

ENHANCEMENT OF CHEMICAL CONSTITUENTS IN HYDROSOL AND RESIDUAL WATER OF *AQUILARIA MALACCENSIS* TETRAPLOID

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Polyploidisation is an important feature of species evolution in plant, animal and fungal kingdoms. Many researches proved polyploid organisms outperform their diploid relatives in several aspects. Thus, many plant breeders in the last century practised induced and/or used natural polyploids to obtain improved plant cultivars. Fresh samples of *A. malaccensis* tetraploid contain higher amount of important agarwood sesquiterpenes compared with diploid counterparts. In this study, an investigation on the presence of important agarwood sesquiterpenes in hydrosol and residual water in diploid and tetraploid *A. malaccensis* was carried out. Three controls were used, namely, diploid seedlings grown in soil, diploid seedlings grown under control conditions (*in vitro*) and tissue culture-derived shoots of diploid for comparison with tetraploid *A. malaccensis*. Results showed that hydrosol water from tetraploid leaf and root samples contained the highest amount of important agarwood sesquiterpenes, i.e. 30.92 and 36.83% respectively. In residual water, the tetraploid *A. malaccensis* contained the highest amount of important agarwood sesquiterpenes in root sample (49.1%). This article showed that polyploidy can be used as a tool to enhance the presence of important agarwood sesquiterpenes in *A. malaccensis*.

Keywords: Agarwood, sesquiterpenes, diploid, polyploids, compounds

INTRODUCTION

Aquilaria malaccensis (Thymelaeaceae) is one of 25 *Aquilaria* species (agarwood-producing species) distributed throughout Peninsular Malaysia, Indonesia, India, Bangladesh and Thailand (Tajuddin & Yusoff 2010, Nor-Azah et al. 2013). It is considered as one of the most expensive natural products existing today and is known among agarwood traders as the ‘black gold of the forest’ used to make valuable products such as pure agarwood oil, cosmetics, medicines and incense (Nor-Azah et al. 2013). How agarwood is formed is not yet fully understood, but the general understanding is that it is formed and permeates the heartwood as a stress response to wounding, microbial infection or both (Nor-Azah et al. 2013, Siti-Suhaila et al. 2015). Sesquiterpenoids and phenylethyl chromone derivatives are the principal compounds found in the agarwood oil, with α -guaiene, δ -guaiene and α -humulene making up the major volatile

compounds (Nor-Azah et al. 2013). In addition to agarwood essential oil, the water-soluble fraction called hydrosol (also known as floral water) is a valuable by-product obtained by hydrodistillation. Hydrosol contains all the agarwood essential oils in low concentrations, making it suitable for making products where pure essential oil would be considered too strong. Agarwood-based products such as soap, perfume water and drinking water usually incorporate hydrosol water and residual water. As wild *A. malaccensis* populations in natural forests shrink and agarwood demand increases, non-conventional methods in agarwood production need to be explored.

Polyploidisation or chromosome doubling is known to have an effect on many physiological properties of a plant, e.g. producing larger reproductive and vegetative plant parts (Mishra et al. 2010, Dhooghe et al. 2011) and enhancing

secondary metabolite production (Gonzalez & Weathers, 2003, Lavania 1988). Plants such as *Artemisia annua*, *Vetiver* and *Papaver* species exhibited increased key secondary metabolite content through polyploidisation (Hyder et al. 2002, Dhooghe et al. 2011, Ismail et al. 2013). Enhanced content of artemisinin, a secondary metabolite in *A. annua*, was achieved by polyploidisation in its hairy roots. *Datura* and *Papaver* plant species showed very high (almost 100%) increases in secondary metabolites after chromosome doubling (Lavania 1988, Dhooghe et al. 2011). The present study was conducted to identify and compare compounds present in hydrosol and residual water by-products of diploid and polyploid *A. malaccensis* to conclude which plant was better quantitatively (number of chemical constituents detected) and qualitatively (number and amount of key chemical constituents) between the two ploidy levels.

