

Enhancement of compounds in hydrosol and residual water of *Aquilaria malaccensis* tetraploid

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ABSTRACT

Aquilaria malaccensis is an agarwood-producing species in the family Thymeleaceae. Agarwood is a fragrant resin used in the manufacture of incense sticks, and in pharmaceutical, perfumery and cosmetic industries. In addition to the resin, hydrosol and residual water by-products from agarwood woodchip distillation are also utilized. Hydrosol contains water-soluble fragrant chemicals used as a tonic drink, in cooking and cosmetics while the residual water is used in spas and aromatic bath treatments. The present study was conducted to identify and compare compounds present in hydrosol and residual water by-products of diploid and polyploid *A. malaccensis*. Four different four-month-old *A.*

25 *malaccensis* plants were compared: soil-grown diploid seedlings (DS), *in vitro*-grown
 26 seedlings (DV), tissue culture-derived plantlets (DC) and artificially induced tetraploid
 27 plantlets (TC). Hydrosol water from TC leaf and root samples were found to contain
 28 higher amounts of compounds compared with other samples. The TC leaf samples were
 29 qualitatively better as key compounds of agarwood such as α - and γ -eudesmol were
 30 detected. TC stem samples also contained higher amounts of key compounds compared
 31 with other samples, while the overall amount of compounds was highest in DS stem
 32 samples. The residual water of TC stem and root samples contained key compounds not
 33 detected in other samples, while DS residual water samples contained the highest total
 34 amount of compounds. *Aquilaria malaccensis* tetraploids performed better than their
 35 diploid counterparts in production of compounds, and thus may be a better planting
 36 material choice for commercial plantations.

37 **Keywords:** *Aquilaria malaccensis*, diploid, tetraploid, compounds

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40 INTRODUCTION

41 *Aquilaria malaccensis* (Thymelaeaceae) is one of 25 *Aquilaria* species (agarwood-producing
 42 species) distributed throughout Peninsular Malaysia, Indonesia, India, Bangladesh and
 43 Thailand [15, 16]. Their agarwood has been traded across Europe, the Middle East and
 44 Asia for more than 2000 years. It is considered to be one of the most expensive natural
 45 products existing today and is known among agarwood traders as the ‘black gold of the
 46 forest’ used to make valuable products such as pure agarwood oil, cosmetics, and
 47 medicines and incense [15]. How agarwood is formed is not yet fully understood, but the
 48 general understanding is that it is formed and permeates the heartwood as a stress response

49 to wounding, microbial infection or both [15, 21]. Agarwood yield increases as the trees
50 get older, and infected trees are able to produce high quality agarwood oil at the age of 20
51 years [4]. The price of agarwood oil increased tremendously from MYR280/tola (12 ml) at
52 the end of 1990s to MYR500/tola recently. AAAAA grade agarwood was recently priced
53 between MYR120,000–360,000/kg. While these high prices benefit traders, the
54 exponential demand for agarwood and illegal harvesting of wild *A. malaccensis* trees from
55 natural forest parks and reserves has caused this species to be listed under CITES (the
56 Convention on International Trade in Endangered Species of Wild Fauna and Flora,
57 Appendix II) [21].

58 Sesquiterpenoids and phenylethyl chromone derivatives are the principal
59 compounds found in the agarwood oil, with α -guaiene, δ -guaiene and α -humulene making
60 up the major volatile compounds [15, 16]. In addition to agarwood essential oil, the water-
61 soluble fraction called hydrosol (also known as floral water) is a valuable by-product
62 obtained by hydro distillation. Hydrosol contains all the agarwood essential oils in low
63 concentrations, making it suitable for making products where pure essential oil would be
64 considered too strong. Agarwood-based products such as soap, perfume water and drinking
65 water usually incorporate hydrosol water and residual water. As wild *A. malaccensis*
66 populations in natural forests shrink and agarwood demand increases, non-conventional
67 methods in agarwood production need to be explored. Chromosome doubling is known to
68 have an effect on many physiological properties of a plant, e.g. producing larger
69 reproductive and vegetative plant parts [5, 6, 7, 10, 11] and enhancing secondary
70 metabolite production [7, 10]. Plants such as *Artemisia annua* L., *Vetiver* and *Papaver*
71 species exhibit increased key secondary metabolite content [6, 8, 9]. Artemisinin content
72 enhancement in *A. annua* was achieved by polyploidization in its hairy roots. *Datura* and

73 *Papaver* plant species showed very high (almost 100%) increases in secondary metabolites
 74 after chromosome doubling [6, 10]. Crops are also known to benefit from the hydrosol
 75 water and residual water, with improvements seen in the yield and quality of some essential
 76 oil crops [24]. Enhancing the compounds in young *A. malaccensis* may help reduce illegal
 77 harvesting of the species. The present study was conducted to identify and compare
 78 compounds present in hydrosol and residual water by-products of diploid and polyploid *A.*
 79 *malaccensis*.

