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Monoterpenoid accumulation in *Melaleuca alternifolia* seedlings

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Abstract

Individual leaves of the commercial terpinen-4-ol type *of Melaleuca alternifolia* were examined both quantitatively and qualitatively for volatile constituents from the emergence of the first true leaves, through to 6-week-old tenth leaf set material. A GC internal standard addition method was used to measure changes in oil composition and the accumulation of volatile constituents expressed on a dry weight, unit leaf area and whole leaf basis. In the early stages of seedling growth, leaves contained higher concentrations of terpinolene, α-pinene and β-pinene and lower concentrations of terpinen-4-ol, sabinene and *cis*-sabinene hydrate than mature leaf. Concentrations of the former constituents fell and the latter rose by the time leaf set 10 was 6 weeks old. Key constituent, 1,8-cineole remained in similar concentration throughout ontogeny. The variation in concentration of other key constituents during early stages of seedling development suggests that caution is required in extrapolating seedling leaf data to mature tree oil quality.

Keywords: *Melaleuca alternifolia*; Myrtaceae; Australian tea tree; Terpinen-4-ol type; Ethanolic extraction; Oil concentration; Seedling quality; Ontogenesis; Pinene; γ-Terpinene; Terpinolene; *cis*-Sabinene hydrate; Terpinen-4-ol

1. Introduction

Melaleuca alternifolia (Maiden and Betche) Cheel (family Myrtaceae), commonly known as Australian tea tree, is a paperbark tree growing to 8 m, flourishing in swampy conditions and adjacent to water courses in eastern Australia. The leaves of the terpinen-4-ol rich chemical variety give an essential oil (1–3%) with medicinal (especially bactericidal and fungicidal) properties that have secured it a place in the commercial market for more than 60 years (Penfold, 1925; Southwell and Stiff, 1989; Southwell and Lowe, 1999). The biological activity has been found to be related to terpinen-4-ol, the major component of the steam-distilled oil (Penfold and Grant, 1925; Southwell et al., 1993; Carson and Riley, 1995). The existence of other chemical varieties of *M. alternifolia* with lower terpinen-4-ol concentrations lacking the appropriate anti-microbial activity has presented quality control problems for the industry. The most significant of these are the 1,8-cineole (Brophy et al., 1989; Southwell, 1999) and terpinolene (Southwell et al., 1992; Butcher et al., 1994; Southwell, 1999) chemical varieties, some of which can contain as little as 1% terpinen-4-ol. Increasing demand for the terpinen-4-ol type oil has advanced the industry beyond natural stand harvesting to plantation production (Brophy et al., 1989; Southwell and Lowe, 1999) which now sources most of the oil currently on the commercial market.

Plantation establishment requires the discriminant selection of the terpinen-4-ol chemical variety. Mass propagation from seed rather than clones is preferred largely due to cost (Baker, 1999). For seedling production, seed collected from mother trees need not reflect mother tree quality as cross-pollination predominates (Butcher et al., 1992). Hence the early determination of transplant seedling quality is vital for successful plantation establishment. To achieve this, a greater understanding of the initiation times for the various biogenetic pathways involved is required.

Earlier work has established a chemical composition difference between flush growth and older leaf on mature trees (Southwell and Stiff, 1989; Southwell, 1999). More recently, we reported the cotyledon leaf volatile oil composition for three *M. alternifolia* chemical varieties (Southwell and Russell, 2002). As seedlings develop by expressing new leaf sets each of which then age to maturity, leaf composition has the potential

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to vary in two dimensions, with respect to both order of leaf-pair emergence (leaf set number) and age-of-leaf.

