aromatic industries worth millions of US dollars (Nor Azah et al., 2008, 2009, 2013; Ismail et al., 2013). Due to the high prices in the global market, illegal harvesting and trading of the agarwood cause diminishing supplies and listing of *A. malaccensis* in CITES (Appendix II) (Antonopoulou et al., 2010; Tajuddin and Yusoff, 2010). *A. malaccensis* plantation is a current effort in Malaysia and other agarwood-producing countries as a means to inhibit illegal harvesting from the wild (Mamat et al., 2010). However, it must be artificially inoculated to trigger the agarwood formation. It is reported that if only 7 to 10% of the trees will produce agarwood, it would be beneficial to develop new breed of *A. malaccensis* that are able to have an indication of its capabilities to form agarwood. The new breed of *A. malaccensis* will be a suitable candidate for plantation and in future help the inhibition of illegal harvesting in the wild. To the authors' best knowledge, the polyploidization method for enhancements of the phytochemicals is the first to be reported on *A. malaccensis*. This study aimed to develop *in vitro* polyploidization protocol and evaluate the phytochemical constituents from fresh plant parts (leaf, stem and roots) samples of seedlings and *in vitro* diploid and polyploid samples using Headspace-Solid Phase Microextraction (HS-SPME) and Gas Chromatography-Mass Spectrometry (GC-MS).

MATERIALS AND METHODS

Plant material

The *A. malaccensis* seedlings were obtained from Seed Technology Laboratory, FRIM; and *in vitro* plantlets were obtained from Tissue Culture Laboratory, FRIM.

Preparation of *in vitro* polyploidization

To induce polyploidization, 30 shoot tips and nodal segment explants were pretreated in modified MS (1962) hormone-free medium for 8-9 days, then immersed in different antimitotic agents [(colchicine - 0, 0.5, 1, 2 M) and (trifluralin - 0, 0.01, 0.05, 0.1 mM)] coupled with 1% DMSO at various exposure time (6, 12, 24, 48, 120 h) with 3 replicates. The explants were shaken at 90 rpm at 22°C under dark treatment. Following the treatments, the explants were rinsed 3 times with sdH_2O, after which they were transferred to fresh medium and incubated under 2000 flux (16L/8D) light regime at 22°C for 8 weeks. By the end of the 8th week, percentage of survival rates of the explants from each treatment was conducted by taking the numbers of survived explants divided with number of explants used times (100). These survived *A. malaccensis* plantlets were analyzed using flow cytometer to confirm the ploidy level using the young leaves emerging from the treated samples (Dolezel et al., 2005, 2007). The flow cytometer (Cyflow space, Partec-Germany) was used with standard (known) 2C DNA nuclei of *Raphanus sativus* cv. 'Saxa' as internal standard. 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 suspensions were prepared by rapidly chopping 1 cm^2 *A. malaccensis* and *R. sativus* leaves samples in Petri dish (16 mm × 90 mm) containing General Purpose Buffer (GPB) using razor blade (Gillette no.5) to release the DNA nuclei into the buffer. The resulting homogenates were removed with 10 mL disposable syringes and filtered through 50 um CellTrics^R (Partec) nylon disposable syringe filters into 3.5 mL sample tubes (Sarstedt) containing 50 ul propidium iodide, PI (1 mg/ml) and 50 ul RNAse (1 mg/ml). Samples were analyzed immediately and data were collected. The coefficient of variation (CV%) of *A. malaccensis* (G_2) samples peaks must be lower than 5% throughout the study (computed by flow cytometer). The absolute DNA amount of a sample is calculated based on the values of the G_2 peak means:

$$\text{Sample 2C DNA content} = \frac{\text{sample mean fluorescence intensity, } G_2 \times \text{standard 2C DNA (pg), } G_1}{\text{standard mean fluorescence intensity, } G_1}$$

