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Volatile oil comparison of cotyledon leaves of chemotypes of *Melaleuca alternifolia*

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Abstract

Oil quantity and quality were measured for the cotyledon leaves of the commercial terpinen-4-ol chemical variety of *Melaleuca alternifolia*. Oil yield obtained by ethanolic extraction was 3.8 micrograms per leaf or 2.6% (dry weight basis). The major components of the oil were α-pinene (7.4%), β-pinene (12.0%) and terpinolene (27.3%). The non-commercial terpinolene chemical variety was found to be rich in 1,8-cineole (12.5%) and terpinolene (25.4%). The non-commercial 1,8-cineole chemical variety was rich in 1,8-cineole (37%) with significant quantities of α-pinene (15.5%), β-pinene (23.3%) and terpinolene (10.9%). The cotyledon leaf composition, when compared with that of mature leaf from the same chemical variety, was found to be biased toward pinene and terpinolene biogenetic pathway constituents and hence not a good indicator of mature tree quality especially for the commercial terpinen-4-ol chemical variety. The implications of these analyses for the determination of *M. alternifolia* plantation quality and the understanding of oil formation, are discussed. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Melaleuca alternifolia*; Myrtaceae; Australian tea tree; Essential oil; Composition; Chemotypes; Cotyledon leaf; Terpinen-4-ol; 1,8-Cineole; Terpinolene

1. Introduction

The genus *Melaleuca* approaches *Eucalyptus* in both abundance and diversity amongst Myrtaceae genera in Australia and neighbouring regions. Commercially useful essential oils are sourced from the broadleaved *M. quinquenervia* (niaouli oil) and *M. cajuputi* (cajuput oil) and the small-leaved *M. alternifolia*–*M. linariifolia* complex (Southwell and Lowe, 1999). One of the key issues of the last decade in the successful commercialisation of *M. alternifolia* has been that of choosing the right chemical variety for plantation establishment. Consequently, a reliable, user-friendly method for the determination of the chemical quality of *Melaleuca* plantations at the earliest possible stage of development would be of utmost value to plantation managers, *Melaleuca* researchers and plant biochemists.

Varieties rich in either 1,8-cineole, terpinolene or terpinen-4-ol are known (Brophy et al., 1989; Southwell et al., 1992; Butcher et al., 1994) with the last type being the only variety suitable for commercial development. The development of micro-analytical extraction methods capable of examining the oil content of single leaves weighing less than 10 mg (Brophy et al., 1989; Southwell et al., 1995; Southwell and Lowe, 1999), has made the analysis of the first emerging cotyledon leaves from a germinant as small as a *M. alternifolia* seedling possible.

This paper reports the results of such an investigation where the cotyledon leaf analysis of the commercial terpinen-4-ol *M. alternifolia* chemotype is compared with the non-commercial 1,8-cineole and terpinolene varieties.

2. Results and discussion

In *M. alternifolia*, oil is formed as early as the cotyledon leaf stage of development with a concentration of 2.6% (3.8 μg per leaf) measured for the terpinen-4-ol chemical variety. The oil was, however, not typical of oil extracted by us from mature trees (Table 1; Brophy et al., 1989; Southwell and Lowe, 1999). The terpinen-4-ol variety contained proportions of α-pinene (12%), β-

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Table 1
Concentration and identity of volatiles in the cotyledon leaves of the three chemotypes of *M. alternifolia*

