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Monoterpenoid accumulation in 1,8-cineole, terpinolene and terpinen-4-ol chemotypes of *Melaleuca alternifolia* seedlings

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Abstract

Individual leaves of the three most common chemotypes *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. The 1,8-cineole and terpinolene chemotypes were investigated and compared with the recently reported commercial terpinen-4-ol chemotype. The 1,8-cineole chemotype was found to accumulate 1,8-cineole and associated *p*-menthanes limonene, terpinen-4-ol and α-terpineol gradually with increasing leaf set number. As with the terpinen-4-ol variety, higher than expected concentrations of the pinenes and terpinolene were found only in the early leaf sets. The terpinolene variety showed two stages of terpinolene accumulation, the first at leaf sets 2–3 similar to the unexpected biosynthesis of terpinolene in the terpinen-4-ol chemotype and the second at leaf sets 8–9 which is characteristic of the terpinolene variety.

Keywords: *Melaleuca alternifolia*; Myrtaceae; Australian tea tree; 1,8-Cineole type; Terpinolene type; Terpinen-4-ol type; Ethanolic extraction; Oil concentration; Seedling quality; Ontogenesis; β-Pinene; 1,8-Cineole; Terpinolene; Terpinen-4-ol

1. Introduction

Australian tea tree, *Melaleuca alternifolia* (Maiden and Betche) Cheel (family Myrtaceae) has been the subject of extensive investigation in recent years (Southwell and Lowe, 1999) due to the medicinal value (Southwell et al., 1993; Carson and Riley, 1995; Markham, 1999) of the essential oil obtained from the terpinen-4-ol chemotype. The existence of a number of chemotypes is well established. Penfold et al. (1948) distinguished three chemotypes based on oils with low, medium or high 1,8-cineole concentration or conversely on oils with high, medium or low terpinen-4-ol concentrations. As more populations were investigated, this distinction was questioned (Lassak, 1988; Brophy et al., 1989; Southwell, 1999). More recently however, Butcher et al. (1994) used principal component analysis to extend the species to five chemotypes by adding two chemotypes rich in terpinolene (Southwell et al., 1992). The new chemotypes both had moderate levels of 1,8-cineole (17–36%) with one favouring terpinolene (28–57%) over terpinen-4-ol (1–2%) and the other terpinen-4-ol (15–20%) over terpinolene (10–18%). A similar natural variation investigation (Homer et al., 2000), followed by a two dimensional correspondence analysis (Lee et al., 2002), added a sixth chemotype. This third terpinolene type gave an oil rich in 1,8-cineole (47–64%) dominating both terpinolene (6–15%) and terpinen-4-ol (<4%). Consequently the selection of the correct chemotype for commercial plantation establishment is critical.

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,

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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).

Although some modern biochemical and genetic work on *M. alternifolia* is commencing (Rossetto et al., 1999a,b, 2000; Doran et al., 2002) there is still much to be learnt from oil analyses about the early stages of terpenoid pathway development.

We have recently reported on the composition of the predominantly monoterpenoid volatile oil in the seedling cotyledon leaves of three chemotypes of *M. alternifolia* (Southwell, 1999; Southwell and Russell, 2002) and followed oil accumulation through to mature seedling leaves with the commercial terpinen-4-ol chemical variety (Southwell, 1999; Russell and Southwell, 2002).

The sequential onset of different biogenetic pathways in these early stages of ontogeny has significant implications for tea tree producers transplanting seedlings from greenhouse to the field. To avoid costly replanting, the discriminant selection of the terpinen-4-ol chemical variety is essential. However, we have shown that the early analysis of seedling leaf volatiles can be misleading. The terpinen-4-ol variety for example, at early stages, could be mistaken for the terpinolene variety because of the predominance of the terpinolene pathway in early leaf-sets (Russell and Southwell, 2002).

