The Sequential Onset of Terpenoid Biogenesis in Seedlings: Implications for *Melaleuca alternifolia* Chemotype Identification Prior to Plantation Establishment

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Abstract

The producers of medicinal and aromatic plants need to ensure that their plantation is stocked exclusively with the desired commercial chemical variety. Planting the wrong variety can only be remedied by costly crop removal and replanting operations. The examination of germinants at successive stages of development gave an indication of the chemical changes that take place between germination and harvest. Using Australian tea tree (*Melaleuca alternifolia*) as an example, terpenoid biogenetic pathways were found to be initiated at different stages of ontogeny.

The cotyledon leaves of commercial terpinen-4-ol chemical variety seedlings were rich in α-pinene (7.4%), β-pinene (12.0%) and terpinolene (27.3%). The non-commercial terpinolene variety was found to be rich in 1,8-cineole (12.5%) and terpinolene (25.4%) and the 1,8-cineole variety rich in 1,8-cineole (37%) with significant quantities of α-pinene (15.5%), β-pinene (23.3%) and terpinolene (10.9%).

Individual leaves of all three chemical varieties were then examined from the emergence of the first true leaves, through to six-week-old leaf-set-ten material. In the terpinen-4-ol variety, the higher concentrations of terpinolene, α-pinene and β-pinene and lower concentrations of terpinen-4-ol, sabinene and *cis*-sabinene hydrate gradually changed, firstly with the emergence of each new leaf set and then again as each leaf set aged. By the time that leaf-set-ten was 6 weeks old, terpinolene, α-pinene and β-pinene levels had fallen, *cis*-sabinene hydrate risen and then fallen and terpinen-4-ol increased so that all components were now present in concentrations similar to those of mature leaf. In the 1,8-cineole and terpinolene chemical varieties, some differences were evident, but early leaves better reflected the chemical quality of the mature tree.

The cotyledon and early seedling 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. Hence pinene and terpinolene biogenesis commences prior to the onset of sabinene hydrate and terpinen-4-ol formation. Consequently early seedling leaf microanalysis is not a good indicator of mature tree quality unless the sequential onset of the biogenetic pathways in tea tree is taken into consideration.

INTRODUCTION

Producers of medicinal and aromatic plants are often confounded by the number of chemical varieties of their target crop (eg basil, fennel; Bauer et al., 1997). When propagation is from seed where cross-pollination predominates, the quality of the parent does not ensure the quality of the progeny. To ensure that resources are not wasted pursuing the wrong variety, the early detection of substandard types is essential. To minimise wasted resources where wrong varieties have been selected, methods for the earliest detection of substandard varieties were examined. For example, producers of tea tree (*Melaleuca alternifolia*) oil in Australia need assurance that their seedling transplants

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will grow into plantation trees rich in terpinen-4-ol rather than terpinolene or 1,8-cineole (Fig. 1.) (Southwell, 1999).

Melaleuca alternifolia (family Myrtaceae), Australian tea tree, grows to 8m in swampy conditions and adjacent to water courses in eastern Australia. The leaves of the terpinen-4-ol variety give an essential oil (1-3%), which has been marketed for its medicinal properties for more than 60 years. The biological activity was found to be related to terpinen-4-ol, the major component of the steam-distilled oil. Other chemical varieties of *M. alternifolia* with lower terpinen-4-ol concentrations lacking the appropriate anti-microbial activity have presented quality control problems for the industry (Southwell and Lowe, 1999). The 1,8-cineole and terpinolene chemical varieties can contain as little as 1% terpinen-4-ol. In addition, with *M. alternifolia,* outcrossing predominates with less than 10% self pollination (Butcher et al*.,* 1992; Baker, 1999). Hence the quality of the parent seed tree may not reflect the quality of the second generation. In Australia, propagation of tea tree from seed is more common than from cuttings or clones (Colton et al., 2000). A typical plantation establishment timetable is shown in Fig. 2. As plantation establishment requires the discriminant selection of the terpinen-4-ol chemical variety, this investigation was aimed at determining the earliest possible stage of plant development that would give a reliable indication of tree quality.

MATERIALS AND METHODS

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^{o} to maxima of approximately 25^{o}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. True leaf sets (pairs) then emerged in the following time sequence (numbered from the first leaf closest to the soil to the final leaf closest to the growing tip) (see Fig. 3) and were harvested accordingly: 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.

Replication

Ten leaves were bulked for each measurement and the mean value recorded. Each value was obtained by sampling and measuring in triplicate. Each leaf set was determined at age 0 weeks (ie freshly emerged), 3 weeks and 6 weeks.

Leaf Weight and Area

For dry weight determination, bulked leaf samples in tared vials were weighed to four decimal places after extraction, GC analysis and drying at 75^{0}C (16 hrs). For area measurement, ten leaves were averaged using a HP Scanjet 3C computer scanner and Deltascan software.

Oil Determination - Quantitative

Ten seedling or twenty cotyledon leaves of each chemical variety (0.0029 g dry weight) were extracted with *n*-tridecane (9.5 μgrams) in ethanol (0.376 g of 0.002% solution). Weight of total oil or component per leaf, per leaf weight and per leaf area 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.

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 x 0.25 mm, 0.2 μm film thickness, mid polarity FSOT column with hydrogen (45 cms/sec) as carrier gas, injection port (split 1:50) at 250°C, flame ionisation detector at 300°C and temperature programming from 60°C (1 minute) 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 oil in ethanol.

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 x 0.25 mm, 0.25 μm film thickness, FSOT column with helium (36 cm/sec) 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 69.9eV. 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.

RESULTS

Cotyledon Leaf

In *M. alternifolia*, oil was found to be formed as early as the cotyledon leaf stage of development with a concentration of 2.6% (3.8μg 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-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.