Headspace-Solid Phase Microextraction (HS-SPME) coupled with Gas Chromatography Mass Spectrometry (GCMS)

The plant parts were chopped and transferred into vials to be analyzed separately. The analyses were conducted at Essential Oil Technology Laboratory at Natural Products Division, FRIM. Chemical analyses of the samples were undertaken by gas chromatography-mass spectrometry using Agilent Technologies 7890A gas chromatograph coupled to a 5975C MSD system equipped with HP-5MS capillary column (30m × 0.25 mm ID, film thickness of 0.25 µm). The electron ionization

system was set with an ionization energy of 70eV. Helium was used as the carrier gas, set at a flow rate of 1.0 mL/min. Injector and ion source temperatures were both set at 250°C. Injection volume was 1 µL (split ratio 50:1). Polydimethylsiloxane (PDMS) fiber was used during the analyses with incubation temperature of 80°C. The temperature program was set initially at 60°C for 10 min, then at 180°C for 1 min, and finally at 3°C per minute.

Identification of components

The components were identified by comparing their mass spectrum with mass spectral library (HPCH 2205.L; Wiley7 NiST05.L; NIST0.5a-L).

RESULTS AND DISCUSSION

Ploidy level changes using antimitotic agents

The average percentage of survival rates for shoot tips was 81.1 and 84.9% against colchicine and trifluralin, respectively. The nodal segments samples had survival rates of 80.3% with colchicine treatment and 87.2% against treatment with trifluralin. When the survived plantlets were tested, the best treatment for ploidy level changes was 0.1 mM trifluralin for nodal segments (7.6%) and 1 mM colchicine for shoot tips (17%) both at 120 h of exposure time (Table 3). The percentage was considered low (especially for nodal segments) than that reported by other plant species which was between 15 and 50% (Dhooghe et al., 2011). Both shoot tips and nodal segments samples were observed to be tolerant towards trifluralin better than colchicine possibly due to the fact that the concentrations used were lower in trifluralin (up to 0.1 mM). Based on earlier reports however, the successful rates of polyploidization is much depended on the explants used and it compromises towards the toxicity of the chemical and genome doubling efficiency of the species (Yang et al., 2006; Allum et al., 2007; Khosravi et al., 2008; Sun et al., 2009; Dhooghe et al., 2011).

Phytochemical constituent evaluation

The general chemical profiles and their chemical constituents obtained from the fresh samples and retention indices are listed in Table 1 (the compounds with >90% matching to the NIST05 library were listed). This study showed that *ex vitro* seedlings have the highest total number of phytochemical constituents as compared to *in vitro* samples, both diploid and tetraploid (Table 1). There were 31 from *ex vitro* seedlings, 7 from *in vitro* diploid and 18 from *in vitro* tetraploid samples (Table 2). Studies had shown that growth conditions and stage of plant development are factors that play a role in the differences in phytochemical production between the *ex vitro* and *in vitro* samples (Achakzai et al., 2009; Nikolova et al., 2013).