We now report the accumulation of monoterpenoids in the terpinolene and 1,8-cineole varieties from germinant to transplant, compare biogenesis with the terpinen-4-ol variety and discuss chemotype differentiation.

2. Results

Leaf weights, areas and oil concentrations were measured for *M. alternifolia*, 1,8-cineole and terpinolene types (Table 1) for comparison with the terpinen-4-ol

Table 1
Weights, areas and oil concentrations (minimum, maximum and mean[a] values) for *Melaleuca alternifolia*, terpinen-4-ol, 1,8-cineole and terpinolene type, seedling leaves

Leaf no.		Terpinen-4-ol type					1,8-Cineole type					Terpinolene type				
		Wt (mg)	Area (mm^2)	μg oil /leaf	μg oil/ mg	μg oil/ mm^2	Wt (mg)	Area (mm^2)	μg oil/ leaf	μg oil/ mg	μg oil/ mm^2	Wt (mg)	Area (mm^2)	μg oil/ leaf	μg oil/ mg	μg oil/ mm^2
1	Min	0.09	2.65	0.48	1.85	0.12	0.17	6.57	0.12	0.46	0.01	0.14	4.63	0.41	2.16	0.07
	Max	0.26	4.00	0.84	5.33	0.24	0.26	10.53	0.28	1.41	0.04	0.22	5.97	0.77	3.64	0.15
	Mean	**0.18**	**3.37**	**0.60**	**3.87**	**0.18**	**0.21**	**8.40**	**0.18**	**0.92**	**0.02**	**0.18**	**5.24**	**0.56**	**3.10**	**0.11**
2	Min	0.80	12.03	3.71	4.64	0.18	0.52	23.59	3.43	5.55	0.14	0.34	9.05	1.40	4.00	0.15
	Max	0.97	20.41	9.20	9.48	0.64	1.08	33.31	6.00	6.59	0.18	0.53	12.49	2.90	8.54	0.32
	Mean	**0.90**	**15.62**	**5.86**	**6.36**	**0.40**	**0.74**	**27.97**	**4.39**	**6.09**	**0.16**	**0.41**	**10.40**	**2.25**	**5.73**	**0.22**
3	Min	0.79	15.25	5.68	7.01	0.30	0.69	27.89	4.90	5.63	0.18	0.68	15.96	5.76	8.47	0.36
	Max	0.86	19.24	6.76	8.56	0.44	1.22	33.06	9.31	7.63	0.28	1.09	22.76	10.33	9.47	0.45
	Mean	**0.81**	**17.24**	**6.16**	**7.59**	**0.36**	**0.97**	**29.68**	**6.63**	**6.79**	**0.22**	**0.92**	**20.49**	**8.35**	**8.97**	**0.40**
4	Min	0.78	15.10	8.63	10.64	0.51	0.91	19.85	6.39	6.59	0.31	0.56	16.11	7.31	8.43	0.37
	Max	1.39	22.12	16.30	11.73	0.77	1.16	27.03	9.83	8.48	0.37	0.98	20.40	8.26	13.57	0.45
	Mean	**1.08**	**19.47**	**12.11**	**11.15**	**0.62**	**1.01**	**22.50**	**7.83**	**7.68**	**0.35**	**0.79**	**18.95**	**7.72**	**10.24**	**0.41**