Seedling Leaf

Leaf Characteristics. Leaf weights, areas and oil concentrations were measured for *Melaleuca alternifolia*, terpinen-4-ol type, seedling leaves at 0, 3 and 6 weeks after emergence. Leaf weights increased to maxima of approximately 1.0mg (dry weight) for 6-week old leaves by leaf set 2 and to approximately 0.5mg for freshly emerged (0 week-old) material by leaf set 4 (Fig. 4). 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. 5). Oil concentrations per leaf, per unit leaf area and per leaf dry weight increased steadily from leaf set 1 through to leaf set 10 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 weeks) at leaf-sets 3-4 and at 6-7. Further small increases occurred as these sets aged to 3 and 6 weeks. A comparison of leaf oil concentration increases per unit dry weight and per unit area is shown (Fig. 6).

Oil Characteristics. The composition of the volatiles in early seedling leaves was found to be significantly different to the composition of mature leaves (Brophy et al., 1989; Southwell, 1999) in natural stand or plantation situations. It was only when leaf set 10 had reached age 6-8 weeks that the volatile oil composition approached that of a mature leaf oil.

Components could be separated into three groups: those that increase in

concentration as seedling leaf develops, those that decrease and those that maintain relatively constant concentration. Only the major components terpinen-4-ol and γ-terpinene were seen to increase significantly in GC % concentration, μg per leaf, μg per unit leaf area and μg per mg. On the other hand, the pinenes, sabinene, terpinolene and cis-sabinene hydrate decrease in concentration and 1,8-cineole and α-terpineol maintain almost constant levels.

For example, major components, α-terpinene, γ-terpinene and terpinen-4-ol increased in proportion from 0.07 (1.4%), 0.25 (4.4%) and 0.0 (0.0%) μg/mg respectively for 0-day-old leaf set 1 material to 0.41 (2.5%), 3.44 (20.8%) and 5.38 (32.5%) μg/mg respectively for 6-day-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 approaching the 6 week values and so have not been included in the Figures and Tables. 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.

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 (eg 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 been absolute if other biogenetic pathways were initiated later and added more metabolites to the pool. Measurement of absolute concentrations as μg metabolites per leaf, per unit leaf area ($10mm^2$) or per mg leaf dry weight however indicated that the absolute concentrations varied as the leaf developed.

The leaf set variation in the concentration of all individual components was measured as area percent from the chromatograms and as μg/leaf, μg/leaf area and μg/leaf weight. The most significant variations were observed for β-pinene, γ-terpinene, terpinolene, *cis*-sabinene hydrate and terpinen-4-ol. *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. 7). A similar significant decrease in β-pinene concentration has been observed with sage where the steam distillation of immature leaves (2-3cms) gave an oil with 20% β-pinene compared with 3% for the fully expanded (>5cm) leaves (Croteau and Karp, 1976). γ-*Terpinene*, the second most abundant (approx. 20%) terpene in commercial tea tree oil (Brophy et al., 1989) increases in concentration ten-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. 8). 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. 9). Although structurally consistent with formation as a deprotonation product of the terpinen-4-yl cation, the minimum concentrations of other similarly derived *p*-menthanes (eg 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. 10). 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 six 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. 11). 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*-sabinene hydrate acetate, the major component decreases (Hallahan and Croteau, 1988).

Leaf set variation in the 1,8-cineole and terpinolene chemical varieties was also measured in an identical manner. The 1,8-cineole chemotype showed only minor concentration variation with sequential leaf sets (Fig. 12). The presence of terpinolene only in the early leaf sets was consistent with the cessation of a terpinolene pathway at approximately 5 weeks. The terpinolene chemotype showed similar variations for 1,8-cineole and limonene. Terpinolene, however, began at high concentrations (~40%), fell to low levels at leaf sets 5-8 and then continued to reach levels typically found in the mature plant (Fig. 13). Hence the sequential onset of terpenoid biogenetic pathways has greatest significance for the terpenen-4-ol chemical variety of *Melaleuca alternifolia* (Fig. 14.).

DISCUSSION

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 (eg 37%, Table 1) is always indicative of the 1,8-cineole chemical variety. One very rich in terpinolene (eg 55%, Table 1) with moderate amounts of 1,8-cineole (eg 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 (Cornwell et al., 1995; Southwell and Stiff, 1989, 1990; Croteau, 1989). 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

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Tables

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	Mature leaves	Cot. leaves	Mature leaves	Cot. leaves	Mature leaves
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.