Generally, secondary metabolites production increased in the presence of higher light intensity compared to shady ones (Mole and Waterman, 1987; Mole et al., 1988). Here, the *ex vitro* seedlings which was grown in an open field with plenty of sunlight had a higher number of phytochemical than the *in vitro* samples grown under fluorescent light in laboratory. In addition, leaves of *in vitro* plantlets usually are not functioning like normal leaves due to high humidity and medium nutrient supplied. The leaves will gradually adapt to the surroundings and later on produce phytochemical constituents which mainly served as defense mechanisms. With correlation to plant development stage, older plants tend to contain higher phytochemical constituents than young plants (Achakzai et al., 2009; Jelinek et al., 2012). In this study, it is also proven that seedlings (12 months old) contain higher numbers of phytochemical constituents as compared to *in vitro* (4 months old) samples due to age differences. Young plants on the other hand contain more of meristematic cells where high cell division, elongation and cell differentiation took place and have to sacrifice the production of secondary metabolites for the growth. In Table 1, leaf and stem of *ex vitro* seedlings had higher number of phytochemical constituents compared to *in vitro* samples. In this study, leaf samples of *ex vitro* seedlings had 8 phytochemical constituents, while the *in vitro* diploid and *in vitro* tetraploid samples have 1 each. In stems of *ex vitro* seedlings, 16 phytochemical constituents were detected while only 2 from *in vitro* diploid and 8 from *in vitro* tetraploid samples were detected. In this case, the differences in age prove to play a role in the production of secondary metabolites (Achakzai et al., 2009; Jelinek et al., 2012). However, between the *in vitro* stem samples, differences in ploidy level have proven to be the factor of higher number of phytochemicals in tetraploid as compared to diploid (Lavania, 1988; Gonzalez and Wheathers, 2003; Sanwal et al., 2010). Interestingly, in roots, samples of *in vitro* tetraploid have higher number of phytochemical constituents (9) than *ex vitro* seedlings (7) and *in vitro* diploid (4). This result had indicated that increase in ploidy level is able to enhance the phytochemicals without taking age of samples as a parameter (Lavania, 1988; Gonzalez and Wheathers, 2003; Sanwal et al., 2010). There has been an extensive study using salicylic acid (SA) or methyl jasmonate (MJ) on callus cells to induce the production of important phytochemicals in *Aquilaria* species (Okudera and Ito, 2009).

Interestingly, this study shows that some of these important compounds are present in tetraploid in *in vitro* stem and roots samples without any treatments of SA and MJ. This proves that polyploidization can be an alternative in the production of secondary metabolites *in vitro* without the use of chemicals. Studies had showed differences in secondary metabolites production from different parts of the plants (Hyder et al., 2002; Springer

Table 1. Phytochemical constituents detected using HS-SPME/GCMS on seedlings, *in vitro* diploid and *in vitro* tetraploid samples.

Compounds	RI	*Ex vitro* seedlings			*In vitro*diploid			*In vitro* tetraploid			Confirmation method(s)
		L	S	R	L	S	R	L	S	R	
Eugenol	1369	-	0.79	-	-	-	-	-	-	-	MS
Geranyl acetate	1382	12.7	-	-	-	-	-	-	-	-	RI, MS
β-Patchoulene	1442	-	-	-	-	-	-	-	-	2.05	MS
β-Elemene	1389	-	-	-	-	-	-	-	1.33	-	RI, MS
Longifolene	1650	-	-	-	-	-	-	-	-	1.04	MS
α-Gurjunene	1648	-	-	-	-	-	-	-	8.61	-	MS
α-*cis*-Bergamotene	1432	-	0.90	-	-	-	-	-	-	-	MS
β-Cedrene	1404	-	-	-	-	-	-	-	-	1.19	MS
β -Caryophyllene	1414	1.19	-	-	-	-	-	-	-	-	MS
α-Guaiene	1435	-	-	6.53	-	-	6.49	-	-	1.09	RI, MS
Aromadendrene	1477	-	-	-	-	2.49	-	-	-	-	MS
α-Humulene	1453	-	-	5.44	-	3.38	-	3.79	-	-	RI, MS
(E)- β-Farnesene	1456	2.92	7.11	4.82	-	-	-	-	-	-	MS
allo-Aromadendrene	1639	-	-	-	-	-	-	-	-	1.24	MS
4,5-di-e*pi*-Aristolochene	1471	-	-	-	-	-	-	-	3.07	2.17	RI, MS
trans-Cadina-1(6),4-diene	1525	3.21	-	-	-	-	-	-	-	-	MS
γ-Gurjunene	1673	-	-	-	-	-	-	-	6.22	14.04	RI, MS
γ-Muurolene	1525	-	-	-	-	-	2.96	-	-	-	MS
γ-Curcumene	1479	-	1.13	-	-	-	-	-	-	-	RI, MS
Germacrene D	1481	0.43	-	-	-	-	-	-	-	-	RI, MS
Curcumene-(ar)	1480	-	1.89	-	-	-	-	-	-	-	MS
δ-Selinene	1645	1.11	-	2.37	-	-	-	-	-	-	MS
α-Zingiberene	1385	-	9.39	-	-	-	-	-	-	-	MS
Valencene	1695	-	-	-	-	-	-	-	4.06	-	RI, MS
Pseudowiddrene	1471	-	-	-	-	-	-	-	1.60	-	MS
α-Muurolene	1408	-	1.86	-	-	-	-	-	-	-	MS
Premnaspirodiene	1639	-	-	-	-	-	-	-	0.52	-	MS
α-Bulnesene	1506	-	-	25.7	-	-	17.5	-	-	3.05	RI, MS
γ-Cadinene	1518	17.1	1.32	-	-	-	-	-	-	-	MS
δ-Cadinene	1523	-	-	-	-	-	3.63	-	-	-	RI, MS
cis-Calamenene	1522	-	2.09	-	-	-	-	-	-	-	MS
Zonarene	1639	-	1.46	-	-	-	-	-	-	-	MS
Citronellyl butanoate	1351	3.14	-	-	-	-	-	-	-	-	MS