MATERIALS AND METHODS

Plant materials

Four-month old tetraploid plantlets (TC) were compared with three types of control (similar age): soil-grown diploid seedlings (DS), *in vitro*-grown seedlings (DV), and *in vitro*-grown tissue culture-derived plantlets (DC). Plant parts (leaf, stem and roots) were separated and subjected to hydrodistillation processes.

Chemical constituents analysis

Plant parts (leaf, stem and root) from all plant samples were weighed at 200 g and placed into individual round-bottom flasks containing 1 L of distilled water. The samples were boiled for 6 hours. Then, at the end of the process, hydrosol and residual water were collected for further analysis. Hydrosol is water condensed in the Clavenger apparatus and residual water refers to water that is left in the flask. The liquid form samples were subjected to HS-SPME followed by GC-MS analysis (Nor Azah et al. 2016, Abd-Majid et al. 2017). The temperature programme was set initially at 60 °C for 10 min and increased to 230 °C at 3 °C min⁻¹. The final temperature was held for 10 min. The chemical constituents was identified by comparing their mass spectrum with database WileyNist05.L and HPCH2205.L.

Statistical analyses

Mean percentage values (average \pm SD) were used to determine the compounds present in the samples of hydrosol water and residual water from tetraploid and diploid *A. malaccensis*. Peaks were identified by comparing the retention times of the samples with those from the National Institute of Standards and Technology database.

RESULTS AND DISCUSSION

Analyses of hydrosol

Hydrosol from TC leaf samples showed the highest amount of sesquiterpenes (50.64%) than all three controls (Table 1). Controls, namely, DS, DV and DC contained 42.68, 8.49 and 11.91% sesquiterpenes respectively. Furthermore, 61.06% of detected sesquiterpenes in TC were important (i.e. key chemical constituents) agarwood sesquiterpenes. Eudesmol is usually found in high quality agarwood essential oil (Tajuddin & Yusoff, 2010, Nor-Azah et al. 2013) and it was found very high in TC leaf samples. DC samples had the second highest amount of important agarwood sesquiterpenes (20.57%) after tetraploid followed by DS (2.39%). No important agarwood sesquiterpenes were detected in DV. The differences in the amount of sesquiterpenes detected could be due to several factors. Firstly, the conditions where the plants were grown were different. Even though the starting materials, seeds for example, were the same, growing the seeds in soil under normal conditions could develop different types and/or amount of sesquiterpenes than those grown *in vitro*. Secondly, it could be due to the functions of the sesquiterpenes produced whereby they were different in terms of chemical profiling and/or amount of sesquiterpenes. The fact that DV samples produced the least amount of total sesquiterpenes (8.49%) was an indication that the sesquiterpenes produced at an earlier stage might be used for defense mechanisms towards pathogens compared with the rest of the samples. This could explain why samples from DC produced less sesquiterpenes, i.e. almost the same amount produced by DV, because less or no pathogen existed in *in vitro* culture. Thirdly, the differences in the amount of sesquiterpenes produced might be due to different ploidy level, whereby tetraploid produced the highest amount

Table 1 Compounds in hydrosol water of *Aquilaria malaccensis* from field-grown seedlings (DS), *in vitro* seedlings (DV), *in vitro* diploid (DC) and tetraploid (TC) plantlets