Many significant correlations between volatile oil composition and plant development have been observed with other species. In two chemotypes of *Mentha sauveolens* for example, 1,2-epoxymenthyl acetate, piperitone oxide and dihydrocarvone undergo significant changes in concentration as the leaf develops (Hendriks and van Os, 1976). Similar changes are observed with constituents limonene, menthol and menthone in *Mentha* × *piperita* (Brun et al., 1991; Voirin and Bayet, 1996; Rohloff, 1999), sabinene, sabinene hydrate and terpinen-4-ol in marjoram (Croteau, 1977; Circella et al., 1995), limonene and linalool in citrus (Attaway et al., 1967; Kekelidze et al., 1989), thymol and γ-terpinene in thyme (Yamaura et al., 1992), 1,8-cineole and linalool in sweet basil (Johnson et al., 1999), thujone, 1,8-cineole, β-pinene and camphor in sage (Croteau and Karp, 1976), sabinene, *Z*-β-ocimene, caryophyllene and germacrene D in origanum (Maarse, 1974), limonene, *trans*-carveol and carvone in caraway (Bouwmeester et al., 1998) and limonene, phellandrene, anethofuran and carvone in dill (Porter et al., 1983). With the lemon-scented chemotypes of *Leptospermum petersonii*, early seedling leaves are devoid of citronellal, neral and geranial which were only detected in leaves more developed than leaf-set 5 (Brophy et al., 2000).

This investigation reports the quantitative and qualitative differences in the formation of volatile constituents in the leaf of the terpinen-4-ol chemical variety of *Melaleuca alternifolia* in subsequent stages of seedling development. Leaf volatile constituents are monitored from the appearance of the first true leaves through 3-month-old leaf on seedlings ready for transplanting to the 2-year-old tree in plantation awaiting first harvest. The sequential onset of different monoterpenoid pathways is described.

2. Results and discussion

The monoterpenoid content variation in developing *Melaleuca alternifolia* seedling leaves was found to be significant. Although an increase in oil concentration as early leaf sets developed was expected (Croteau, 1977), the dramatic decreases in α-pinene, β-pinene and terpinolene and increases in γ-terpinene, *cis*-sabinene hydrate and terpinen-4-ol concentrations were not. Consequently, caution was found to be necessary in interpreting the results of early-ontogeny oil analyses as an indicator of mature plant oil quality.

2.1. Leaf characteristics

Leaf weights, areas and oil concentrations were measured for *M. alternifolia*, terpinen-4-ol type, seedling leaves at 0, 3 and 6 weeks after emergence (Table 1). Leaf weights increased to maxima of approximately 1.0 mg (dry weight) for 6-week old leaves by leaf set 2 and to approximately 0.5 mg for freshly emerged (0 week-old) material by leaf set 4 (Fig. 1). Leaves were observed to change in shape from obovate cotyledon and first leaf set through to spathulenate (leaf set 2) and oblanceolate (leaf set 3) to lanceolate for leaf sets 4–6 and beyond. Leaf areas peaked at approximately 15–20 mm^2 for leaf sets 2–4 before falling to 10–15 mm^2 for leaf sets greater than 6 (Fig. 2). Oil concentrations per leaf, per unit leaf area and per leaf dry weight increased steadily from leaf set 1 through to leaf set 10 (Table 1) maintaining a constant level by leaf sets 14–18. Concentrations in the freshly emerged leaves were higher, presumably due to the lower leaf weight. Oil content per leaf showed a two-stage accumulation of oil in fresh leaf-sets (ie at 0

Table 1
Weights, areas and oil concentrations (minimum, maximum and mean[a] values) for *Melaleuca alternifolia*, terpinen-4-ol type, seedling leaves