5	Min	0.60	10.85	8.07	11.27	0.57	0.62	12.48	7.15	8.11	0.44	0.79	10.92	6.13	6.98	0.43
	Max	0.91	18.04	12.32	14.49	0.74	1.13	20.67	9.16	13.63	0.68	1.01	15.73	7.05	7.76	0.56
	Mean	**0.79**	**15.24**	**10.22**	**13.07**	**0.68**	**0.86**	**15.81**	**8.25**	**10.15**	**0.54**	**0.91**	**13.06**	**6.63**	**7.34**	**0.52**
6	Min	0.73	10.23	9.77	12.26	0.86	0.75	12.78	6.19	8.25	0.48	0.84	9.60	6.25	7.44	0.65
	Max	0.95	13.15	11.65	13.38	0.96	1.02	17.03	11.66	12.15	0.69	0.91	9.96	8.12	9.55	0.82
	Mean	**0.83**	**11.84**	**10.62**	**12.79**	**0.90**	**0.91**	**15.58**	**8.97**	**9.76**	**0.57**	**0.87**	**9.81**	**7.47**	**8.61**	**0.76**
7	Min	0.80	12.45	15.93	19.51	1.24	0.76	11.61	8.58	10.86	0.74	0.78	9.71	6.25	7.81	0.62
	Max	0.96	15.12	22.36	24.30	1.51	0.79	12.61	10.11	13.13	0.81	0.98	11.66	9.42	9.90	0.81
	Mean	**0.89**	**14.14**	**19.01**	**21.24**	**1.34**	**0.77**	**12.22**	**9.46**	**12.24**	**0.77**	**0.85**	**10.46**	**7.80**	**9.11**	**0.74**
8	Min	0.76	12.16	14.43	18.14	1.18	0.71	11.73	10.32	12.29	0.83	0.77	7.82	5.21	6.77	0.67
	Max	1.20	18.43	21.77	21.26	1.32	0.84	14.24	14.14	17.25	0.99	1.02	9.68	7.70	8.19	0.88
	Mean	**0.97**	**15.25**	**18.73**	**19.46**	**1.23**	**0.79**	**12.82**	**11.78**	**14.95**	**0.92**	**0.91**	**8.75**	**6.77**	**7.40**	**0.77**
9	Min	0.94	13.79	16.52	16.78	1.20	1.06	14.74	10.20	8.72	0.69	10.7	10.99	7.36	6.87	0.67
	Max	1.41	17.40	23.66	17.57	1.36	1.29	18.20	13.58	12.81	0.85	1.43	14.83	13.23	9.25	0.89
	Mean	**1.18**	**15.46**	**20.24**	**17.16**	**1.30**	**1.17**	**16.32**	**12.15**	**10.45**	**0.75**	**1.30**	**13.07**	**10.77**	**8.17**	**0.81**
10	Min	0.83	9.94	13.53	16.30	1.29	1.02	14.00	11.85	9.26	0.73	1.29	11.89	10.06	7.80	0.85
	Max	1.25	16.17	20.78	16.72	1.36	1.31	16.58	14.59	13.39	0.97	15.3	14.73	13.92	9.10	0.95
	Mean	**1.05**	**13.15**	**17.46**	**16.55**	**1.33**	**1.14**	**15.22**	**12.86**	**11.42**	**0.85**	**1.37**	**13.07**	**11.55**	**8.39**	**0.88**