Figures

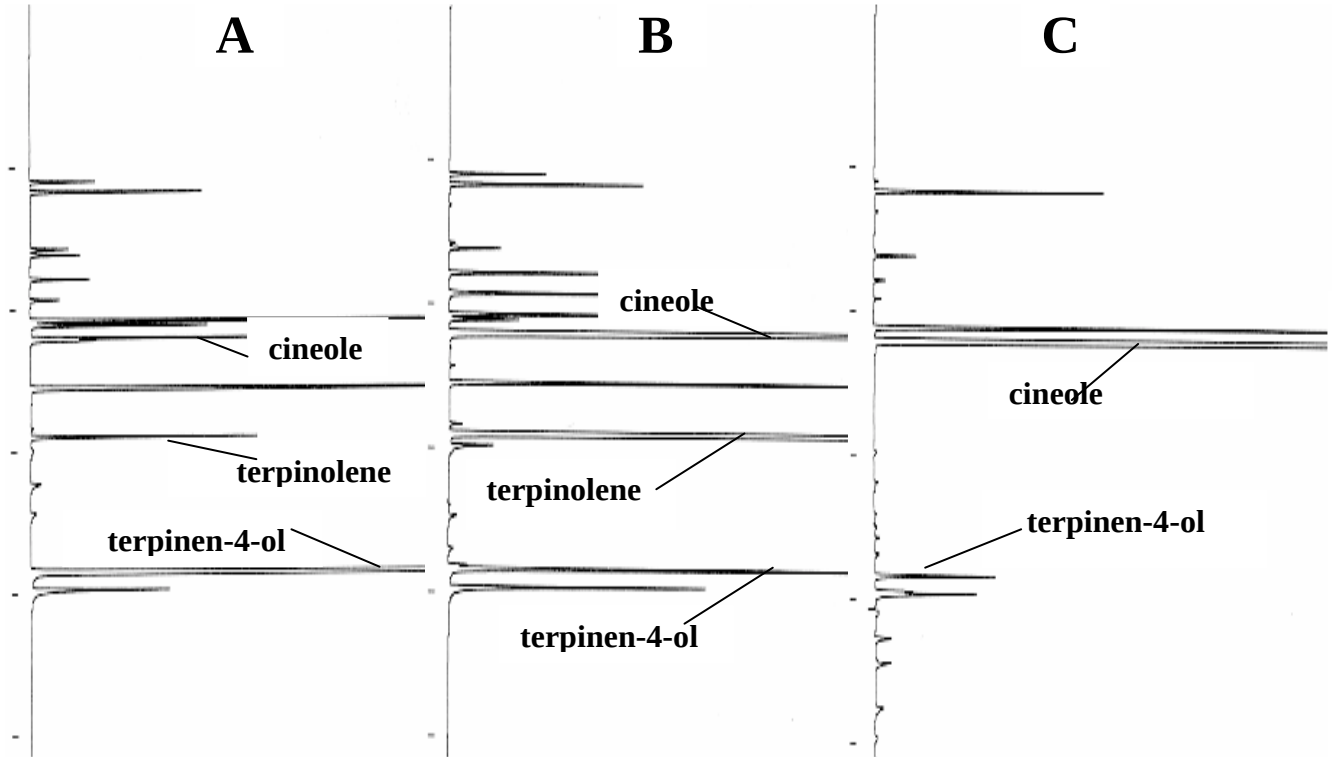

Fig. 1. Comparative gas chromatograms of *Melaleuca alternifolia* volatile oils from the terpinen-4-ol (A), terpinolene (B) and 1,8-cineole (C) chemical varieties.

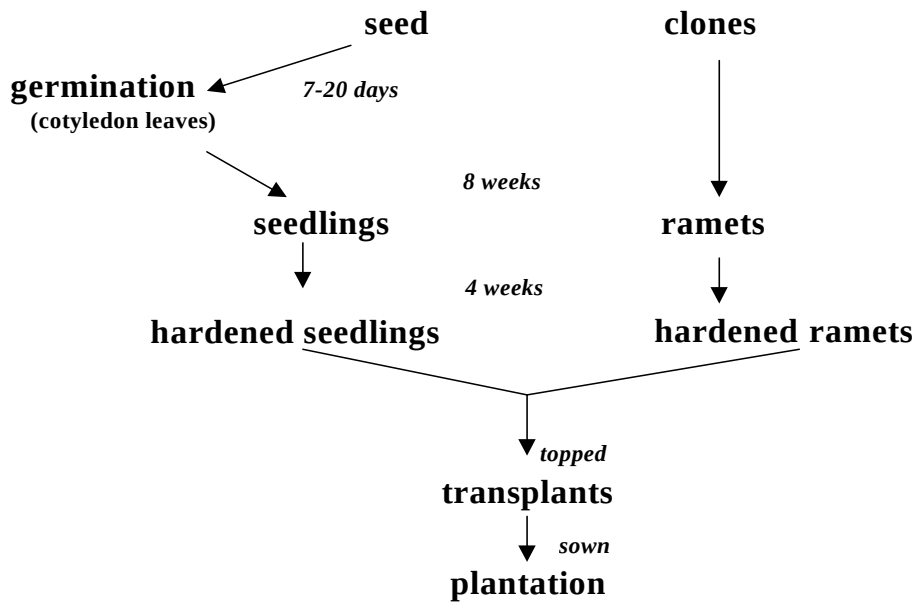

Fig. 2. Typical tea tree plantation establishment timetable.

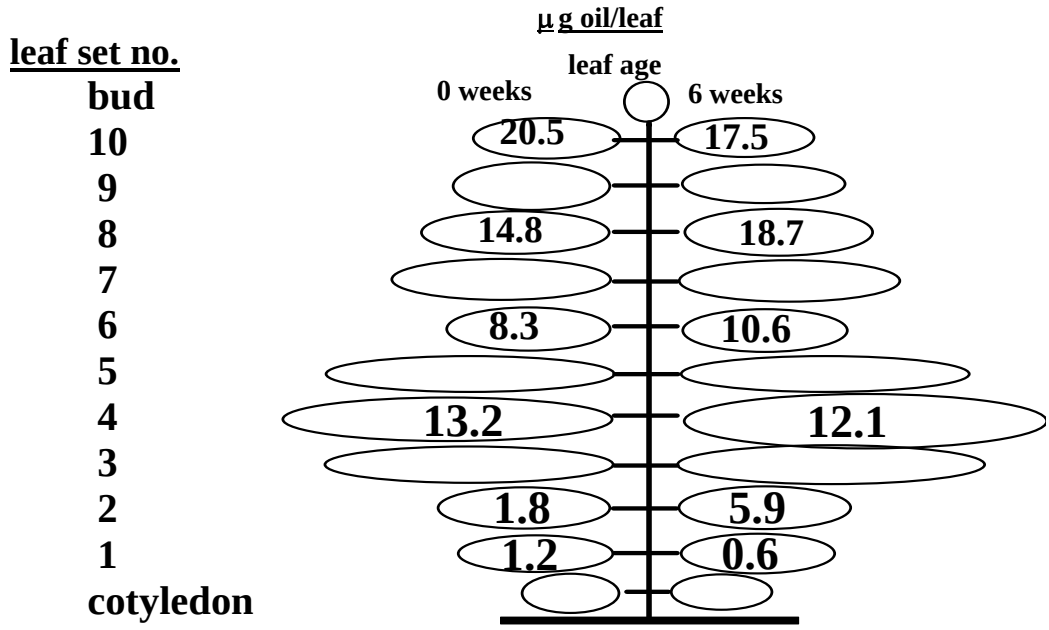

Fig. 3. Schematic diagram of tea tree seedling with oil concentrations (µg/leaf)