Leaf set no.		Weight	Area	Oil concentration		
		(mg)	(mm^2)	μg/leaf	μg/mg (dry wt)	μg/mm^2
1	Min	0.09	2.65	0.48	1.85	0.12
	Max	0.26	4.00	0.84	5.33	0.24
	Mean	0.18	3.37	0.60	3.87	0.18
2	Min	0.80	12.03	3.71	4.64	0.18
	Max	0.97	20.41	9.20	9.48	0.64
	Mean	0.90	15.62	5.86	6.36	0.40
3	Min	0.79	15.25	5.68	7.01	0.30
	Max	0.86	19.24	6.76	8.56	0.44
	Mean	0.81	17.24	6.16	7.59	0.36
4	Min	0.78	15.10	8.63	10.64	0.51
	Max	1.39	22.12	16.30	11.73	0.77
	Mean	1.08	19.47	12.11	11.15	0.62
5	Min	0.60	10.85	8.07	11.27	0.57
	Max	0.91	18.04	12.32	14.49	0.74
	Mean	0.79	15.24	10.22	13.07	0.68
6	Min	0.73	10.23	9.77	12.26	0.86
	Max	0.95	13.15	11.65	13.38	0.96
	Mean	0.83	11.84	10.62	12.79	0.90
7	Min	0.80	12.45	15.93	19.51	1.24
	Max	0.96	15.12	22.36	24.30	1.51
	Mean	0.89	14.14	19.01	21.24	1.34
8	Min	0.76	12.16	14.43	18.14	1.18
	Max	1.20	18.43	21.77	21.26	1.32
	Mean	0.97	15.25	18.73	19.46	1.23
9	Min	0.94	13.79	16.52	16.78	1.20
	Max	1.41	17.40	23.66	17.57	1.36
	Mean	1.18	15.46	20.24	17.16	1.30
10	Min	0.83	9.94	13.53	16.30	1.29
	Max	1.25	16.17	20.78	16.72	1.36
	Mean	1.05	13.15	17.46	16.55	1.33

[a] Means of 10-leaf collections of three replicates for each of 3 leaf ages (0, 3, 6 weeks).

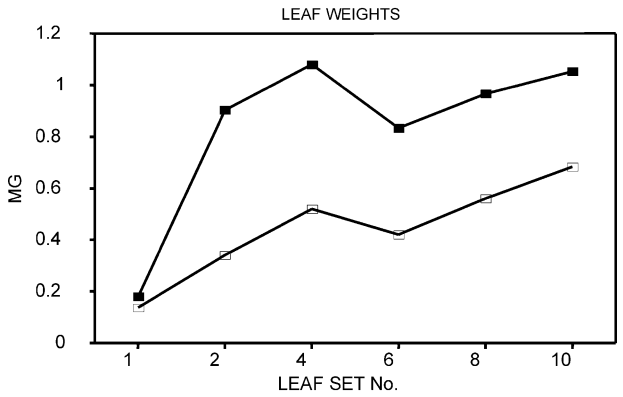

Fig. 1. The mean weights (mg) of individual leaves along a tea tree seedling stem for leaf sets from 1–10 at age 0 (□) and age 6 (■) weeks.

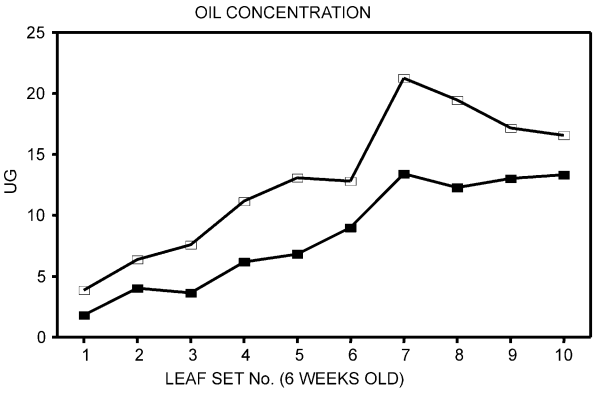

Fig. 3. The mean oil concentrations (□ μg oil/mg dry wt, ■ μg oil/10 mm^2 surface area) of individual leaves along a tea tree branch for leaf sets from 1–10 for 6-week-old *M. alternifolia*, terpinen-4-ol type.