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

type (Russell and Southwell, 2002). Leaf weights were similar across chemotypes showing accelerated expansion at leaf sets 1–3 and 8–9 increasing to maxima of approximately 0.8 and 1.2 mg (dry weight) respectively for 6-week old leaves (Fig. 1A).

The terpinen-4-ol type 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. The leaves of the terpinolene variety were a similar size but more obovate and those of the 1,8-cineole type larger and more elliptical. Leaf areas for both the terpinolene and terpinen-4-ol types 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. 1B). The 1,8-cineole type however was clearly distinguished by a 50% larger leaf area (30 mm^2) at leaf sets 2–3. By leaf set 7 however, these leaves were the same size as those of the other varieties.

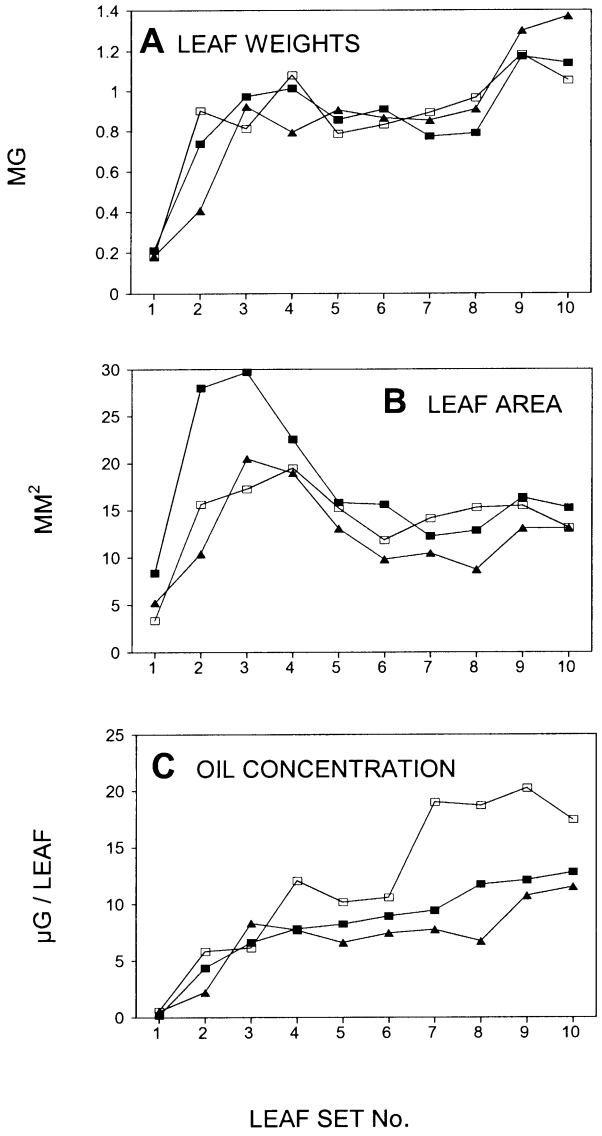

Fig. 1. The mean (A) weights (mg), (B) areas (mm^2) and (C) concentrations (μg/leaf) for leaf sets from 1 to 10 at leaf age 6 weeks for the 1,8-cineole (■), terpinen-4-ol (□) and terpinolene (▲) chemotypes of *M. alternifolia*.

Oil concentrations increased steadily for all three chemotypes from leaf set 1 through to leaf set 10 (Table 1) maintaining a relatively constant level by succeeding leaf sets. Concentrations in the freshly emerged leaves (0 weeks old) were often higher than for 6 week old leaf (e.g. 40% for 1,8-cineole type at leaf sets 5 and 10) indicating oil accumulation prior to the completion of biomass increase. Oil content per leaf (Fig. 1C) again showed a two-stage accelerated accumulation of oil in 6-week old leaf sets at leaf sets 1–4 and at 6–9. Leaf oil concentration increased along the stem (Fig. 1C) for all three chemotypes with the terpinen-4-ol type plateauing at 17, the 1,8-cineole type at 13 and the terpinolene type at 12 μg/leaf.

In the 1,8-cineole type, the concentration of 1,8-cineole, expressed as both oil (μg) per leaf (Fig. 2A1) and proportion (%) (Fig. 2B1), increased steadily from <1 μg/leaf at leaf set one to >6 μg/leaf by leaf set 10. This increase in concentration may not however be indicative of a proportional increase with respect to total oil. 1,8-Cineole concentration, when measured as a proportion of the total oil, decreased from 50 to 33% as the leaf expanded during leaf sets 1–3 and then increased gradually until the second phase of leaf expansion (leaf sets 8–9) when % concentration increased again to 50% (Fig. 2B1). β-Pinene, present in only trace amounts in the mature leaf of this variety, contributed strongly (23%) to leaf set 1, much less (8%) to leaf set 2 with a gradual subsequent decrease to 1% by leaf set 10. Terpinolene, also present in only trace amounts in the mature leaf of this variety, contributed 5% in leaf sets 1–4 and then decreased abruptly to only traces from leaf set 5. 1,8-Cineole minor component congeners limonene, α-terpineol and terpinen-4-ol appear only at leaf set two (Fig. 2.2) indicating that they may not be by-products of the 1,8-cineole pathway which is active for the first leaf set.