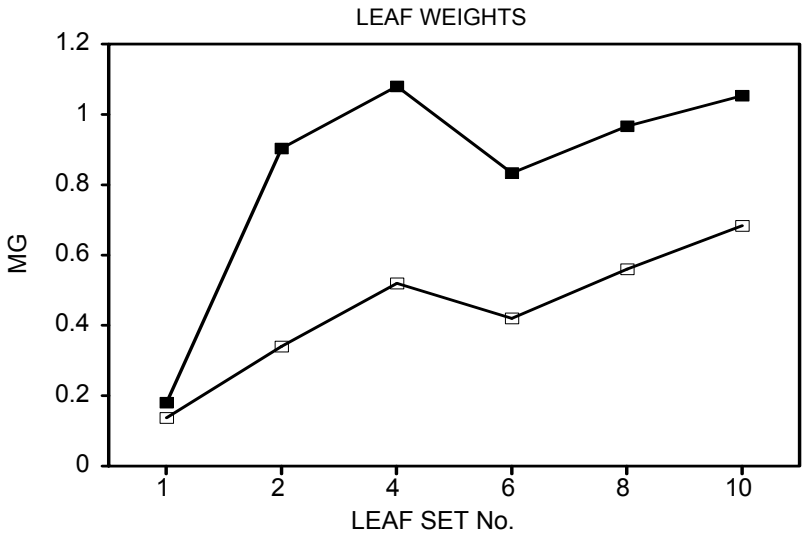

Fig. 4. The mean weights (mg) of individual leaves along a tea tree seedling stem for leaf sets from 1 – 10 at age 0 (□) and age 6 (■)weeks.

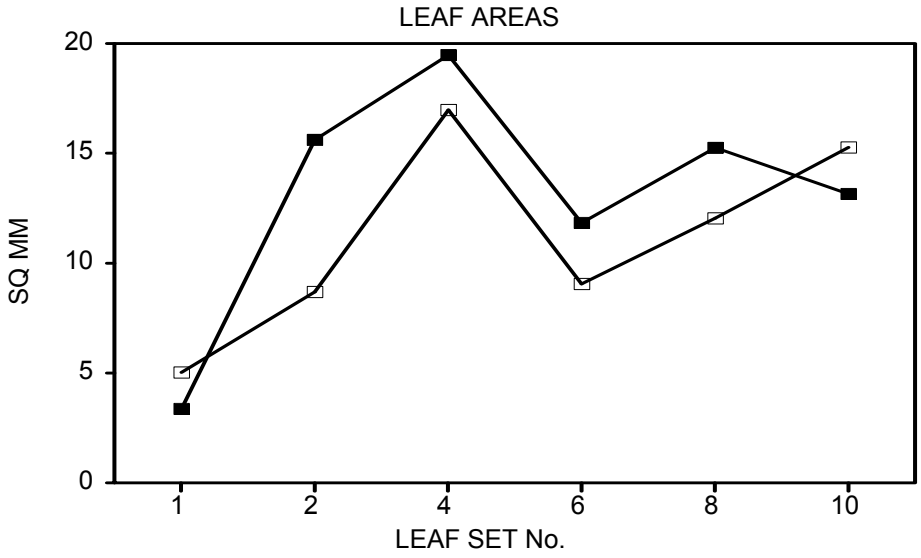

Fig. 5. The mean areas (mm^2) of individual leaves along a tea tree seedling stem for leaf sets from 1 –10 at age 0 (□) and age 6 (■) weeks.

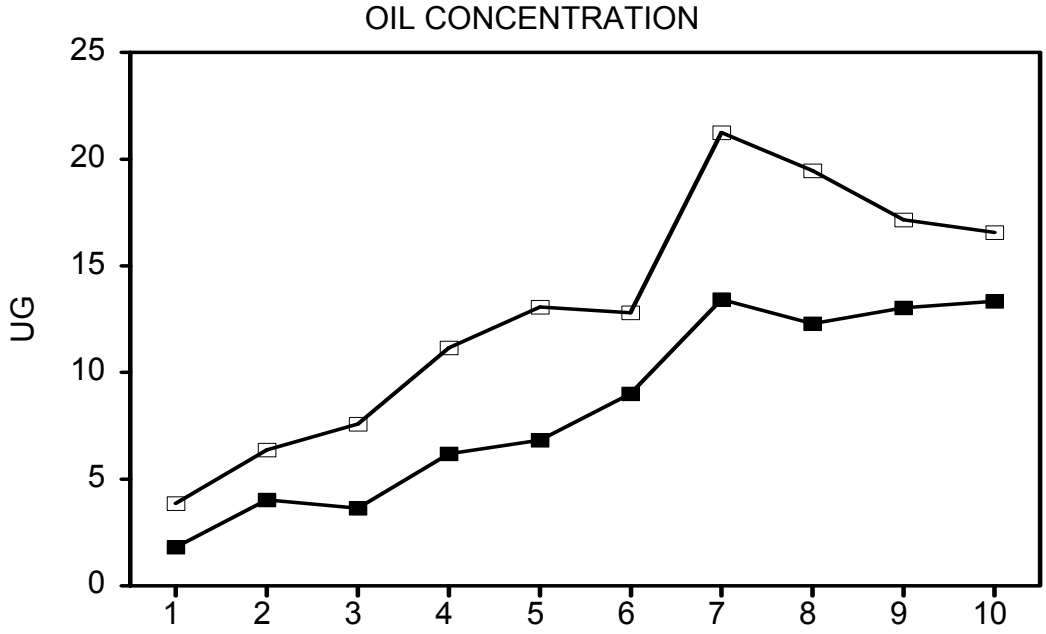

Fig. 6. The mean oil concentrations (□µg oil/ mg dry weight, ■µg oil/ $10mm^2$ surface area) of individual leaves along a tea tree branch for leaf sets from 1 – 10.