Constituent	RI	Terpinen-4-ol type area % (μg/g)		Cineole type area %		Terpinolene type area %	
		Cotyledon leaves	Parent type	Cotyledon leaves	Parent type	Cotyledon leaves	Parent type
α-Thujene	930	0.7 (185)	1.0	0.4	0.1	tr	1.0
α-Pinene	**937**	**7.4 (1954)**	**2.6**	**15.5**	**3.4**	**tr**	**1.3**
Sabinene	976	0.2 (53)	0.3	tr	0.4	tr	nr
β-Pinene	**979**	**12.0 (3168)**	**0.7**	**23.3**	**0.6**	**0.8**	**0.3**
Myrcene	993	Tr	0.9	1.9	2.1	0.9	1.4
α-Phellandrene	1005	1.1 (290)	0.5	2.2	0.2	tr	3.2
α-Terpinene	1019	0.9 (238)	10.5	–	0.3	tr	0.8
p-Cymene	1028	0.1 (26)	1.5	–	–	tr	–
Limonene	1031	0.8 (211)	0.9	5.9	8.6	1.5	2.6
β-Phellandrene	1032	0.5 (132)	1.0	–	0.2	0.5	0.8
1,8-Cineole	**1035**	**0.5 (132)**	**2.0**	**37.0**	**66.1**	**12.5**	**13.5**
γ-Terpinene	1062	2.2 (581)	21.5	2.4	0.9	2.0	2.8
trans-Sabinene hydrate	1071	0.2 (53)	tr	–	–	–	–
Terpinolene	**1091**	**27.3 (7207)**	**3.6**	**10.9**	**1.0**	**25.4**	**55.3**
Linalool	1101	0.3 (79)	0.1	–	0.1	–	–
cis-Sabinene hydrate	1101	1.3 (343)	tr	–	–	–	–
Terpinen-4-ol	**1185**	**1.0 (264)**	**37.4**	**1.1**	**2.0**	**0.4**	**1.2**
trans-Piperitol	1200	0.3 (79)	tr	–	–	–	–
α-Terpineol	1196	0.4 (106)	2.6	4.7	7.6	1.2	2.3
cis-Piperitol	1212	0.2 (53)	tr	–	–	–	–
Caryophyllene	1435	0.9 (238)	0.6	0.6	0.2	1.1	0.6
Aromadendrene	1456	1.1 (290)	1.8	0.8	1.1	0.9	1.8
allo-Aromadendrene	1478	1.0 (264)	0.7	1.2	0.4	1.2	0.6
Ledene	1512	3.0 (792)	1.3	1.9	1.1	1.6	1.1
Bicyclogermacrene	1513	4.3 (1135)	0.8	0.8	0.1	0.6	0.2
δ-Cadinene	1536	0.6 (158)	1.5	tr	0.6	Tr	1.3
Globulol	1604	1.0 (264)	0.5	1.3	0.2	0.7	0.6
Viridiflorol	1612	0.5 (132)	0.2	0.6	0.2	0.6	0.2

tr, trace; nr, not resolved.

pinene (15%) and terpinolene (12%) far in excess of the parent genotype, along with low levels of γ-terpinene (4%) and a zero level of terpinen-4-ol. The 1,8-cineole variety contained high proportions of 1,8-cineole (40%) as expected along with elevated levels of α-pinene (15%), β-pinene (20%) and terpinolene (10%). On the other hand, the terpinolene variety contained trace levels of α-pinene, β-pinene, and terpinen-4-ol and moderate levels of 1,8-cineole (12.5%) and terpinolene (25.4%) which were consistent with the parent tree. Sesquiterpene component concentrations were also significant in the cotyledon leaves.

Although early leaf composition was significantly different to that of the mature leaf, the differences were consistent enough for plantation quality to be predicted solely on the grounds of cotyledon leaf composition. A cotyledon leaf rich in α-pinene (7.4%), β-pinene (12.0%) and terpinolene (27.3%) and low in 1,8-cineole, terpinen-4-ol, sabinene, *cis*-sabinene hydrate and *trans*-sabinene hydrate is always indicative of the terpinen-4-ol chemical variety mature seedling. A cotyledon leaf rich in the above along with substantial quantities of 1,8-cineole (e.g. 37%, Table 1) is always indicative of the 1,8-cineole chemical variety. One very rich in terpinolene (e.g. 55%, Table 1) with moderate amounts of 1,8-cineole (e.g. 13%) and low concentrations of α-pinene and β-pinene is always indicative of the terpinolene chemical variety mature tree.