80

81 MATERIALS AND METHODS

82 *Plant materials*

83 Four different four-month-old *A. malaccensis* plant materials were compared: soil-grown
 84 diploid seedlings (DS), *in vitro*-grown seedlings (DV), *in vitro*-grown tissue culture-derived
 85 plantlets (DC) and *in vitro*-grown artificially-induced tetraploid plantlets, polyploids (TC).
 86 Plant parts (leaf, stem and roots) were separated and subjected to hydro distillation
 87 processes.

88

89 *Hydro distillation process*

90 Plant parts (leaf, stem and root) from all plant samples were weighed at 20 g and placed
 91 into individual round-bottomed flasks containing 0.5 L of distilled water. The samples were
 92 boiled for 6 hours at 100 °C. The distilled agarwood compounds were collected in a
 93 Clevenger column. Hydrosol water (water condensed and trapped together with agarwood
 94 oil in the Clevenger column) and residual water (water that was left in the flask) was
 95 evaluated to determine the compounds present.

96

97 *Gas Chromatography-Mass Spectrometry*

98 Samples collected in the Clevenger column were heated at 60 °C for 10 minutes at a rate of
 99 3 °C per minute. The temperature was gradually increased by 68–77 °C to 230 °C and held
 100 at that temperature for 10 minutes. Gases that were released from samples were then
 101 injected into a gas chromatography-mass spectrometry (GC-MS) instrument. Different
 102 compounds vaporized at different times ('retention time', RT). GC-MS was used to analyse
 103 compounds detected at their respective peaks. The mass spectrometer peaks that were
 104 considered for analyses were those that at least more than 90 percent matched to the
 105 library. The peaks were identified by comparing with mass spectrometer of the HPCH
 106 2205.L, Wiley7 NiST05. And NIST0.5a.L. Retention times are used to calculate relative
 107 retention times—dividing the retention time of a compound by the retention time of an
 108 internal standard. Tables of retention indices (RI) were used by comparing experimentally
 109 found retention indices with known values. The RI is given by the equation:

110

111

$$112 \quad I_x = 100 \times [n + (N-n) \{(\log trU - \log trn) / (\log trN - \log trn)\}]$$

113 x : the name of the target compound

114 $n0$: n-alkane $C_{n0}H_{2n0+2}$ directly eluting before x

115 $n1$: n-alkane $C_{n1}H_{2n1+2}$ directly eluting after x

116 RT : retention time (in any unit such as minutes, seconds, etc.)

117 RI : retention index (pure number without unit)

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120

121 *Statistical Analyses*

122 Mean percentage values ($\pm$ SD) were used to determine the compounds present in the
123 samples using solid-phase microextraction (SPME)-GCMS. Peaks were identified by
124 comparing the retention times of the samples with those from the National Institute of
125 Standards and Technology database.

126

127 **RESULTS AND DISCUSSION**

128 **Analyses of hydrosol**

129 Hydrosol from TC leaves showed the highest peak area for compounds with more than 10
130 fold higher than that from DC, DS, and DV (Table 1). In TC leaf samples, five important
131 compounds were detected, i.e. α -, γ - and 10-epi- γ -eudesmol, epi- α -muurolol and δ -
132 selinene. Eudesmol are found in high quality agarwood essential oil (15, 16). Several
133 important compounds were detected in TC leaf samples, but in smaller amounts, i.e. α -
134 and γ -muurolene, α -selinene and γ -gurjunene. Two important compounds were detected in
135 DS leaf samples, i.e. α - and γ -muurolene. Chromosome doubling in *TC* leaves may have
136 resulted in the high amounts of compounds detected in the hydrosol from those samples.

137 Among stem samples, DS had the highest peak area of compounds followed by
138 DC, TC and DV (Table 1). However, DS stems contained a very high level of detectable
139 compounds, only 1.91% of these were important compounds while the rest were aliphatic
140 hydrocarbons except for allo-aromadendrene. On the other hand, DC contained highest
141 level of important compounds followed by TC. Compounds such as α - and γ -gurjunene
142 were detected in DC stems and in lower amounts in TC stems. Other important
143 compounds detected in TC stems were γ -muurolene, α - and δ -selinene, and cis- β -guaiene.