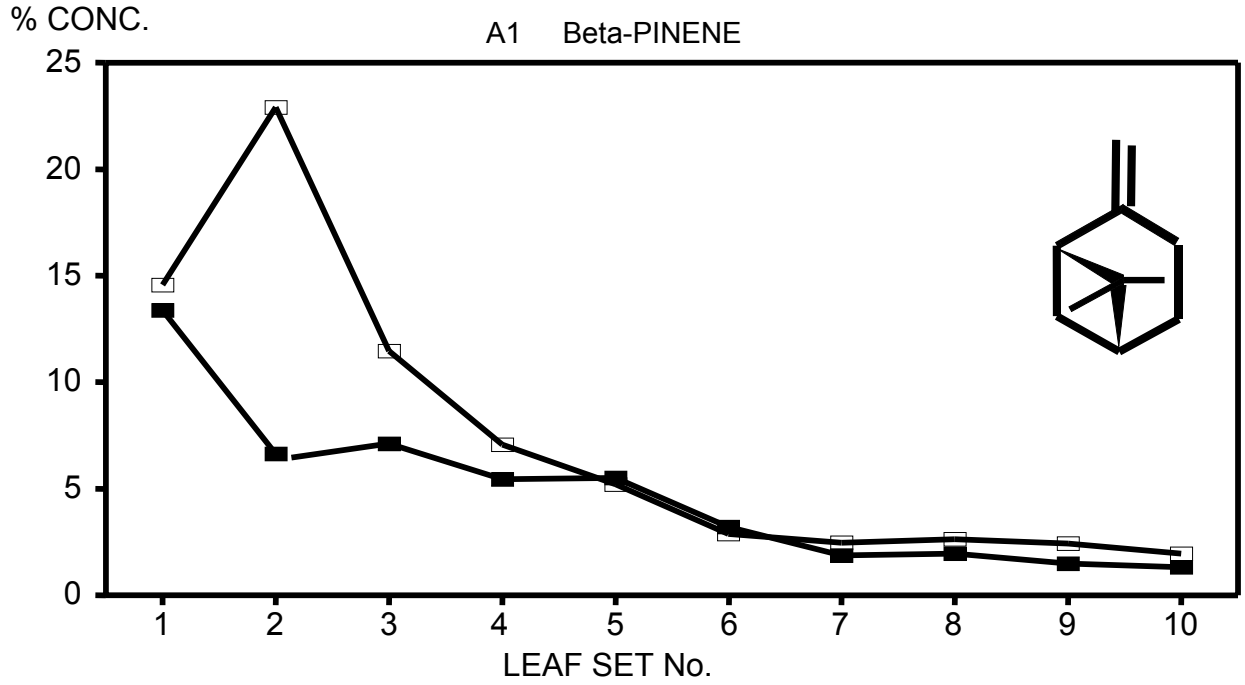

Fig. 7. Variation in β-pinene content (%) of *Melaleuca alternifolia*, terpinen-4-ol type, seedling leaves at emergence (□) (age 0 weeks) and age 6 weeks(■).

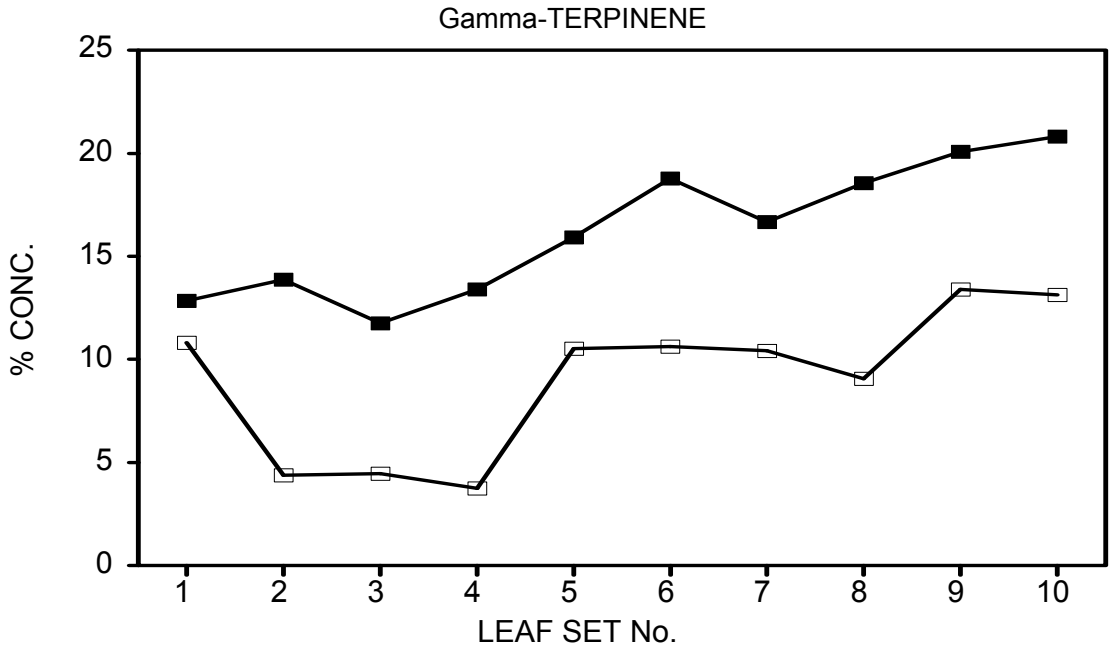

Fig. 8. Variation in γ-terpinene content (%) of *Melaleuca alternifolia*, terpinen-4-ol type, seedling leaves at emergence (□) (age 0 weeks) and age 6 weeks(■).