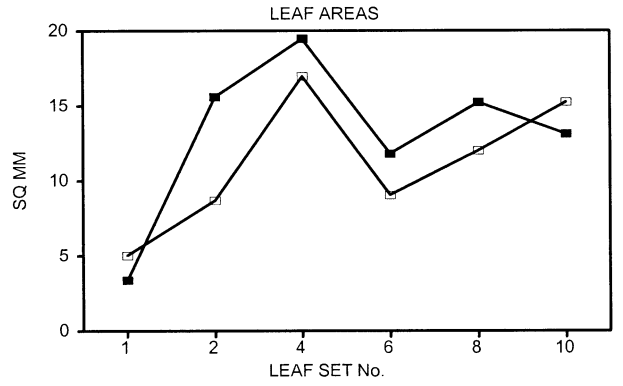

Fig. 2. The mean areas (mm^2) of individual leaves along a tea tree seedling stem for leaf sets from 1—10 at age 0 (□) and age 6 (■) weeks.

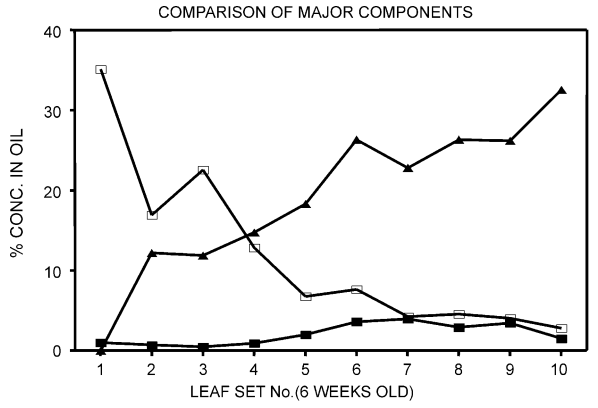

Fig. 4. The mean concentrations (%) for 1,8-cineole (■), terpinen-4-ol (▲) and terpinolene (□) in leaf sets 1–10 for 6-week-old *M. alternifolia*, terpinen-4-ol type.

weeks) at leaf-sets 3–4 and at 7–9. Further small increases occurred as these sets aged to 3 and 6 weeks. Leaf oil concentration increased along the stem (Fig. 3).

2.2. Oil characteristics

The composition of the volatile constituents in early seedling leaves was found to be similar to the composition of the cotyledon leaves (Southwell and Russell, 2002) but significantly different to the composition of mature leaves (Brophy et al., 1989; Southwell and Stiff, 1989; Southwell, 1999) found in natural stands or plantations. It was only when leaf set 10 had reached age 6–8 weeks (seedling age approximately 4 months) that the volatile oil composition approached that of a mature leaf oil.

Components were separated into three groups: those that increased in concentration as seedling leaf developed (e.g. terpinen-4-ol), those that decrease (e.g. terpinolene) and those that maintain relatively constant concentration (e.g. 1,8-cineole) (Fig. 4). The major components terpinen-4-ol and γ-terpinene, both presumably produced enzymically or non-enzymically from the terpinen-4-yl cation, were seen to increase significantly in concentration (%), micrograms per leaf, micrograms per unit leaf area and μg per mg per leaf. The pinenes, sabinene, terpinolene and *cis*-sabinene hydrate decreased in concentration while cineole and α-terpineol remained almost constant.

For example, the major components, α-terpinene, γ-terpinene and terpinen-4-ol increased in concentration from 0.07 (1.4%), 0.25 (4.4%) and 0.0 (0.0%) μg/mg respectively for 0-week-old leaf set 1 material to 0.41 (2.5%), 3.44 (20.8%) and 5.38 (32.5%) μg/mg respectively for 6-week-old leaf set 10 material. Not all terpenoid biogenetic pathways seem to have been initiated by the early leaf set 1—age 0-week stage. At age 3-weeks, analysis indicated concentrations that were approaching the 6-week values and so have not been included in the figures. The rapid weight and area increases evident between leaf sets 1 and 3 were sometimes reflected as unusual peaks or valleys in the plots of component concentrations (e.g. % terpinolene in Fig. 4).