In the terpinolene type, the 1,8-cineole concentration (μg/leaf) increased steadily with increasing leaf sets but much more rapidly at the leaf set 2–3 stage (Fig. 2A3) than for the 1,8-cineole variety where the greatest acceleration in 1,8-cineole accumulation occurred at leaf sets 8–9 (Fig. 2A1). In contrast, the concentration of terpinolene, the other major component, was low between the early (leaf sets 2–3) and late (leaf sets 8–9) terpinolene accumulation phases (Fig. 2.3). The concentration variation of terpinen-4-ol, a minor component in this variety, mirrored the terpinolene variation but at lower concentrations (Fig. 2.3).

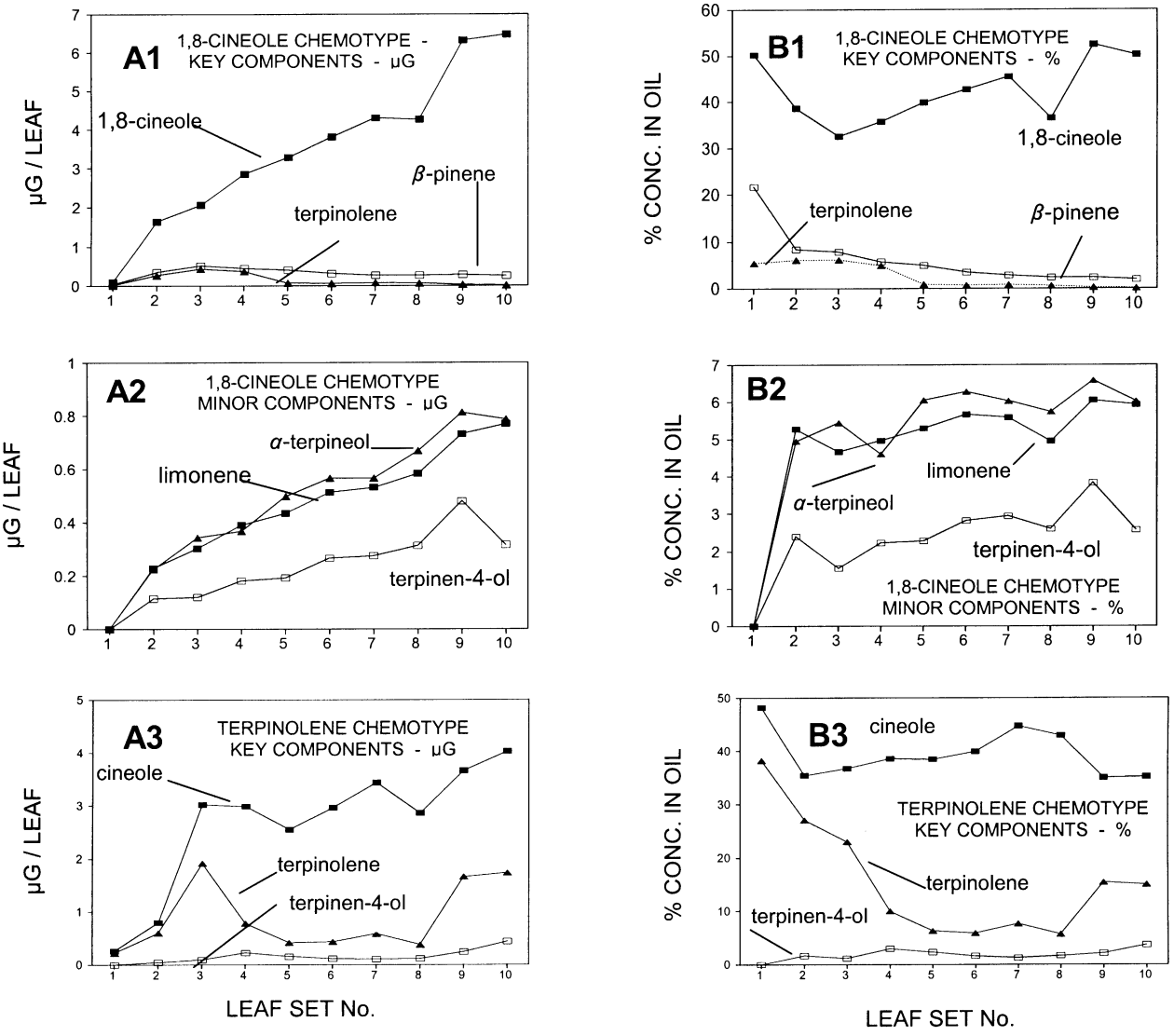

Fig. 2. The mean concentrations (A µg/leaf, B proportion %) of (1) key components 1,8-cineole (■), β-pinene (□) and terpinolene(▲) from the cineole chemotype, (2) minor components limonene (■), terpinen-4-ol (□) and α-terpineol (▲) from the cineole chemotype and (3) key components 1,8-cineole (■), terpinen-4-ol (□) and terpinolene (▲) from the terpinolene chemotype of the extract volatiles of leaf sets 1–10 for 6-week-old *M. alternifolia* seedlings.

3. Discussion

The monoterpenoid content variation in developing *M. alternifolia* seedling leaves was found to be significant for all of the three 1,8-cineole, terpinolene and terpinen-4-ol varieties. Although the variation in concentrations of key constituents in the 1,8-cineole and terpinolene variety leaf sets was not as great as in the terpinen-4-ol variety sets (Russell and Southwell, 2002), differences were noted that need reporting to avoid confusing the chemotypes at early seedling stages. For example, with the terpinolene variety, terpinolene begins at high concentrations