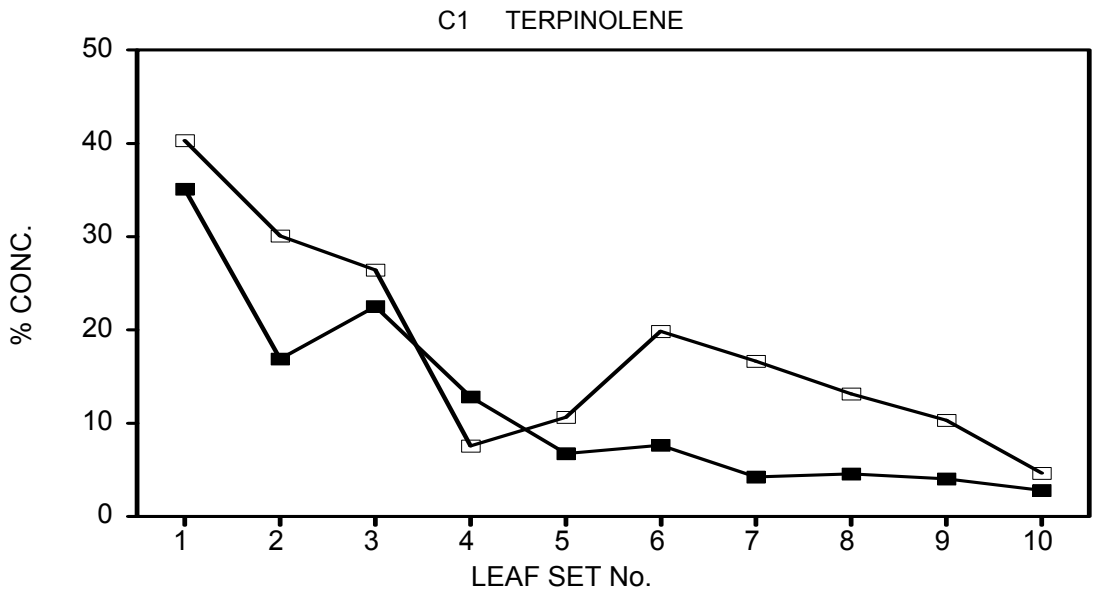

Fig. 9. Variation in terpinolene content (%) of *Melaleuca alternifolia*, terpinen-4-ol type, seedling leaves at emergence (□) (age 0 weeks) and age 6 weeks(■).

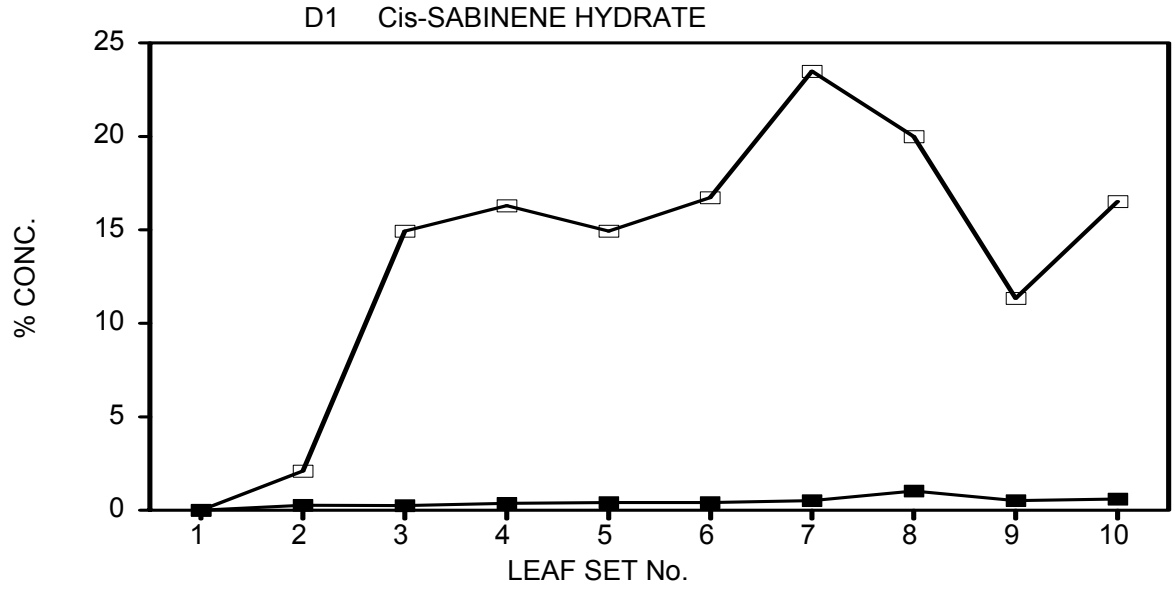

Fig. 10. Variation in *cis*-sabinene hydrate content (%) of *Melaleuca alternifolia*, terpinen-4-ol type, seedling leaves at emergence (□) (age 0 weeks) and age 6 weeks(■).

