VARIATION IN AND BREEDING FOR OIL YIELDS IN LEAVES OF *EUCALYPTUS CAMALDULENSIS*

by

John Charles Doran
Department of Forestry
Australian National University

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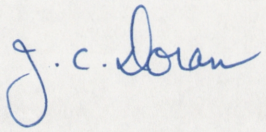
Except where specific reference is made to the work of other people, the research in this thesis is original.



J.C. Doran
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ABSTRACT

This thesis reports an investigation into the variation in essential oil yields, especially 1,8-cineole, in the leaves of the tropical red gums, *Eucalyptus camaldulensis* and *E. tereticornis*. Genetic parameters for yields of the principal leaf monoterpenoids were estimated and a breeding strategy devised for improved oil yields in a commercial population.

Initially, methods were examined for the efficient sampling of and extraction of oil. Ethanolic extraction followed by gas liquid chromatography was found to provide rapid and accurate determination of the principal terpenoids in red gum oils. Oil yields of mature leaves in the crowns of both young (2 yr) trees growing in plantations and veteran trees in natural stands were not influenced by position in the crown nor did month of collection appear to influence yields in natural stands.

A reasonably reliable assessment of rankings of individual trees for 1,8-cineole yield was obtained as early as two years after planting in a fast-growing trial of control-pollinated crosses of Petford *E. camaldulensis*. Two years was also the time required for a reliable indication of oil composition. Further studies of non-genetic sources of variation in young plants (1-4 yr) of *E. camaldulensis* showed leaf maturation and environmental factors to have a substantial influence on yields of the principal monoterpenoids including 1,8-cineole. The highest-yielding plants maintained their ranking in all trials, even though oil yields fluctuated widely in some conditions. Reliable selection of high oil-yielding genotypes is assured as long as comparisons within sites are based on leaves of the same physiological age and selections across sites are tested in a common environment.

Significant variation in oil yields was found between and within tropical provenances of *E. camaldulensis* and *E. tereticornis* in their natural habitat and in young (3.75 yr) fast-growing provenance/progeny trials in Zimbabwe. The oils of only a few provenances showed potential for commercial exploitation. Of these, Petford *E. camaldulensis* stood out because of the widespread use of this provenance in the seasonally-dry tropics. Individual trees amongst Petford provenance produced much more oil of higher quality than the average trees of the population.

Estimates of genetic parameters (heritabilities, additive and dominance genetic effects, genetic and phenotypic correlations, genotype x environment interactions and modes of inheritance) for yields of the principal leaf monoterpenoids in open-pollinated progeny tests (3.75 yr) in Zimbabwe and a control-pollinated progeny test (1 yr) in Queensland showed that they are under strong genetic control.

The potential for improving the economics of oil production in Petford *E. camaldulensis* by selection and breeding is substantial. 'Nucleus' breeding is suggested as an efficient and practical means of improving oil yields in a situation where *Eucalyptus* oil production is secondary to wood production.

Until now it has been very difficult to produce commercial yields of cineole-rich oil in the lowland tropics. This study shows this is now feasible and suggests a way in which the new knowledge can be applied.

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CHAPTER 1. INTRODUCTION

1.1 *Eucalyptus* oil

Eucalypt leaves contain oil-bearing glands that if disturbed give off aromatic odours sometimes characteristic of individual species or even of local populations within species. These essential oils are complex mixtures of volatile organic compounds of possible value in industry although their natural roles are not known. *Eucalyptus* oil is obtained commercially in a relatively simple process involving steam distillation of the leaves.

To be of interest for commercial development, a species should normally contain abundant oil, at least 1.5% oil on fresh foliage, equal to approximately 3% on dry weight. One or two important chemicals should predominate in the oils obtained. Oil-producing species are commonly grouped into three broad categories depending on the type of oil they produce. These types are the medicinal, industrial and perfumery/flavouring oils details of which are given in Chapter 2. Medicinal oils containing not less than 70% of 1,8-cineole, the principal therapeutic agent, are the most sought after commercially and command the highest price. Crude, rectified or blended cineole-rich oils and pure 1,8-cineole are used in preparations for the relief of cold and influenza symptoms (e.g. inhalants, chest rubs, lozenges). Other uses include liniments, gargles, dentrifices, soaps, disinfectants and as a solvent for spot and stain removal (Penfold and Willis 1961; Abbott 1977). A major future use of cineole could be as a component of petrol-ethanol fuel blends (Ammon *et al.* 1986).