Some monoterpenoid and sesquiterpenoid biogenetic pathways are functioning as soon as the first cotyledon leaves appear but the components present indicate that not all pathways have commenced. Pinene, terpinolene and 1,8-cineole formation has commenced. Compounds associated with the sabinene hydrate–terpinen-4-ol–γ-terpinene pathways seem to be formed at later stages of ontogeny. This is of particular interest when compared with previous studies on the ontogenetical changes in the monoterpenoids of *M. alternifolia* leaf (Southwell and Stiff, 1989) where the predominance of thujane constituents (sabinene, *cis*- and *trans*-sabinene hydrates) in the brighter green flush growth of mature trees gave way to a predominance of *p*-menthanes (γ-terpinene and terpinen-4-ol) in the darker green mature leaf. Possible explanations for this include a chemical hydration of sabinene and the sabinene hydrates, secondary enzymic activity to form γ-terpinene and terpinen-4-ol and the cessation of the thujane biogenetic pathway and concomitant increase in γ-terpinene/terpinen-4-ol biosynthesis (Southwell and Stiff, 1989, 1990; Cornwell et al., 1995). The last of these explanations is strongly supported by the sequential onset of the different pathways reported in this *M. alternifolia* cotyledon leaf investigation. The onset of this pathway, the development of related pathways and the implications of this ontogenetic variation are currently under investigation and will be the subject of further communications.

3. Experiments

3.1. Plant material

M. alternifolia seed was obtained from the CSIRO Division of Forestry, Australian Tree Seed Centre for the 1,8-cineole (Seedlot Gr 70), terpinolene (Seedlot St 127) and terpinen-4-ol (Seedlot DL 655) chemical varieties. Propagation was carried out in an ambient temperature glasshouse when temperatures were ranging from minima of approximately 15 °C to maxima of approximately 25 °C. Seed was sown in light commercial potting mix in seedling trays standing in water for bottom irrigation. Germination commenced after 17 days and cotyledon leaves were harvested 8–18 days after emergence.

3.2. Oil determination—quantitative

Twenty cotyledon leaves of the terpinen-4-ol chemical variety only (0.0029 g dry weight) were extracted with n-tridecane (9.5 mg) in ethanol (0.376 g of 0.002% solution). Weight of total oil or component per leaf was then calculated from the resultant GC integral using the pre-determined 0.92 response factor for tea tree oil with respect to the n-tridecane internal standard. The similarity between extract analysis and steam distillation (Brophy et al., 1989; Baker et al., 2000) enabled a direct comparison with the steam volatile oil from the terpinen-4-ol (95-531) and 1,8-cineole (T990728) distillation and terpinolene (90-189) chemotype extraction.

3.3. Oil determination—qualitative

Fifty to sixty cotyledon leaves of the terpinolene and 1,8-cineole chemical varieties were required for extraction with ethanol to obtain a solution concentrated enough for analysis. The leaf oils were analysed and constituents quantified using a Hewlett Packard 5890 chromatograph, 3393A Integrator, 7673A autosampler and an Alltech AT 35 60 m×0.25 mm, 0.2 μm film thickness, mid polarity FSOT column with hydrogen (45 cm/s) as carrier gas, injection port (split 1:50) at 250 °C, flame ionization detector at 300 °C and temperature programming from 60 °C (1 min) to 250 °C at 10 °C/min. Integration percentages were determined by area normalization of total FID response from the injection of a solution of oil in ethanol. GC/MS investigations were performed similarly using a Hewlett Packard 6890 instrument fitted with an HP5-MS 30.3 m×0.25 mm, 0.25 μm film thickness, FSOT column with helium (36 cm/s) as carrier gas, injection port (split 1:50) at 250 °C, mass selective detector (HP 5973) at 250 °C (source) and 150 °C (quad) with transfer line 280° and ion source filament voltage of 69.9 eV. Component identification was made on the basis of mass spectral fragmentation, retention time comparison with authentic constituents and mass spectral and retention matching with commercial (NIST, Wiley and Adams) libraries.

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