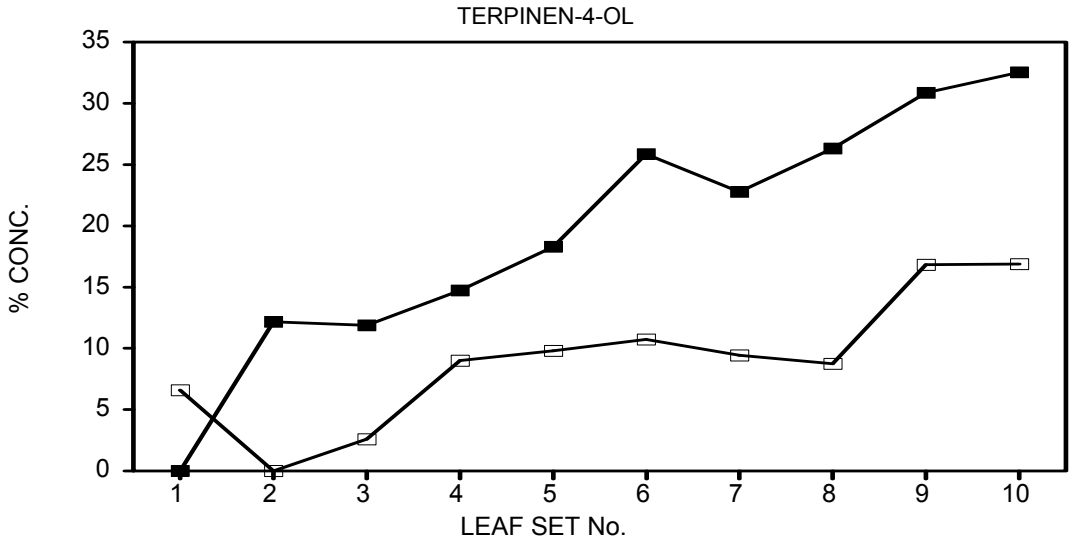

Fig. 11. Variation in terpinen-4-ol content (%) of *Melaleuca alternifolia*, terpinen-4-ol type, seedling leaves at emergence (□) (age 0 weeks) and age 6 weeks(■).

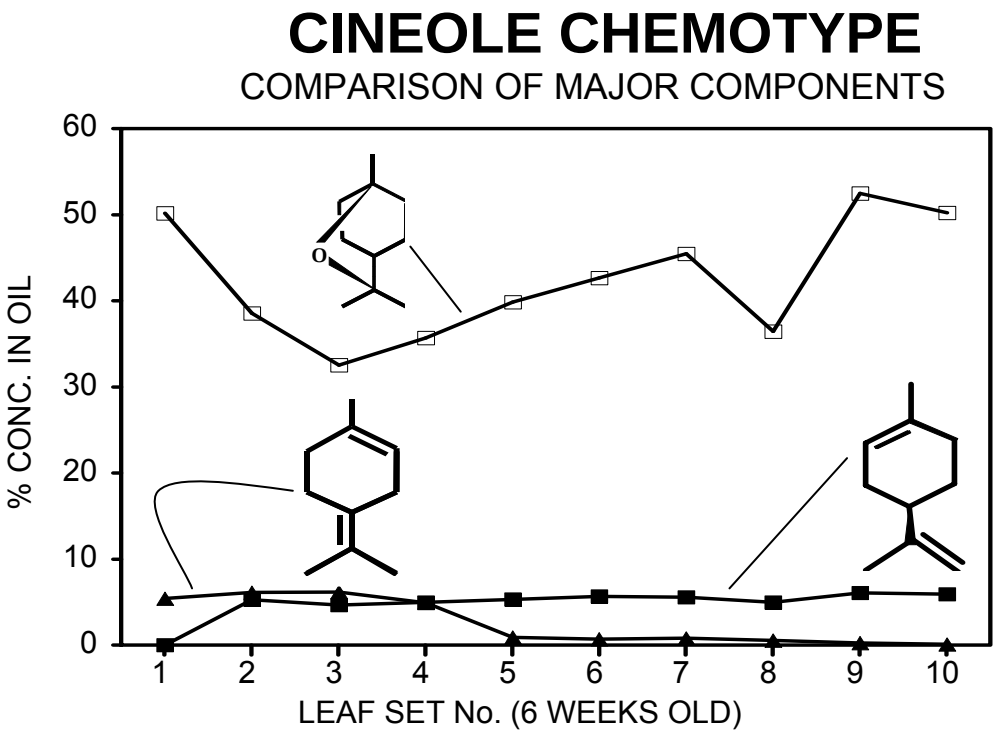

Fig. 12. Variation in limonene (■), 1,8-cineole (□) and terpinolene (▲) content (%) of *Melaleuca alternifolia*, 1,8-cineole type, seedling leaves at age 6 weeks.