The yield of oil from the leaves and its chemical constitution varies greatly, not only between and within species but also according to the type of leaf harvested and its physiological age, how the leaf is handled prior to distillation and on environmental conditions (Penfold and Willis 1961). Of the more than 600 species of *Eucalyptus* probably less than 20 have ever been exploited commercially for oil production (Lassak 1988). Only a handful of species, marginal in the quality of their oils, are suitably adapted for growth in the lowland tropics (e.g. *E. exserta*, *E. tereticornis*). This is a problem for tropical countries wishing to grow well-adapted, fast-growing species to produce *Eucalyptus* oil.

1.2 Importance of the tropical red gums

Eucalyptus camaldulensis and *E. tereticornis*, two closely-related species of the red gum group of eucalypts, are extremely important forest crop plants. As exotics both species are used for a wide range of purposes including shade and shelter, agroforestry and industrial wood production. The primary wood products are posts, poles, firewood, charcoal and pulp. Field trials throughout the seasonally-dry tropics invariably highlight the superior growth characteristics of red gum provenances from a region of northern Queensland between 14° and 18° S latitude and 144° and 145° E longitude. This region contains many well-known provenances including the Petford provenance of *E. camaldulensis*. This provenance is conspicuous in consistently having a faster growth rate than other provenances in numerous trials. Consequently Petford has become one of

the most important seed sources for plantations throughout the tropics (Midgley *et al.* 1990).

1.3 Background to this study

The present study of the oils of the tropical red gums has its origins in a reforestation program using fast-growing red gums to replace degraded forest in the central Terai of Nepal (White 1988). White established several red gum provenance trials and observed that a number of provenances had characteristic oils. Petford provenance was especially distinctive because of its more pungent odour, characteristic of cineole-rich oils. Subsequent tests by the Nepal Department of Medicinal Plants confirmed that some provenances produced oils rich in cineole (S.B. Malla pers.comm.). If these versatile species, suitable for reforestation primarily for stem wood production on some of the poorest soils in the world can also be exploited for their essential oils much economic and social benefit might follow. The collection and extraction of the oil is technologically simple and operational at the village and farm level.

Yield and quality in *Eucalyptus* oils depends largely on biomass production, oil concentration in the leaves and compositional characteristics. A preliminary analysis of the yield of 1,8-cineole and other major compounds in the leaves of natural populations of the red gums in northern Australia (see Exp.1, Chapter 6) showed substantial variability in these traits within and between populations. Because such variation is frequently strongly inherited, considerable scope for selection to improve oil yields and quality seemed possible. However, due to a lack of information on key genetic parameters pertaining to oil traits in eucalypts, it was not possible to predict the benefits of such a program or to recommend with confidence a breeding strategy to improve both wood and oil traits in unison.

1.4 Objectives of this study

The principal objectives of this study were to determine the variability, heritability and potential for improvement by selection and breeding of 1,8-cineole yield in the leaves of the tropical red gums and especially in the Petford provenance of *E. camaldulensis*. Additional aims were to investigate the effects of season, physiological stresses, ontogeny and age of leaves on oil yields and the implications of these non-genetic sources of variation for tree breeding. It was intended that the results of the research would provide the basis for recommendations on how breeding for oil production in Petford *E. camaldulensis* might be best integrated into a main-stream breeding program aimed at improving health and growth.