Table 1. Cont'd.

α-Calacorene	1550	-	1.04	-	-	-	-	-	-	-	MS
Elemol	1563	-	3.11	-	-	-	-	-	-	-	MS
Tridecenol (2E)	1773	-	-	-	19.4	-	-	-	-	-	MS
10-e*pi*-γ-Eudesmol	1629	-	2.96	-	-	-	-	-	-	-	MS
γ-Eudesmol	1645	-	11.9	1.87	-	-	-	-	-	-	MS
α-Eudesmol	1670	-	-	-	-	-	-	-	18.2	-	MS
α-Cadinol	1670	-	26.3	8.95	-	-	-	-	-	-	MS
Cadelene	1689	-	3.36	-	-	-	-	-	-	-	MS
Aristolone	1634	-	-	-	-	-	-	-	-	2.73	MS
TOTAL		8	16	7	1	2	4	1	8	9	

Components are listed in order of their relative retention content >0.1%, linear retention indices. RI were determined relative to the retention times on the HP-5MS capillary column of a homologous series of C6-C18 n-alkanes, Identification: MS by comparison of the MS with those of the HPCH 2205.L; Wiley7 NiST05.L; NIST0.5a-L library (>90% matching from the library) – L – leaf; S – stem; R - root.

Table 2. Total phytochemical constituents of seedlings, *in vitro* diploid and *in vitro* polyploid from leaf, stem and roots of *A. malaccensis*.

Plant part	*Ex vitro* seedlings (12 months old)	*In vitro* diploid (4 months old)	*In vitro* tetraploid (4 months old)
Leaf	8	1	1
Stem	16	2	8
Roots	7	4	9
TOTAL	31	7	18

et al., 2002; Kowalski and Wolski, 2006). In this study, the production of phytochemical differs from one part of the plant to another. The major phytochemical constituents detected in *ex vitro* seedlings were leaf: γ-cadinene (17.1%), geranyl acetate (12.7%), *trans*-Cadina-1(6),4-diene (3.21%), citronellyl butanoate (3.14%), and (E)-β-farnesene (2.92%); stem: α-cadinol (26.3%), γ-eudesmol (11.9%), α-zingiberene (9.39%) and (E)-β-farnesene (7.11%), cadelene (3.36%), elemol (3.11%) and 10-e*pi*-γ-Eudesmol (2.96%); roots: α-bulnesene (25.7%), α-cadinol (8.95%), α-guaiene (6.53%), α-humulene (5.44%) and (E)-β-farnesene (4.82%) and δ-selinene (2.37%). In the *in vitro* diploid samples, the phytochemical constituents detected were: leaf: tridecenol (2E) (19.4%); stem: α-humulene (3.38%) and aromadendrene (2.49%); roots: α-bulnesene (17.5%), α-guaiene (6.49%), δ-cadinene (3.63%) and γ-muurolene (2.96%), while in the tetraploid samples the phytochemical constituents detected were leaf: α-humulene (3.79%); stem: γ-eudesmol (18.2%), α-gurjunene (8.61%), γ-gurjunene (6.22%), valencene (4.06%) and 4,5-di-e*pi*-aristolochene (3.07%); roots: γ-gurjunene (14%), α-bulnesene (3.05%), aristolone (2.37%), 4,5-di-e*pi*-aristolochene (2.17%), didehydro-cycloisolongifolene (2.11%) and β-patchoulene (2.05%).