144 The roots of all samples showed the presence of γ -gurjunene with the highest level
145 found in TC followed by DS, DV and TC (Table 1). Several important compounds were
146 detected in DS roots, i.e. *cis*- β -guaiene and α -agarofuran, while DC contained low levels of
147 α -muurolene and aromadendrene compounds detected at a high level in DC was eugenol,
148 which although not an important sesquiterpene in agarwood oil, is valuable as an antiseptic
149 and anesthetic compound in the pharmaceutical industry. Eugenol can be combined with
150 zinc oxide to form zinc oxide eugenol, which has restorative and prosthodontic applications
151 in dentistry. TC contained low levels of other important compounds, i.e. α - and γ -
152 muurolene, α - and δ -selinene, and *cis*- β -guaiene.

153

154 **Analyses of residual water**

155 The residual water of only DV and TC contained compounds, with higher levels found in
156 compounds from TC (Table 2). DV leaf samples had the highest total peak area and the
157 important compounds α -guaiene and γ -eudesmol were present. TC leaves contained a
158 lower level of detectable compounds with a sole important chemical constituent, γ -
159 muurolene. Among the stem samples, a total peak area of 5.90% was detected in DV and
160 1.83% in TC. γ -Eudesmol is an important chemical constituent that was detected in DV.
161 Again, no sesquiterpenes were detected in DS and TC. Among the root tissue samples, DV
162 contained a higher total peak area than TC compounds but almost 50% of the total
163 compounds detected in TC were important, compounds i.e. α -muurolene (0.91%) and δ -
164 selinene (Table 2). DV did not have any important compounds, and no compounds were
165 detected in DS and TC residual water root samples. The low or undetectable amounts of
166 compounds in residual water indicate most of the chemicals were released during hydro

167 distillation. It is notable that a few important compounds were found in DV and TC
168 residual water samples compounds after hydro distillation.

169 The thicker cell walls of DC compared with those of TC may have resulted in
170 retention of these chemical constituents during hydro distillation. In line with the higher
171 levels of compounds found in TC as compared with diploid plants in the present study,
172 previous research has also shown that polyploidization can enhance secondary metabolite
173 production in plants [e.g. 6, 4 and 10]. Another major factor influencing secondary
174 metabolite production is growth condition. Seedlings grown under natural conditions
175 contained the high level of total volatile compounds compared to plant tissues from those
176 grown under control conditions (*in vitro*) [1, 20]. Production of secondary metabolites can
177 also increase in the presence of higher light intensity whereby many such compounds are
178 important in plant defense and produced in response to external threat stimuli [12, 13].
179 Therefore lower levels may be produced in *in vitro* plants that are unlikely to be attacked
180 or damaged by biotic and/or abiotic factors.

181 The *in vitro* tetraploids in our study produced high levels of important
182 sesquiterpenes, indicating higher gene expression levels in the tetraploid than in the diploid
183 plants. The large quantities of important compounds detected in the hydrosol from all
184 plant samples showed that hydro distillation can efficiently extract compounds from *A.*
185 *malaccensis* compounds. The higher yields of secondary metabolites demonstrated by the
186 tetraploid *A. malaccensis* in our study are consistent with those reported for the
187 tetraploid rose (*Rosa damascene*), which is widely used to produce rose oil and rose
188 water in quantity, and is probably triparental in origin [17,23]. Our results indicate that
189 while the total quantity of compounds was about the same in diploid and tetraploid *A.*

190 *malaccensis*, the composition of these compounds was of better quality in tetraploids,
 191 which had relatively higher levels of important sesquiterpenes. Cells with higher ploidy
 192 levels have larger genome sizes, and are expected to have higher levels of transcription than
 193 cells with lower ploidy levels [2, 5, 10]. Therefore, gross expression of most or some of
 194 these genes would be expected to increase linearly with ploidy level. Previous studies have
 195 demonstrated that different parts of plants produce different secondary metabolites [11, 19,
 196 20, 22] and the results of our study are in line with this different compounds were detected
 197 in the leaves, stems and roots of diploid and tetraploid *A. malaccensis*. The enhanced
 198 production of important compounds in *in vitro* tetraploids can potentially add more value
 199 to *A. malaccensis* than its diploid counterparts. Commercial planting of tetraploid *A.*
 200 *malaccensis* yielding higher amounts of these important compounds than the current
 201 diploid plantings.