CHAPTER 2. REVIEW OF LITERATURE PERTAINING TO COMMERCIAL SOURCES, USES, FORMATION AND BIOLOGY OF *EUCALYPTUS* OILS

2.1 Introduction

Essential oils are volatile isolates obtained from plants by steam distillation or by mechanical processes and, being largely insoluble in water, separate spontaneously from the aqueous phase and can be collected. Many plants synthesise and store essential oils in specialised secretory structures such as glands and ducts. These occur in plant organs including the flowers, fruit, leaves, roots, bark and wood. Oils obtained by steam distillation include those of *Eucalyptus, Melaleuca* (tea tree) and *Mentha* (peppermint) while oils of *Citrus* such as orange, lemon and grapefruit are obtained by pressing the pericarps of their fruits. It is the quickly volatilising essential oils, most obvious from the blue haze that hangs over the Australian bush in summer or by their smell after rain or when leaves are crushed, that give the eucalypts their characteristic aromatic odours. Small globular cavities called glands filled with oil composed of odorous terpenes such as cineole, phellandrene and piperitone are distributed more or less abundantly throughout the leaf parenchyma of most *Eucalyptus* species. These glands can be readily seen as small pin-pricks of transmitted light if a single leaf is held towards the sun. It is incorrect to assume that all eucalypts have visible oil glands in their leaves as they are obscure, or apparently absent, from many tropical species and most desert bloodwoods (Brooker and Kleinig 1990). Figure 2.1 provides examples of oil glands in the leaves of several species. Depending upon species, the concentration of oil in the leaves ranges from undetectable or mere trace amounts to 0.1–5% of oil, or even up to 7%, on a fresh weight basis.

Essential oils also occur in the stem barks of many species such as *E. macarthurii* and *E. aromaphloia.* Depending on species, oil glands can occur in the root bark, the stem pith, phloem, petioles, midrib, peduncle and floral buds, and even in the young eucalypt fruits in some cases to the extent of 1% (e.g. *E. sideroxylon*) (Carr and Carr 1969; Carr *et al.* 1970). The term 'essential' oil is thought to have been derived from the work of a Swiss medical reformer of the middle ages, Bombastus Paracelsus von Hohenheim (1493-1541), who coined the term quinta essentia (quintessence). His theory was that it is the last possible and most sublime extractive, the quinta essentia (the fifth essence), which represents the efficient part of every drug and that the isolation of this extractive should be the goal of pharmacy (Urdang 1949).

It is not possible here to provide an exhaustive list of the many essential oils available today. Of the 64 most important commercial oils extracted from plant species, belonging to 20 botanical families, *Eucalyptus* oils come in the top 20 in world trade terms. The halcyon days of the industry came in the late 19th century with an explosion in work on preparations of essential oils, chemical work on their constituents and commercial applications. There are indications that the world demand for essential oils and natural, rather than synthetic, products is again increasing. The industry is presently characterised by product shortages, rising prices and a thirst for new and exciting products. There is an air of optimism in the industry that perhaps better times are ahead. This international demand will also affect the *Eucalyptus* oil industry.

