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# Preferred Age for Assessment of Qualitative and Quantitative Characteristics of the Essential Oil of Tea Tree (*Melaleuca alternifolia*) Seedlings Prior to Plantation Establishment

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An analytical method for determining the quality and hence the chemical variety status of tea tree transplants is described. The key to the procedure was found to be the leaf age of the test material. Investigation at very early development stages was seen to give misleading results due to the sequential onset of different monoterpenoid biogenetic pathways. For example, in the first few leaves, the high concentration of terpinolene in the terpinen-4-ol variety suggests that the terpinolene variety is under investigation. However, 1,8-cineole percent concentrations in plantation tree leaf were ~1.6 times lower than those measured for seedlings prior to transplant. Consequently, the use of a plantation cineole indicator is proposed for estimating plantation cineole from seedling leaf analyses. Although recent investigations enable the chemotype status to be predicted with some certainty, it is now proposed that analysis of leaf set 10 at the age of 6 weeks (seedling age ~17 weeks) provides an unambiguous analysis and correlates seedling quality with mature plantation quality. In addition, the oil yield of mature tea tree leaf was found, by steam distillation, to be ~5 times higher than that of seedling leaf.

**KEYWORDS: Tea tree; *Melaleuca alternifolia*; chemotypes; 1,8-cineole type; terpinolene type; terpinen-4-ol type; transplant quality; leaf microanalysis; gas chromatography**

## INTRODUCTION

Australian tea tree, *Melaleuca alternifolia* (Maiden & Betche) Cheel, has been harvested intensively for the past 20 years (*1*). To cope with this industry expansion, leaf previously sourced from natural stands in the New South Wales (NSW) northern rivers region had been obtained either from stands growing further afield or from cultivated stands (*2*). Both of these approaches were confounded by quality control problems because of the existence of chemotypes with low concentrations of terpinen-4-ol, the component responsible for the bioactivity of the medicinal oil steam-distilled from the leaves (*3*−*6*). With the abandonment of harvesting most natural stands, the quality of seedlings used for plantation establishment has become critical.

As the industry propagates extensively from seed (*7*), plantation purity can be a problem because *M. alternifolia* is principally an out-crossing species. Estimates of the degree of self-crossing vary from 7% (*8*) to 14% (*9*) to 6−28% (*10*). Consequently, cultivation is fraught with danger as even seed collected from a reliable mother tree may have been pollinated from a low terpinen-4-ol chemotype tree. Indeed, several *M. alternifolia* plantations have inadvertently planted high-1,8-cineole type variants. Such faux pas are preventable by the early analysis of seedling leaf before transplanting.

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Earlier investigations have shown that this approach is not straightforward. In addition to a change in terpenoid composition as flush growth develops into mature leaf (*11*), the cotyledon leaves (*12*) and early seedling leaves (up to leaf set 10 at the age of 6 weeks) (*13*) contain terpenoids in vastly different proportions to mature leaf.

Of the numerous procedures available for the assessment of the quality of volatile leaf oils, microwave-assisted microextraction was found to be superior for analyzing *M. alternifolia* seedlings. Steam distillation, the procedure that most accurately reflects essential oil quality, requires leaf quantities, throughput times, and processing equipment often unavailable or inappropriate for the rapid analysis of large numbers of seedlings. To overcome these barriers, microextraction methods have been developed (*2*, *11*, *14*, *15*). The value of the microwave-assisted macro version of this procedure was shown for tea tree with excellent correlation between ethanolic extraction and 2-h distillations for the measurement of both total oil and 1,8-cineole and terpinen-4-ol concentrations (*16*). In contrast, procedures using headspace analysis (*17*, *18*) either give vastly different component percentage results or require a complex correction formula.

This investigation reviews these quantitative and qualitative differences in volatile oil formation in the leaves of the terpinen-4-ol variety of *M. alternifolia* and identifies the best stage of seedling development for which analytical procedures can reliably determine the quality of subsequent plantations.