Compound	RI	Leaf samples:				Stem samples:				Root samples:			
		DS	DV	DC	TC	DS	DV	DC	TC	DS	DV	DC	TC
Allo-Aromadendrene	1517	-	-	-	-	1.91 ± 3.3	-	-	0.54 ± 1.0	-	-	-	-
3Z-Hexenol		-	1.32 ± 2.0	-	-	-	-	-	-	-	-	-	-
Aromadendrene	1529	-	-	-	-	-	-	-	2.40 ± 4.2	-	-	0.43 ± 1.0	-
Bornyl acetate	1274	-	4.06 ± 1.9	-	-	2.94 ± 5.1	0.66 ± 0.2	-	7.77 ± 7.5	0.21 ± 2.5	-	-	-
Methyl-γ-ionone	1294	-	-	-	-	-	-	-	0.21 ± 0.4	-	-	-	-
Geranyl propanoate	1376	-	-	-	-	-	-	0.52 ± 0.9	0.25 ± 0.4	-	-	-	-
Sibirene	1400	-	-	-	-	-	-	-	-	0.28 ± 3.2	-	-	-
β-Copaene	1419	-	-	-	-	-	-	-	-	-	-	-	0.09 ± 0.4
α-Cadinol	1652	-	-	-	-	-	-	-	-	-	-	-	-
γ-Amorphene	1471	-	-	-	-	-	-	-	-	-	-	-	-
<i>trans</i> -Cadin-1(6),4-diene	1475	1.23 ± 2.1	-	-	-	-	-	-	-	-	-	-	-
α-Agarofuran	1511	-	-	-	-	-	-	-	-	-	-	-	-
γ-Cadinene	1513	0.84 ± 1.5	-	0.77 ± 1.3	-	-	-	-	1.82 ± 3.2	-	-	-	-
<i>cis</i> -α-Guaiene	1516	-	-	-	-	-	-	-	-	2.82 ± 4.8	-	-	0.03 ± 0.0
β-Calacorene	1564	-	-	-	-	3.97 ± 3.8	-	-	-	-	-	-	-
<i>cis</i> -Calamenene	1520	-	-	-	-	2.50 ± 2.2	0.28 ± 2.2	-	-	-	-	0.29 ± 0.5	0.47 ± 0.8
<i>trans</i> -Calamenene	1522	-	-	0.66 ± 1.2	-	-	-	-	-	-	-	-	-
Z-Isocugenol acetate	1531	-	-	-	-	-	-	-	-	-	-	6.0 ± 10	-
9- <i>epi</i> -(E)-Caryophyllene	1556	-	-	-	-	-	-	-	1.14 ± 1.8	-	-	-	-
1,8-Cineole	1011	-	-	-	-	-	1.70 ± 1.9	-	-	-	0.79 ± 0.6	-	-
Z-Isosafrole	1336	-	0.21 ± 0.8	-	-	-	-	-	-	-	-	-	-
Neo-isopulegol	1144	-	0.66 ± 0.5	-	-	-	-	-	-	-	-	-	-
Isobornyl acetate	1274	-	-	-	-	5.68 ± 9.9	-	-	2.03 ± 3.5	-	-	-	-
Citronellal	1143	-	1.12 ± 3.5	-	-	0.91 ± 1.6	-	-	-	-	-	-	-
Isopulegol	1145	-	-	-	-	-	-	-	-	0.08 ± 1.0	-	-	-
Iso-isopulegol	1155	-	-	-	-	-	-	-	-	0.16 ± 3.6	-	-	-
<i>cis</i> -Thujopsadiene	1465	-	-	-	-	1.15 ± 2.0	-	-	-	-	-	-	-
ar-Curcumene	1479	-	-	-	-	-	-	-	-	-	-	-	-
<i>cis</i> -α-Ambrinol	1439	-	0.39 ± 1.0	-	-	-	-	-	0.67 ± 1.2	-	-	-	-

continued

of total sesquiterpenes and the higher amount of important agarwood sesquiterpenes could be due to polyploidisation, i.e. chromosome doubling effect. Other researches also mentioned of the effects polyploidisation towards secondary metabolites (sesquiterpenes) produced were higher compared with its diploid counterparts.

In the stem samples, sample from DS had the highest amount of total sesquiterpenes (94.09%) followed by DC (20.19%), TC (19.95%) and DV (3.29%) samples (Table 1). However, DS stems contained very low amount of important agarwood sesquiterpenes (1.06%). The highest amount of important agarwood sesquiterpenes was obtained from DC (80%) followed by TC (45.11%). Seedlings grown in soil produced the highest amount of sesquiterpenes, possibly for defense mechanism as very high amount was found in hydrosol water of DS. In root samples, the highest amount of sesquiterpenes detected was from DS (82.07%), followed by DC (53%), TC (12.57%) and lastly from DV (7.63%). However, of this, important agarwood sesquiterpenes detected was only 8.5%. Important sesquiterpenes was higher in TC (36.83%) and DV (13.11%). The least amount of important agarwood sesquiterpenes was detected in DC (1.87%). This could be due to the fact that most of the sesquiterpenes produced by DS are for defense mechanism, and less was required from other samples due to *in vitro* growing conditions.