CONCLUSION AND RECOMMENDATION

Based on the data obtained, the phytochemical

Table 3. Percentage of survival rates (%Sr.) and ploidy level changes (%Pc.) in *A. malaccensis* shoot tip and nodal segment explants after treated separately with 2 antimitotic agents, colchicine and trifluralin.

Explant	Hours	Colchicine (mM)							
		0.0		0.5		1.0		2.0	
		%Sr.	%Pc.	%Sr.	%Pc.	%Sr.	%Pc.	%Sr.	%Pc.
Shoot tip	6	100	0	97.8	0	100	0	92.2	0
	12	98.9	0	94.4	0	93.3	0	77.8	0
	24	95.9	0	94.4	0	65.6	0	74.4	0
	48	97.8	0	82.2	0	82.2	0	54.4	0
	120	95.6	0	88.9	0	78.9	4	61.1	0
Nodal segment	6	98.9	0	91.1	0	100	0	93.3	0
	12	95.6	0	91.1	0	87.8	0	84.4	0
	24	95.6	0	85.6	0	82.2	5.4	74.4	0
	48	93.3	0	76.7	0	55.6	0	60	0
	120	96.7	0	74.3	0	43.3	7.6	26.7	0

Explant	Hours	Trifluralin (mM)							
		0.0		0.01		0.05		0.1	
		%Sr.	%Pc.	%Sr.	%Pc.	%Sr.	%Pc.	%Sr.	%Pc.
Shoot tip	6	100	0	100	0	100	0	100	0
	12	95.6	0	87.8	0	92.2	0	85.6	0
	24	96.7	0	92.2	0	83.3	0	85.6	0
	48	93.3	0	86.7	0	82.2	0	73.3	0
	120	93.3	0	82.2	0	31.1	0	38.0	0
Nodal segment	6	100	0	100	0	97.8	0	100	0
	12	94.4	0	93.3	0	92.2	0	90	0
	24	98.9	0	92.2	0	88.9	0	80	0
	48	98.9	0	88.9	0	85.6	0	67.8	0
	120	91	0	81	0	59	0	43.3	17.0

constituents in *in vitro* plantlets of *A. malaccensis* increased as the ploidy level increased, provided the samples are at the same age and growth conditions. The *in vitro* tetraploid *A. malaccensis* stem and roots samples showed higher number of phytochemical constituents compared to its diploid counterparts. The leaf samples however gave similar result due to growth conditions provided under control environment. The study also showed that *in vitro* plantlets are able to produce phytochemical constituents at young age and may facilitate studies on *Aquilaria* species that were conducted using mature tree samples. The phytochemical constituents can be enhanced on polyploidy *in vitro* plant samples without the use of chemicals such as SA and MJ as it has been demonstrated using callus.

Further investigation of phytochemical constituents between seedlings and *in vitro* plantlets when they are at the same age, and the photosynthesis activities as well as photorespiration would be useful to better understand the plant system between field grown *A. malaccensis* and *in vitro A. malaccensis*. The new breed of *A. malaccensis* may help the industry on selection of good and/or elite clones for commercial plantation purposes.

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