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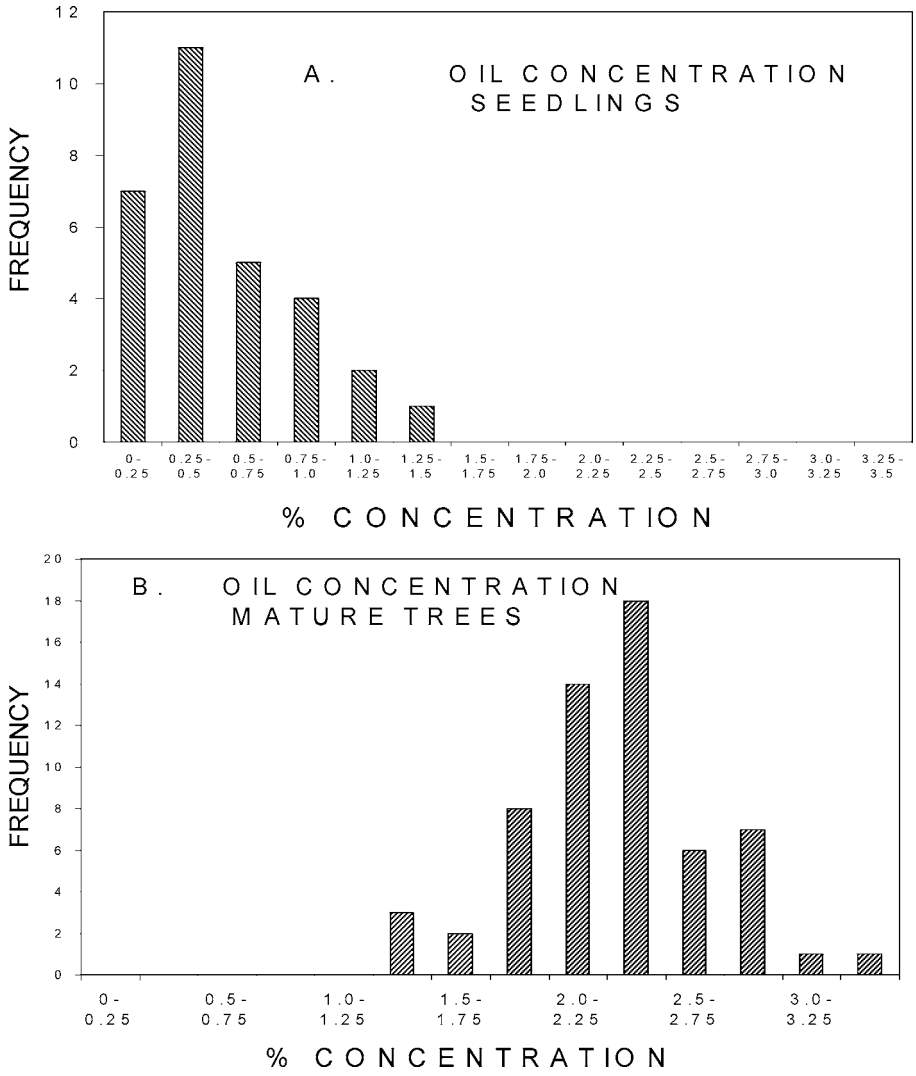


**Figure 1.** Leaf oil concentration histogram for *M. alternifolia* seedlings (A, $n = 30$) and plantation trees (B, $n = 60$).

## MATERIALS AND METHODS

**Plant Material.** *M. alternifolia* terpinen-4-ol variety seed was obtained from the CSIRO Division of Forestry, Australian Tree Seed Centre. Seed-lot DL 7 gave progeny (80) with low 1,8-cineole content (~1%) and seed-lot 655 progeny (63) with higher 1,8-cineole content (~10%). Both seed-lots were kept separate from sowing through analysis. Propagation was carried out in an ambient temperature glasshouse when temperatures ranged from 15 to 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): 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. After 17 weeks, the seedlings were then transplanted into a red Krasnozem soil field trial at the Wollongbar Agricultural Institute.

**Sampling.** For the seedling−mature plantation tree oil yield comparison (**Figure 1**), 500−1000 g of fresh bulk tips was sampled from well-mixed representative bulk leaf harvests. For the seedling emergence to 4 months investigation (**Figure 2**), one leaf from each of 10 seedlings was bulked for each oil concentration measurement. For the young transplanted saplings, 5.5−44 months (**Figure 2**), 10 leaves from the same 10 randomly selected saplings were bulked for each measurement. At 44 months, the final reading was from 1 g of leaf from each of these same 10 saplings (now small trees). For the variation in component concentration with seedling development investigation (**Figure 3**), 10 leaves, one from each of 10 seedlings,