in early leaf sets to fall to low values for intermediate leaf sets and then increase to mature leaf values by leaf-sets 9 and 10. Consequently, for all three chemotypes, caution was found to be necessary in interpreting the results of early-ontogeny oil analyses as an indicator of mature plant oil quality.

For all three chemotypes, 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 et al., 1992; Southwell, 1999) found in natural stands or plantations. The terpinolene type was similar to the terpinen-4-ol type in that 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. The 1,8-cineole type however was less variable at an earlier leaf stage.

The best and most practicable comparison of constituent concentrations between chemotypes was seen in plots of % of the key constituent measured for the same leaf set number of the three different chemotypes. 1,8-Cineole concentrations for both the 1,8-cineole and terpinolene varieties decrease for early leaf sets and then remain relatively constant until later leaf sets. At this mature leaf stage, 1,8-cineole concentrations increase for the 1,8-cineole chemical variety and decrease for the terpinolene variety (Fig. 3A). Terpinolene decreases in proportion (%) for all chemotypes except for the abrupt increase at leaf set 8 for the terpinolene variety (Fig. 3B). This secondary enzymic activity distinguishes the terpinolene variety from the 1,8-cineole variety. Terpinen-4-ol increases steadily with advancing leaf sets irrespective of whether the final concentration is high or low (Fig. 3C). β-Pinene decreases from very high to very low concentrations except in the terpinolene variety where levels remain low for the total development of the seedling (Fig. 3D).