In observing percentage concentration changes, α-pinene (20%), β-pinene (23%) and terpinolene (40%) were the dominant components at 0 weeks for early leaf set material. This was in contrast to the mature leaf (e.g. leaf set 10 at age 6 weeks) where α-pinene was typically 4%, β-pinene 2% and terpinolene 3%. The higher proportions in the younger leaf may not have indicated an absolute change if other biogenetic pathways were initiated later and added more metabolites to the pool. Measurement of metabolites (μg) per leaf, per unit leaf area (10 mm^2) or per mg dry weight however indicated that the absolute concentrations also varied as the leaf developed. The varying proportions (% of volatile constituents) and concentrations (μg/leaf) of the key components β-pinene, γ-terpinene, terpinolene, *cis*-sabinene hydrate and terpinen-4-ol in seedling ontogeny, are shown in Fig. 5.

β-Pinene, a minor (0.3%) component in commercial tea tree oil (Brophy et al., 1989), contributes in excess of 20% (or 0.6 μg/leaf) to the volatile constituents of early seedling leaves and 2% (0.2 μg/leaf) to later seedling leaves (Fig. 5A). A similar significant decrease in β-pinene concentration has been observed with sage where the steam distillation of immature leaves (2–3 cms) gave an oil with 20% β-pinene compared with 3% for the fully expanded (>5 cm) leaves (Croteau and Karp, 1976).

α-Pinene, another product of pinene synthase (Croteau, 1987; Lewinsohn et al., 1992) was similarly recorded at elevated concentrations in early leaf sets consistent with early enhanced pinene synthase activity. The preference for (+)-α-pinene formation in *M. alternifolia* (Leach et al., 1993; Cornwell et al., 1995; Southwell, 1999) suggests the involvement of a (+)-pinene synthase that is capable of binding to the left-handed screw-sense isomer of geranyl pyrophosphate (GPP). This then affords the 3*R* enantiomer of linalyl pyrophosphate (LPP) as acyclic precursor (Croteau, 1987) with the same stereochemistry that dominates the pinene synthesis in citrus, fennel and sage (Croteau, 1987; Croteau et al., 1989; Wheeler et al., 1990) but with the opposite stereochemistry of grand fir, *Abies grandis* (Gijzen et al., 1992; Lewinsohn et al., 1992; Bohlmann et al., 1997) and lodgepole pine *Pinus contorta* (Savage et al., 1995). This is consistent with a preferential binding of left handed screw form of GPP to form the bound 3R-LPP as also envisaged for the production of *cis*-sabinene hydrate in *M. alternifolia* (Southwell and Stiff, 1990). In grand fir, the wounding of stems induces monoterpene cyclases of which (-)-pinene synthase is the most significant (Gijzen et al., 1992).

α-Terpinene, the third most abundant (approx. 10%) constituent in commercial tea tree oil (Brophy et al., 1989), does not occur in leaf extracts in concentrations equivalent to leaf oils (Brophy et al., 1989; Southwell and Stiff, 1989; Cornwell et al., 1995). In this investigation, α-terpinene never exceeded 4% confirming lower concentrations by extraction procedures and supporting suggestions that α-terpinene arises largely as an artefact in *M. alternifolia*.

γ-Terpinene, the second most abundant (approx. 20%) terpene in commercial tea tree oil (Brophy et al., 1989) increases in concentration 10-fold from approximately 0.4 μg/leaf (5%) in early young leaf sets to approximately 4 μg/leaf (20%) for older leaf-set 10 material (Fig. 5B). Although the presence of a γ-terpinene cyclase has been observed in the induced calli of grand fir (Lewinsohn et al., 1994), γ-terpinene has also been proposed as an artefact or breakdown product of *cis*-sabinene hydrate (Cornwell et al., 1995) following deprotonation of the terpinen-4-yl cation or a product of secondary metabolism subsequent to the action of sabinene hydrate cyclase (Southwell and Stiff, 1989).