Analyses of residual water

The residual water sesquiterpenes were only detected from DV and TC (Table 2), with higher levels found in the leaf of DV (49.98% of sesquiterpenes), of which 14.31% were important agarwood sesquiterpenes. DC samples contained 19.63% total sesquiterpenes and only 3.45% was important agarwood sesquiterpenes. In stem samples, a total peak area of 5.90% was detected in DV and 1.83% in TC. Unfortunately, none of the sesquiterpenes were important agarwood sesquiterpenes. Among the root tissue samples, DV contained a higher amount of sesquiterpenes (17%) than TC (3.95%). However, TC root samples contained high amount of important agarwood sesquiterpenes (49.1%) while they were detected in DV root samples. The low or undetectable amounts of compounds in residual water indicated that most of the chemicals were released during hydrodistillation. Higher levels

of sesquiterpenes detected in TC compared with diploid plants in the present study, may be due to polyploidisation which enhances secondary metabolite production in plants (Lavania 1988, Chakrabarty et al. 1994, Dhooghe et al. 2011). Another major factor influencing secondary metabolite production is growth condition. Seedlings grown under natural conditions contain high levels of total volatile compounds compared with plant tissues grown under control conditions (*in vitro*) (Achakzai et al. 2009, Nikolova et al. 2013). Production of secondary metabolites can also increase in higher light intensity whereby many such compounds are important in plant defense and produced in response to external threat stimuli (Mole & Waterman 1987, Mole et al. 1988). Therefore, lower levels may be produced in *in vitro* plants that are unlikely to be attacked or damaged by biotic and/or abiotic factors.

The *in vitro* tetraploids in our study produced high levels of important sesquiterpenes, indicating higher gene expression levels in the tetraploid than in the diploid plants. The large quantities of important compounds detected in the hydrosol from all plant samples showed that hydrodistillation can efficiently extract compounds from *A. malaccensis*. The higher yields of secondary metabolites by the tetraploid *A. malaccensis* in our study are consistent with those reported for the tetraploid rose (*Rosa damascene*), which is widely used to produce rose oil and rose water, and is probably triparental in origin (Iwata et al. 2000, Yokoya et al. 2000). Our results indicated that while the total quantity of compounds was about the same in diploid and tetraploid *A. malaccensis*, the composition of these compounds was of better quality in tetraploids, which had relatively higher levels of important sesquiterpenes.

Cells with higher ploidy levels have larger genome sizes, and are expected to have higher levels of transcription than cells with lower ploidy levels (Albuzio et al. 1978, Lavania 1988, Dhawan & Lavania 1996). Therefore, gross expression of most or some of these genes would be expected to increase linearly with ploidy level. Previous studies have demonstrated that different parts of plants produce different secondary metabolites (Springer et al. 2002, Mishra et al. 2010, Nikolova et al. 2013) and the results of our study are in line with these different compounds detected in the leaves, stems and roots of diploid and tetraploid

Table 2 Compounds in residual water of *A. malaccensis* from field-grown seedlings (DS), *in vitro* seedlings (DV), *in vitro* diploid (DC) and tetraploid (TC) plantlets