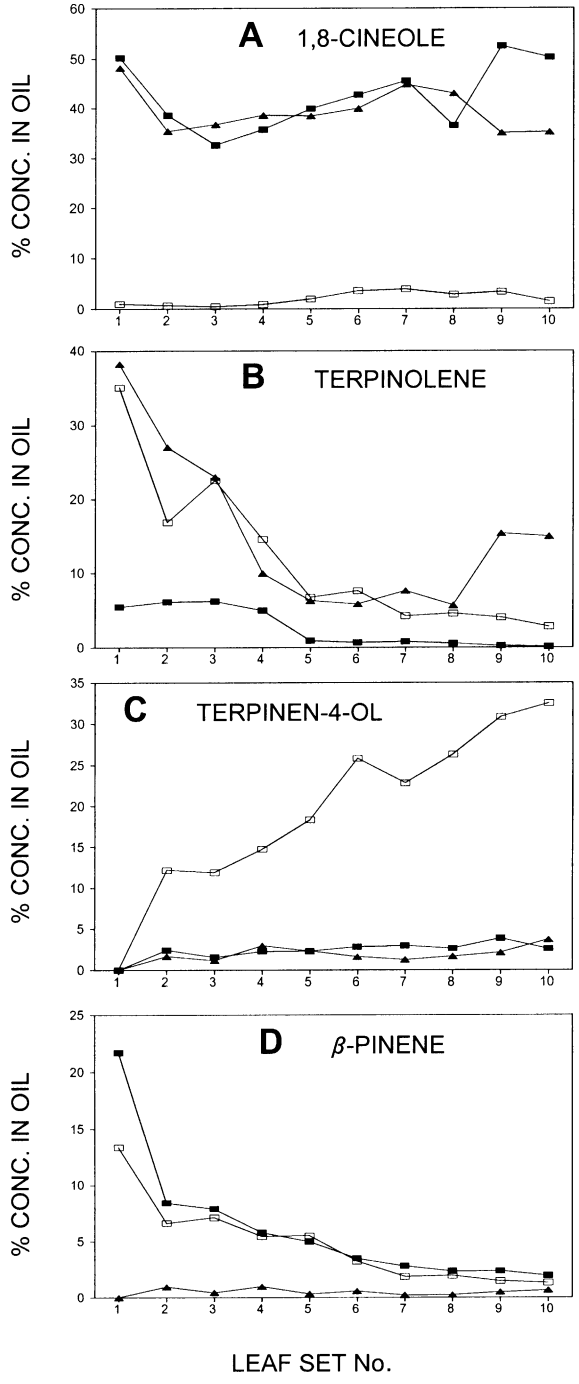

Fig. 3. Proportion (%) of (A) 1,8-cineole, (B) terpinolene, (C) terpinene-4-ol and (D) β-pinene in the leaf extract volatiles of leaf sets 1–10 for 6-week-old *M. alternifolia* 1,8-cineole (■), terpinen-4-ol (□) and terpinolene (▲) type seedlings.

All leaf and oil measurements were made at emergence (age zero weeks) and at age three and six weeks. As with the terpinen-4-ol variety (Russell and Southwell, 2002), the 1,8-cineole and terpinolene types were similar in development at both 3 and 6 weeks of age, with little, if any, development taking place between the former and latter stages of growth. On occasions a concentration decrease occurred between 0 and 3 weeks reflecting increasing leaf weight, area and oil content (Fig. 4). At times oil components did not increase as rapidly as leaf weight or area and so the proportion of oil was seen to decrease (e.g. Fig. 4B).

The 1,8-cineole and terpinolene varieties of *M. alternifolia* can be clearly distinguished from the commercial terpinen-4-ol variety on the grounds of monoterpenoid biogenesis during early leaf stages. Characteristic of the