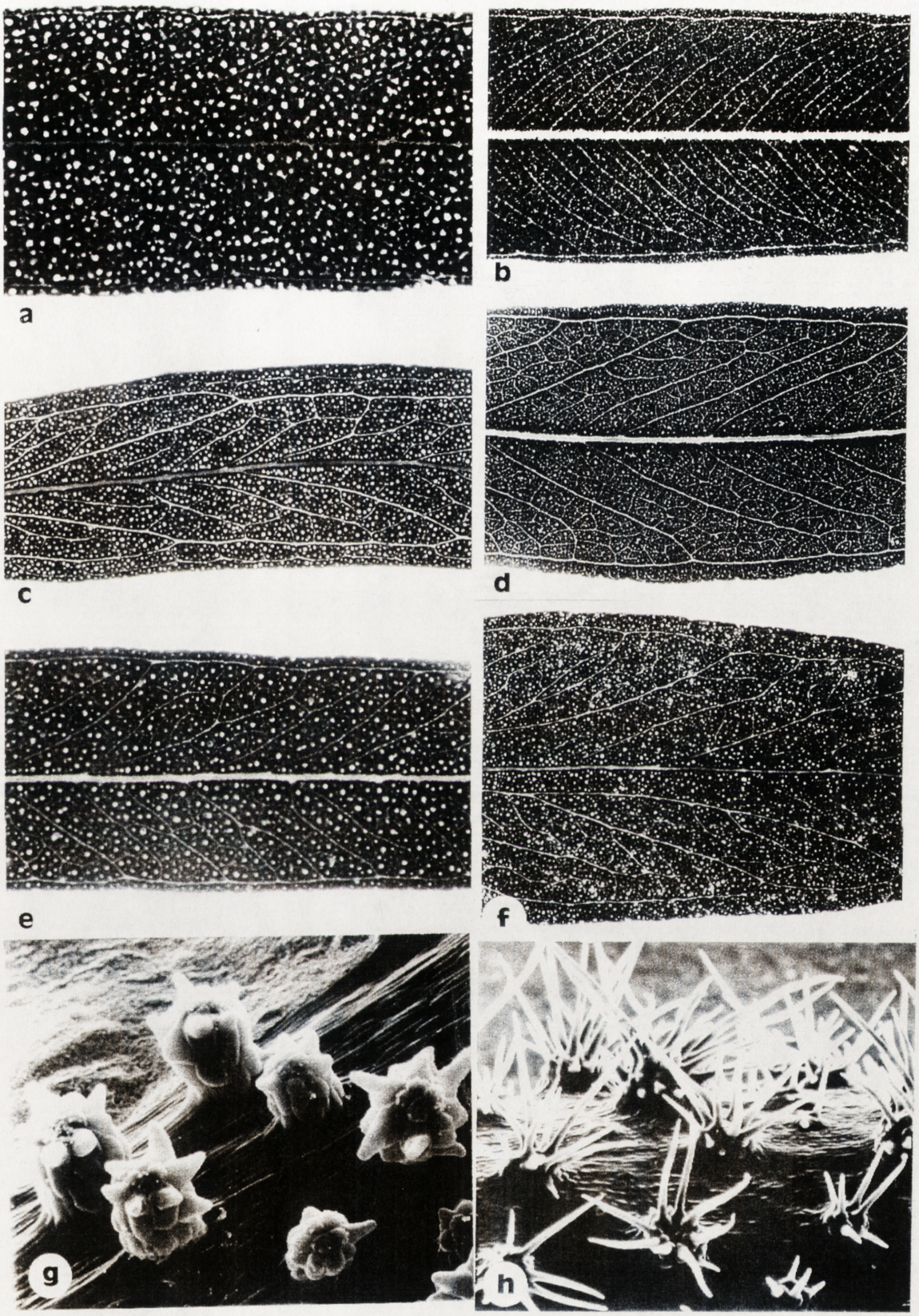


Figure 2.1 a-f Selection of eucalypt leaves showing distribution of oil glands; a - *E. polybractea*, b - *E. citriodora*, c - *E. radiata*, d - *E. globulus*. e - *E. badjensis*, f - *E. dives*. g and h Scanning electron micrographs of leaf surfaces showing stellate hairs associated with oil glands and cap cells; g - *E. abbreviata*, h - *E. olsenii*.

2.2 Uses of *Eucalyptus* oils

Oil-producing species are commonly grouped into three broad categories depending on the principal end use of their oil, viz. medicinal, industrial and perfumery/flavouring oils. While this provides a convenient way of labelling species and chemotypes, it should be appreciated that a number of oils span all three categories of end use. Oils and their uses are given exhaustive treatment by several authors including Baker and Smith (1920) and Penfold and Willis (1961).

In overview, the main points for each category are –

Medicinal oils - The active therapeutic agent and the principal constituent of medicinal oils is 1,8-cineole. The medicinal quality of the oil is specified by minimum standards which are defined in the British, United States and other pharmacopoeias and standards (e.g. Australian Standards 2113 and 2115 of 1977). The specifications of the British Pharmacopoeia are often used as the international bench-mark. They require a medicinal oil to contain not less than 70% cineole and to be practically free of α- and β-phellandrene. (The B.P. test detects amounts in excess of about 0.3% - E.V. Lassak pers. comm.) While some controversy surrounds the relevance of the phellandrene test in the B.P. regulations (Penfold and Morrison 1950), it nevertheless remains and is an influence on the species and oils that qualify for medicinal use in world trade.

Although many species contain 1,8-cineole in their oils, only a limited number combine a composition high in 1,8-cineole with consistently high total oil yields and are suitable for commercial exploitation. Oils are graded and priced on their 1,8-cineole content (70–75%, 80–85% and pure 1,8-cineole) and blending and rectification have become universal practices applied to adjust oil characteristics to meet market requirements. Abbott (1977) blames the common and sometimes dubious practice of blending oils of different qualities and sources (often including oils from genera other than eucalypts) on the market's obsession, after the 70% cineole content requirement has been met, with an oil's smell, colour and price, rather than its suitability for a particular end product. The odour of a 1,8-cineole-rich oil is modified by the presence of small quantities of other compounds which may enhance the smell (e.g. cuminal (cumin seed-like), neral and citronellal (lemon-like) etc.) or irritate the senses (e.g. the horrible choking odour of isovaleric aldehyde). Colour also can be adversely affected by the presence of undesirable, dark coloured high boiling constituents.