Terpinolene, like the pinenes, was found to be present in much higher concentrations in early leaf sets (max. 1.8 μg/leaf, 40%) than in mature leaves beyond leaf set 10 (0.5 μg/leaf, 3%) (Fig. 5C). Although structurally consistent with formation as a deprotonation product of the terpinen-4-yl cation, the minimum concentrations of other similarly derived *p*-menthanes (e.g. α- and γ-terpinene) suggests the early activation of a terpinolene synthase. The existence of terpinolene chemotypes of both *M. alternifolia* and *M. trichostachya* (Southwell et al., 1992) supports this supposition.

cis-Sabinene hydrate was found to increase rapidly from zero levels to 3 μg/leaf (24%) only in freshly emerged leaf material. By the time these leaves had aged 3 weeks, concentrations were greatly reduced and after 6 weeks were close to zero (Fig. 5D). This decrease in concentration with age has been reported in *M. alternifolia* mature tree flush growth (Southwell and Stiff, 1989) and is in contrast with the constant concentrations reported for the sabinene hydrate cyclase products from marjoram (Hallahan and Croteau, 1988, 1989). In addition, the *cis*:*trans* sabinene hydrate ratios of >7:1 were consistent with *M. alternifolia* mature tree flush growth (Southwell and Stiff, 1989) and similar to those reported for marjoram (Hallahan and Croteau, 1988, 1989; Novak et al., 2000).

Terpinen-4-ol also increased from minimal concentrations, especially when leaf set 1 was 6 weeks old, to levels consistent, for leaf-set 10 at age 6 weeks (5.7 μg/leaf, 35%), with mature leaf. In contrast with *cis*-sabinene hydrate however, terpinen-4-ol levels increased substantially as the leaf aged (Fig. 5E). This inverse relationship, between terpinen-4-ol and *cis*-sabinene hydrate concentrations over time is consistent with the along-the-branch analyses previously reported (Southwell and Stiff, 1989) that traced the ontogeny of leaves along a single branch. The composition of young marjoram tissue has been reported to increase in the concentration of most constituents whilst that of *cis*-