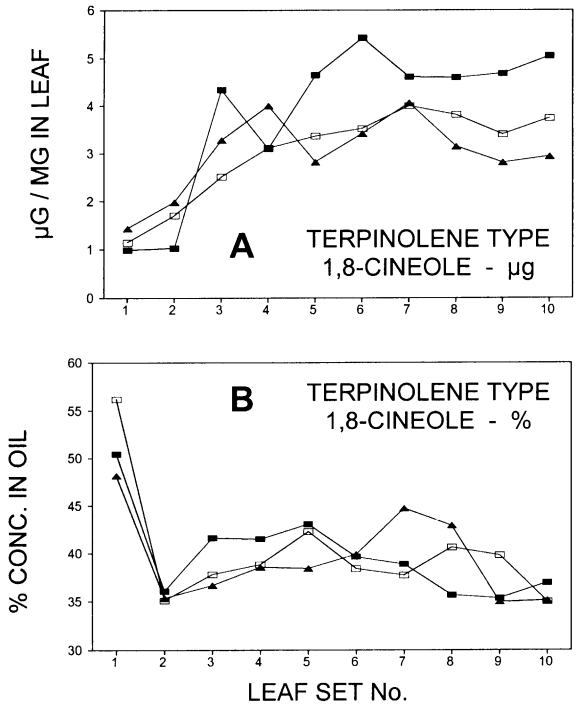

Fig. 4. The mean concentrations (A µg/mg leaf, B proportion %) of 1,8-cineole in the extract volatiles of seedling leaf sets 1–10 for 0 (■), 3- (□) and 6- (▲) week-old *M. alternifolia* terpinolene type seedlings.

1,8-cineole variety was (1) a larger leaf at the leaf sets 2–4 leaf-set stage, (2) a consistently high proportion of 1,8-cineole (%) from early to late leaf sets despite a gradually increasing concentration when measured on a per leaf basis (μg/leaf) and (3) moderate proportions of the pinenes and terpinolene in early leaf sets only.

In the same way in the terpinolene variety, the proportion of 1,8-cineole (%) remained high in all leaf sets with a concentration (μg/leaf) change that showed rapid accumulation at leaf sets 2–3 with a smaller increase at sets 8–9 (Fig. 2.3). Terpinolene accumulation ceased when terpinolene concentration decreased after the initial 1–3 leaf set increase. A second phase of accumulation commenced at leaf set 8 (Fig. 2.3). The first accumulation phase is similar to that observed in the terpinen-4-ol variety with the second characteristic of the terpinolene variety. This may be indicative of formation from two different precursors or two different enzymes as exemplified by terpinolene formation from terpinolene synthase in Grand fir, *Abies grandis,* (Bohlmann et al, 1999) and from (+)-sabinene synthase in common sage, *Salvia officinalis* (Wise et al., 1998) where terpinolene is known to be a significant minor product (Dewick, 2002). In *M. alternifolia,* the proportion of terpinolene remained relatively constant in a similar way to the 1,8-cineole percentage (Fig. 3A) and in contrast to the declining proportion in the terpinen-4-ol variety (Fig. 3B). The terpinolene variety is also characterised by very low proportions of β-pinene at all stages of leaf ontogeny despite the presence of pinene in early 1,8-cineole and terpinen-4-ol leaf sets (Fig. 3D).

Computer-aided cluster techniques encourage a proliferation of chemotypes with cluster boundaries often close or overlapping (Butcher et al., 1994; Lee et al., 2002). More thorough sampling both within and between populations can blur boundaries as has been observed with *Eucalyptus punctata* ssp. *punctata* (Southwell, 1973). This may well be the case with *M. alternifolia* chemotypes as further samples and populations are investigated and as cross-pollination between types continues. These and related investigations confirm the significance of the terpinen-4-ol, 1,8-cineole and terpinolene rich chemotypes of *M. alternifolia* and suggest that a plethora of intergrades and intermediate types are to be expected. Consequently, the measurement of monoterpenoid constituent proportions by gas chromatographic leaf extract analysis was found to be a simple and reliable method for the determination of *M. alternifolia* chemotype status when understood in the light of the above variation in monoterpenoid synthase activity.

4. Experimental

M. alternifolia seed was obtained from the CSIRO Division of Forestry, Australian Tree Seed Centre as previously described (Southwell and Russell, 2002). Propagation, sampling, measurement of leaf weight and areas, quantitative and qualitative oil determinations were all performed as outlined for the terpinen-4-ol variety investigation (Russell and Southwell, 2002).

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