202

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275

Table 1. Compounds in hydrosol water of *A. malaccensis* from field-grown seedlings, *in vitro* seedlings, and *in vitro* diploid and tetraploid 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±3.30	-	-	0.54±0.94	-	-	-	-
Aromadendrene	1599	-	-	-	-	-	-	-	2.40±4.16	-	-	-	-
Bicyclogermacrene	1494	-	-	-	-	-	-	-	-	-	-	-	3.20±0.74
α -Cadinol	1632	-	-	-	-	-	-	-	-	-	19.25±0.78	-	-
<i>trans</i> -Cadin-1(6),4-diene	1475	1.23±2.14	-	-	-	-	-	-	-	-	-	-	0.47±0.98
γ -Cadinene	1513	0.84±1.46	-	0.77±1.34	-	-	-	-	0.12±0.21	-	-	-	-
β -Calacorene	1564	-	-	-	-	3.97±3.84	-	-	-	-	-	-	-
<i>cis</i> -Calamenene	1590	-	-	-	-	2.50±2.90	0.98±2.23	-	-	-	0.31±0.01	-	-
<i>trans</i> -Calamenene	1592	-	-	0.66±1.15	-	-	-	-	-	-	-	-	-
(E)-Caryophyllene	1408	-	-	-	-	-	-	-	-	-	-	-	1.31±0.20
9- <i>epi</i> -(E)-Caryophyllene	1536	-	-	-	-	-	-	-	1.14±1.97	-	-	-	-
1,8-Cineole	1011	-	-	-	-	-	-	-	-	-	11.81±3.88	-	-
Citronellyl Acetate	1346	-	-	-	-	-	-	-	-	-	-	-	1.41±1.20
Cuparene	1502	-	-	-	-	-	-	-	-	-	-	-	0.32±5.54
<i>ar</i> -Curcumene	1479	-	-	-	-	-	-	-	0.67±1.17	-	-	-	-
Curazene	1493	-	-	-	-	-	-	-	-	-	-	-	1.09±1.02
Elemol	1538	-	-	-	-	-	-	1.24±2.14	-	-	3.93±0.43	-	-
10- <i>epi</i> - γ -Eudesmol	1563	-	-	-	0.58±1.00	-	-	-	-	-	-	-	-
α -Eudesmol	1635	-	-	-	12.34±21.4	-	-	-	-	-	-	-	-
γ -Eudesmol	1632	-	-	-	13.97±6.01	-	-	1.17±2.02	-	-	6.11±1.98	-	-
Geranyl Butanoate	1377	-	-	-	-	-	-	-	-	-	-	-	5.96±2.84
Germacrene D	1492	-	-	-	-	-	-	-	-	-	-	-	0.44±0.98
α -Guaiane	1535	-	-	-	-	-	-	-	0.63±1.08	-	0.27±2.76	-	-
β -Gurjunene	1542	-	-	-	-	-	-	-	1.03±0.95	-	-	-	-
γ -Gurjunene	1530	-	-	0.29±0.50	-	-	-	1.66±2.88	0.32±0.89	-	-	-	-
α -Gurjunene	1509	-	-	-	-	-	-	9.33±12.12	0.72±1.24	-	-	-	-
Hedycaryl	1546	-	-	-	15.39±6.41	-	-	0.45±0.78	-	-	-	-	-
E- β -Ionone	1487	-	0.40±1.20	-	-	-	-	-	-	-	-	-	-
Longifolene	1679	-	-	-	-	-	-	-	5.50±5.01	-	-	-	-
Methyl dodecanoate	1594	-	-	-	0.16±0.27	-	-	-	-	-	-	-	-
Methyl γ -ionone	1479	-	-	-	-	-	-	-	0.31±0.40	-	-	-	-
<i>epi</i> - α -Muurolol	1572	-	-	-	3.71±6.42	-	-	-	-	-	7.55±2.04	-	-
α -Muurolene	1441	0.69±1.20	-	1.13±1.96	-	-	-	-	-	-	-	-	-
γ -Muurolene	1425	0.33±0.56	-	0.79±1.37	-	-	-	-	-	-	0.38±1.09	-	3.45±0.32
Pentadecane	1500	-	-	-	-	-	-	-	0.47±0.82	-	-	-	-
Prenanaspodiene	1669	-	-	-	-	-	-	-	0.63±0.18	-	-	-	-
Rosifolol	1600	-	-	-	-	-	-	0.91±0.58	-	-	-	-	-
Selin-11-en-4- α -ol	1667	-	-	-	-	-	-	3.35±5.88	-	-	-	-	-

DS=field-grown seedlings, DV=*in vitro* seedlings, DC=*in vitro* diploid, TC=tetraploid plantlets, RI=retention index on the HP-5MS capillary column of a homologous series of C_6 - C_{18} *n*-alkanes; - : absent.