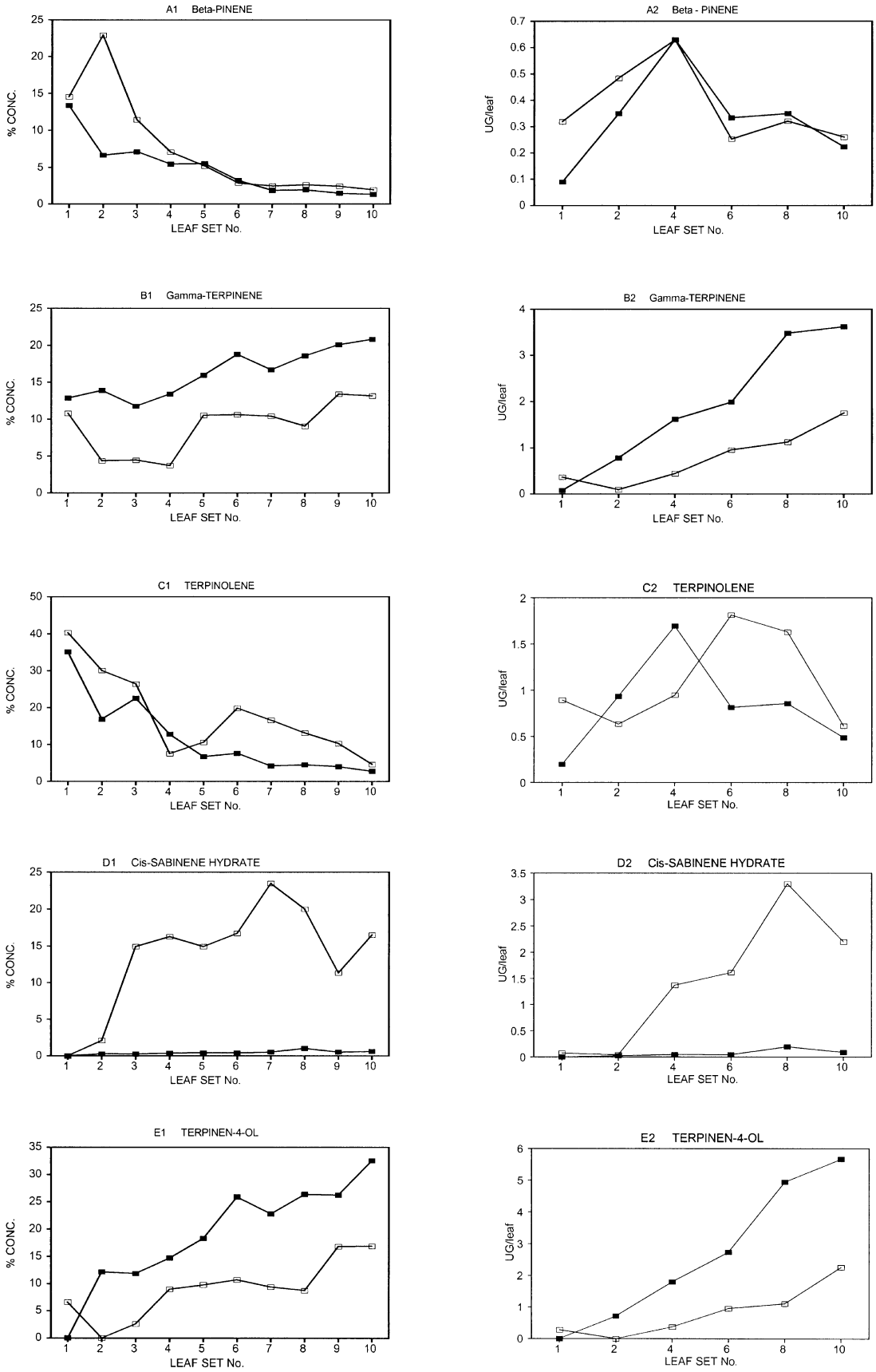

Fig. 5. Variation in the concentrations (% [1], μg/leaf [2]) of key components β-pinene (A), γ-terpinene (B), terpinolene (C), *cis*-sabinene hydrate (D) and terpinen-4-ol (E) of individual leaves along a tea tree seedling stem for leaf sets from 1–10 at age 0 (□) and age 6 (■) weeks.

sabinene hydrate acetate, the major component decreases (Hallahan and Croteau, 1988).

1,8-Cineole (Fig. 4) and α-terpineol, minor constituents, showed small concentration increases to mature leaf levels for both increasing age and increasing leaf set numbers, especially for later leaf sets. These trends were similar to those for γ-terpinene and terpinen-4-ol. Although 1,8-cineole is achiral, the enantiomeric excess strongly in favour of (+)-4*R*-α-terpineol and (+)-4*R*-limonene (Leach et al., 1993; Southwell, 1999) suggests that in *M. alternifolia*, as in sage (Croteau et al., 1994), the cyclase binds preferentially to the left-handed screw form of GPP to form (3*R*)-LPP en route to the 4*R*-terpinyl cation which, on termination with water forms 1,8-cineole and predominantly (+)-4*R*-α-terpineol. This stereochemistry is consistent with the above-mentioned biosynthesis of the pinenes and previously recorded observations regarding the formation of the sabinene hydrates and terpinen-4-ol in *M. alternifolia* (Southwell and Stiff, 1990).