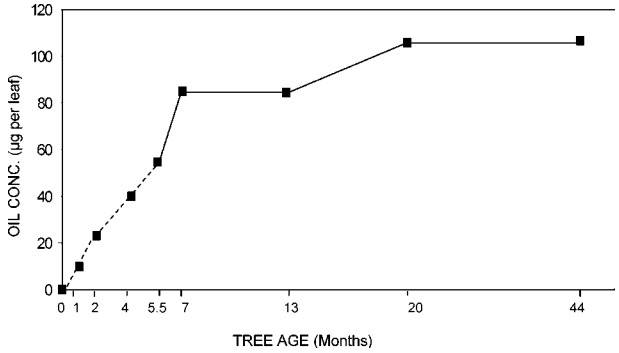


**Figure 2.** Increase in mean leaf oil yield for *M. alternifolia* from sowing through transplanting (- - -) to 3-year-old mature trees (—).

were sampled at 6 weeks of age, from leaf set 1 (cotyledon) to leaf set 10 (seedling age ~17 weeks). These were determined in triplicate. For the development of the cineole estimator (**Figure 4**), 20 seedlings were sampled at 3 months and 3 years and the mean 1,8-cineole concentrations plotted using an XL line-of-best-fit regression analysis.

**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 of microwave irradiation (700 W) followed by 24 h at 20 °C. Concentrations of total oil and individual components per leaf were 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

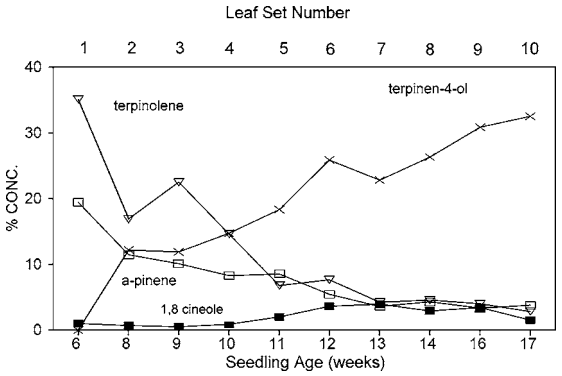


**Figure 3.** Mean concentrations of key tea tree oil constituents [terpinolene (▽), terpinen-4-ol (×), 1,8-cineole (■), and α-pinene (□)] in different leaf sets 6 weeks after emergence.

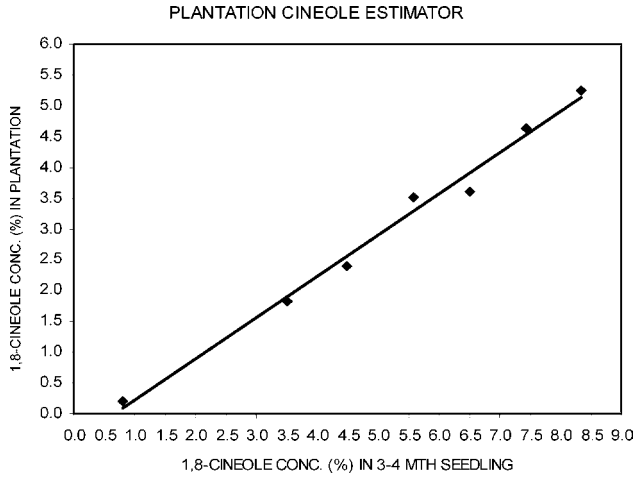


**Figure 4.** Relationship between 1,8-cineole concentration in 3–4-month seedlings and in the same transplanted plantation trees at 3 years of age.

then concentrated in an oven at 75 °C for 16 h for dry leaf weight determination.

The leaf extracts were analyzed and constituents quantified using a Hewlett-Packard 5890 chromatograph, a 3393A Integrator, a 7673A autosampler, and an Alltech AT35, 60 m × 0.25 mm, 0.2 μm film thickness, midpolarity 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 the total FID response from the injection of a solution of extract in ethanol.

Steam distillation procedures were performed as previously described (*2*).

**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 at 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 (Aldrich, BDH, Ajax), and mass spectral and retention matching with commercial libraries (*19*−*21*).