Compound	RI	Leaf sample				Stem sample				Root sample			
		DS	DV	DC	TC	DS	DV	DC	TC	DS	DV	DC	TC
<i>cis</i> -Calamenene	1520	-	0.31 ± 0.0	-	-	-	-	-	-	-	-	-	0.85 ± 6.01
Elemol	1558	-	3.93 ± 0.4	-	-	-	-	-	-	-	-	-	0.40 ± 0.31
Alfa-Guaiene	1535	-	0.27 ± 2.8	-	-	-	-	-	-	-	-	-	-
α -Cardinol	1652	-	19.25 ± 0.8	-	-	-	-	-	-	-	-	-	-
α - <i>epi</i> -Muurolol	1572	-	7.55 ± 2.0	-	-	-	-	-	-	-	-	-	-
Germacrene D	1492	-	-	-	0.44 ± 0.9	-	-	-	-	-	-	-	0.76 ± 2.32
1,8-Cineole	1011	-	11.81 ± 4.0	-	-	-	-	-	-	-	-	-	-
Viridiflorene	1496	-	-	-	-	-	-	-	-	-	-	-	-
Alfa-Pinene	932	-	-	-	-	-	0.3 ± 0.0	-	-	-	-	-	-
Geraniol	1256	-	-	-	1.31 ± 1.0	-	-	-	-	-	-	-	-
Isopulogenol	1145	-	-	-	-	-	-	-	-	-	-	-	-
Geranial	1265	-	-	-	0.67 ± 0.8	-	-	-	-	-	-	-	-
Geranyl butanoate	1377	-	-	-	5.96 ± 2.8	-	-	-	-	-	-	-	-
Spathulenol	1577	-	-	-	-	-	0.70 ± 0.05	-	-	-	-	-	-
7- <i>epi</i> -Sipiperfol	1345	-	0.08 ± 0.0	-	-	-	-	-	-	-	-	-	-
Citronellyl acetate	1346	-	-	-	1.41 ± 1.2	-	-	-	-	-	-	-	-
Isobornyl acetate	1283	-	-	-	-	-	-	-	-	-	-	-	-
Tetradecane	1388	-	-	-	-	-	-	-	1.83 ± 0.52	-	-	-	-
Bornyl acetate	1274	-	-	-	-	-	2.78 ± 0.3	-	-	-	-	-	-
Epi-Caryophyllene	1408	-	0.29 ± 0.3	-	1.31 ± 0.4	-	-	-	-	-	-	-	-
Trans-Calamenene	1521	-	-	-	-	-	1.25 ± 0.01	-	-	-	-	-	-
Bicyclogermacrene	1424	-	-	-	3.2 ± 0.7	-	-	-	-	-	-	-	-
Curzerene	1493	-	-	-	1.09 ± 1.0	-	-	-	-	-	-	-	-
Citronellol	1223	-	-	-	-	-	-	-	-	-	-	-	-
Trans-Cadina-1916),4-diene	1519	-	-	-	0.47 ± 0.9	-	-	-	-	-	-	-	-
Cuparene	1502	-	-	-	0.32 ± 5.5	-	-	-	-	-	-	-	0.91 ± 0.33
α -Muurolene*	1441	-	-	-	-	-	0.87 ± 0.89	-	-	-	-	-	-
γ -Eudesmol*	1639	-	-	-	-	-	-	-	-	-	-	-	-
γ -Gurjunene*	1530	-	1.16 ± 0.21	-	-	-	-	-	-	-	-	-	-
γ -Muurolene	1425	-	0.381.1	-	3.45 ± 0.3	-	-	-	-	-	-	-	1.03 ± 0.86
δ -Selinene*	1519	-	-	-	0.32 ± 0.01	-	-	-	-	-	-	-	3.95
Total sesquiterpenes identified (%)		0.00	49.98	0.00	19.63	0.00	5.9	0.00	1.83	0.00	17.00	0.00	
Important agarwood sesquiterpenes (%)		0.00	14.31	0.00	3.45	0.00	0.87	0.00	0.00	0.00	0.00	0.00	1.94

RI = retention index; chemical constituents were identified by comparing their mass spectrum with database WileyNist05.L and HPCHE2205.L; - : not detected

A. malaccensis. The enhanced production of important compounds in *in vitro* tetraploids can potentially add more value to *A. malaccensis* than its diploid counterparts.

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