Sabinene, (also a minor component) on the other hand, decreased in concentration both with increasing age and increasing leaf set numbers, especially with later leaf sets, in a manner similar to *cis*-sabinene hydrate.

These investigations indicate that seedling leaf volatile oil analyses are not indicative of the quality of mature leaf and hence could be misleading as a determination of the quality of a potential plantation unless sampling was from leaf set 10 material of age 6-weeks or older. As for marjoram (Croteau, 1977), young rapidly expanding leaves of *M. alternifolia* synthesize monoterpenes most rapidly and this ability decreases as the leaves expand. Pinene and terpinolene synthases seem most active in early leaf sets. Sabinene hydrate cyclase is then activated for the production of *cis*- and to a lesser extent *trans*-sabinene hydrate. The concentrations of these products fall rapidly as each leaf set ages. It has been suggested (Cornwell at al., 1995) that the *p*-menthanes terpinen-4-ol, α- and γ-terpinene, terpinolene and *p*-cymene are unlikely to be enzymic products but rather artefacts derived from *cis*-sabinene hydrate, *trans*-sabinene hydrate and sabinene. From a quantitative viewpoint however, the mean leaf content of the thujane precursors (<5 μg/leaf) does not account for the quantities of *p*-menthanes (>11 μg/leaf) detected. Hence the latter are, to some extent at least, the products of terpinene synthases.

3. Experimental

3.1. Plant material

M. alternifolia, terpinen-4-ol type, seed was obtained from the Australian Tree Seed Centre, CSIRO Forestry and Forest Products, Canberra (Seedlot DL 655). 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 when cotyledon leaves appeared. True leaf sets (pairs) then emerged in the following time sequence (sets are numbered from the first leaf pair closest to the soil to the final pair closest to the growing tip) and were harvested accordingly: leaf set 1 (11 days after germination), 2 (19 days), 3 (23 days), 4 (31 days), 5 (40 days), 6 (51 days), 7 (54 days), 8 (59 days), 9 (67 days), 10 (75 days). The seedlings were then transplanted out into a red Krasnozem soil field situation at the Wollongbar Agricultural Institute.

3.2. Sampling

Ten leaves were bulked for each measurement and the mean value for each leaf recorded. This sampling and measuring procedure was completed in triplicate. Each leaf set was sampled at age 0 weeks (ie freshly emerged), 3 weeks and 6 weeks. Consequently, data in Table 1 are means of 90 leaves (10 leaves×3 ages×3 replicates) and that plotted in Figs. 1–5 are the means of 30 leaves (10 leaves×3 replicates all at same age).

3.3. Leaf weight and area

Leaf samples were bulked, oven dried and weighed after extraction. For area determinations, replicates of 10 leaves were collected, immediately placed on a Petri dish and photocopied to produce a permanent record of leaf area before dehydration caused leaf area shrinkage. Areas were then measured using a HP Scanjet 3C computer scanner and Deltascan software and mean values calculated.

3.4. Oil determination–quantitative

Ten fresh leaves were added to an accurately weighed solution of *n*-tridecane (0.02 mg/g) in ethanol (1 ml) in a tared vial. Extraction commenced with 10 s microwave irradiation (700 W) followed by 24 h at 20 °C. Weight of total oil and individual components per leaf was calculated from the resultant GC integral using the predetermined response factor (0.92 for tea tree oil) with respect to the *n*-tridecane internal standard. Each vial was then concentrated at 75 °C for 16 h for dry weight determination.

The leaf extracts were analysed and constituents quantified using a Hewlett Packard 5890 chromatograph, 3393A Integrator, 7673A autosampler and an Alltech AT35 60 m×0.25 mm, 0.2 μm film thickness, mid polarity FSOT column with hydrogen (45 cms/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 the total FID response from the injection of a solution of extract in ethanol.

3.5. Oil determination—qualitative

For constituent identification, 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 °C 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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