## RESULTS AND DISCUSSION

The method of choice for the determination of oil concentration was found to be steam distillation except where small sample sizes or large sample numbers were involved. For example, *M. alternifolia* cotyledon leaf analyses required a microextraction method due to small leaf size (∼1 mg) (*12*). Breeding projects requiring regular multiple analyses also need to bypass the distillation step. Oil concentrations and component identities and concentrations were measured in a time efficient manner using solvent extraction procedures that were set up to reflect concentrations measured by oil distillation (*16*). Fresh leaf weight oil concentrations ranged from 0.1 to 1.4 mL/100 g (mean = 0.54, standard deviation = 0.32, $n$ = 30) for pretransplant seedlings and increased to 1.3−3.3 mL/100 g (mean = 2.20, standard deviation = 0.40, $n$ = 60) for typical plantation trees (**Figure 1**). When measured on a concentration per dry leaf basis, oil content increased stepwise after transplanting, from 50−60 to >100 μg/leaf (**Figure 2**) at age 20 months and onward. These findings then complete the picture of oil accumulation in tea tree, which commences at <5 μg/leaf for cotyledon (*12*) and early leaf sets and increases to ∼20 μg/leaf by the time leaf set 10 had reached 6 weeks of age (*13*). Accumulation continues on to 40−70 μg/leaf at transplant age (∼16−20 weeks) and ceases above 100 μg/leaf between 16 and 40 months. This is consistent with producers' observations that oil yields improve significantly between the first (9−12 months), second (20−24 months), and third harvests (30−36 months) (*22*−*24*).

The key constituents for tea tree quality are terpinen-4-ol and 1,8-cineole. As the former is the bioactive constituent (*4*−*6*), >30% is acceptable (*15*, *25*) with ∼40% desirable. On the other hand, 1,8-cineole is considered to be undesirable as it reflects the high-cineole undesirable chemical variety. In addition, 1,8-cineole concentrations increase as terpinen-4-ol concentrations decrease. Hence, although cineole concentrations as high as 15% pass international standards (*25*), the market likes to trade in cineole concentrations of <5% (*6*, *7*). That there are no other reasons for limiting 1,8-cineole to these low concentrations is seen in investigations, including skin irritancy clinical trials, that have shown that 1,8-cineole is not detrimental to tea tree oil (*26*).

Although plantation oil yields are difficult to predict at the transplant stage, oil quality is more straightforward. Our earlier investigations have highlighted the pitfalls possible if producers assume that cotyledon (*12*) or early leaf sets (*13*) of pretransplant seedlings are a true indicator of the chemical quality of subsequent plantation trees. In this paper, measurement of oil concentrations (percent) of key components α-pinene, 1,8-cineole, terpinolene, and terpinene-4-ol in seedling leaves confirmed remarkable concentration changes during the first 17 weeks of seedling development (**Figure 3**). Terpinolene and α- and β-pinene concentrations decreased while terpinen-4-ol increased and 1,8-cineole remained low following a 10−16 week low-level enhancement. After this stage, significant changes in the concentrations of terpinen-4-ol, terpinolene, and 1,8-cineole were not observed. To answer producer questions regarding the earliest possible seedling age at which analysis would give a reliable indication of plantation quality, oil components were monitored through transplant age (∼16 weeks) for all three chemical varieties and beyond to 3-year-old trees for the commercial terpinen-4-ol chemical variety. In all three varieties, the 1,8-cineole concentrations remain relatively constant, especially after transplant age. In the terpinen-4-ol variety, only trace levels of cineole are present until a seedling age of ∼10 weeks, after which contributions of up to 5% are maintained for the next 4−5 weeks. As this is the age when most seedlings are determined for pretransplant quality, any subsequent variation in this concentration is critical. The determination of 1,8-cineole at both 12 weeks (seedling) and then at 3 years (mature

tree) for 143 trees from both low (1%) and higher (10%) 1,8-cineole seed-lots showed a consistent 50% reduction (**Figure 4**). Such a relationship can be used as a plantation cineole indicator for estimating plantation cineole from seedling leaf analyses.

Consequently, these investigations have shown that the analysis of late-stage seedlings for chemical quality is best done prior to transplant at 16−20 weeks of age. This is most appropriate if seedling growth allows for "topping" (i.e., the trimming of the tops of seedlings prior to transplanting) leaves, which can be used for a bulk, laboratory-scale steam distillation. Otherwise, an ethanolic extract analysis of a cross section of representative seedlings is required.

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