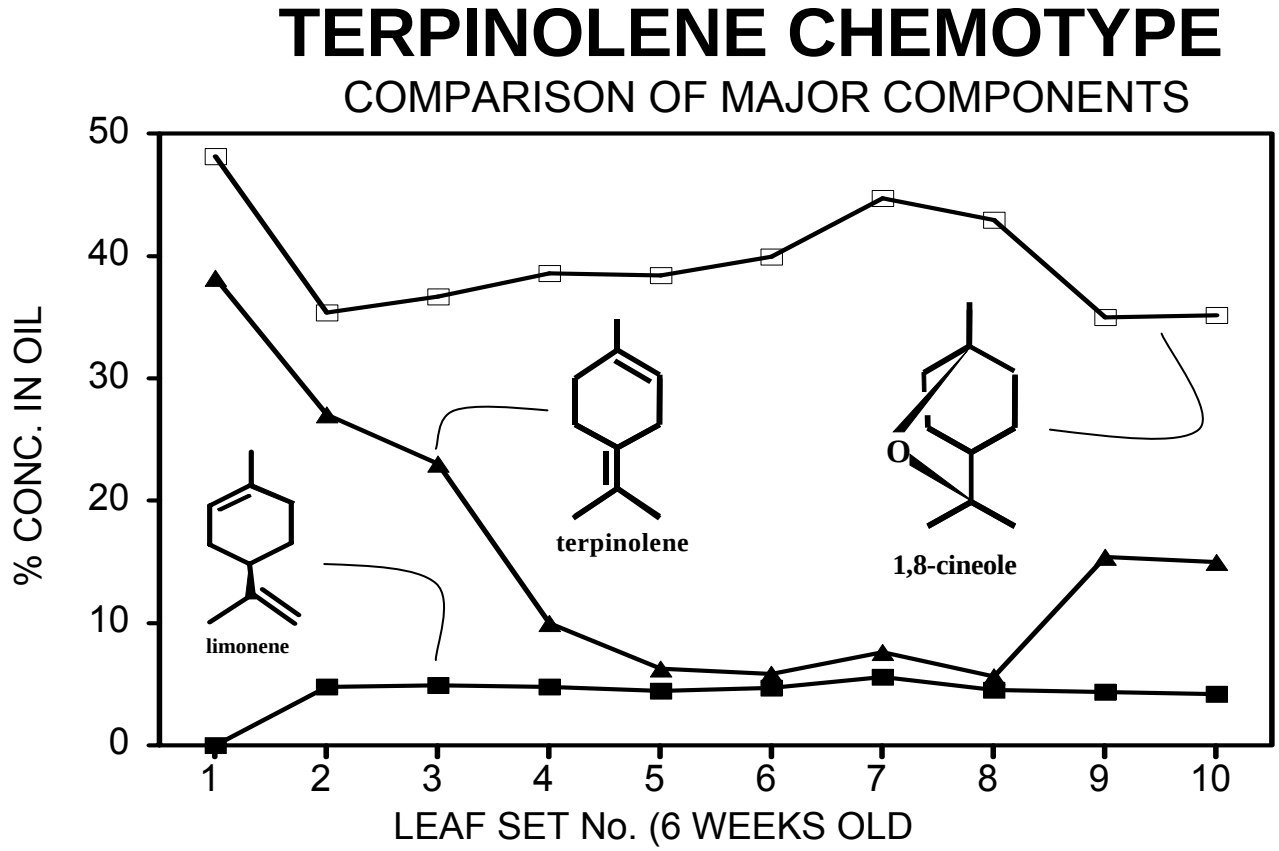

Fig. 13. Variation in limonene (■), 1,8-cineole (□) and terpinolene (▲) content (%) of *Melaleuca alternifolia*, terpinolene type, seedling leaves at age 6 weeks.

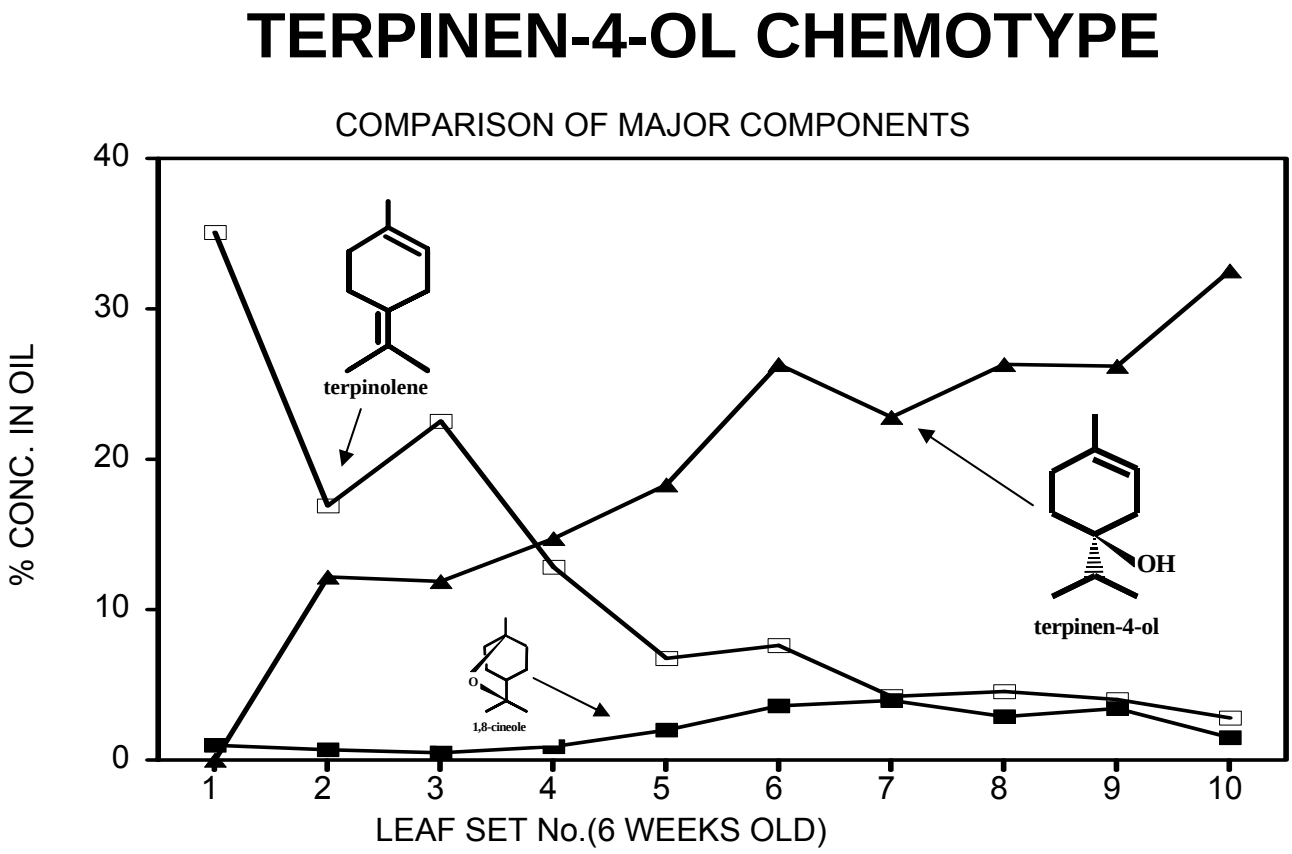

Fig. 14. Variation in terpinene-4-ol (▲), 1,8-cineole (■) and terpinolene (□) content (%) of *Melaleuca alternifolia*, 1,8-cineole type, seedling leaves at age 6 weeks.