Rectification is done by redistillation in a vacuum in which the fore-run and still residues which may themselves be useful for industrial purposes are removed. By rectification, the disagreeable low boiling point fractions of the oil (including the highly irritant isovaleric aldehyde) are removed and the high boiling residues (mainly higher alcohols) are left in the still. The middle fraction, which has improved 1,8-cineole content, odour and colour, is marketed for medicinal use. By this means, species that would otherwise be unsuitable for this purpose can be utilised successfully (e.g. *E. globulus* and *E. smithii*).

The applications of medicinal-grade *Eucalyptus* oil are many and varied. Pharmacological action is that of a mild irritant of the nasal and bronchial mucosa which stimulates a mucous secretion (Candy 1977) thus assisting in the clearing of blocked nasal passages and bronchial tubes. The oils are, therefore, widely used as inhalants with steam and in other preparations for the relief of cold and influenza symptoms. Because of its

refreshing odour and its efficiency in killing bacteria, it also finds application as an antiseptic. Other uses include liniments, gargles, dentrifices, soaps, and as a solvent for spot and stain removal. A major future use of 1,8-cineole could be as a component of petrol–ethanol fuel blends (Ammon *et al.* 1986) and many see potential for much wider industrial applications such as an industrial solvent and cleaning agent (Abbott 1986).

Industrial oils - Industrial *Eucalyptus* oils contain principally piperitone and α-phellandrene as their main constituents. At present, phellandrene-rich oils are used exclusively for scenting of inexpensive disinfectants and industrial liquid soaps, while piperitone from *E. dives* (Type, the piperitone variant) is used for the production of synthetic menthol, used both as a flavouring agent and as an additive to various medicinal preparations (Lassak 1988). Present usage is very limited compared to the past (see Penfold and Willis 1961). With the demand for essential oils and other 'natural' products on the increase around the world and the possibility that some of the past uses may again find favour, it is worth mentioning some of these against their principal sources and constituents.

As indicated earlier practically all *Eucalyptus* oils for medicinal purposes are rectified before sale. The first runnings consist of volatile aldehydes (principally isovaleric), once used in disinfectants and sheep dip, and various other monoterpenes, including α- and β-pinene which are used in paint manufacture and as oil paint thinner under the name 'vegetable turpentine'. α- or β-pinene is also used as a starting material for the syntheses of numerous terpene derivatives used in perfume and flavouring industries (Lassak pers. comm.). The potential of *E. tereticornis* (known locally as *Eucalyptus* hybrid) plantations in India as a source of pinenes was noted by Shiva *et al.* (1984). Still residues rich in cuminal, phellandral, cryptone and p-isopropylphenol (which all possess marked germicidal properties) were once much sought after for the manufacture of disinfectants and germicides. Phellandrene-rich oil once saw much wider application as a general solvent and with piperitone and other constituents it sold as a blended *Eucalyptus* oil for mineral (zinc and lead) flotation. Piperitone also saw wider use as the raw material for the manufacture of synthetic thymol, a fungicide.

Perfumery/flavouring oils - Very few species of eucalypt have ever been exploited to supply oils for the perfumery/flavouring industries. The lemon-scented gum, *E. citriodora*, is rich in citronellal, which has limited use directly as a perfume but which is mainly used to produce other more valuable perfumes. *E. staigeriana*, lemon-scented ironbark, is a source of citral and is, therefore, useful in the compounding of lemon flavours (Lassak 1988). Brazil is the world's major producer of these oil types with production of *E. citriodora* oil amounting to 700 tonnes annually (Hillis 1986).

The somewhat rose-scented geranyl acetate-rich leaf and bark oils of *E. macarthurii* were once produced on a relatively limited scale for perfumery purposes. The still residues, following rectification, were rich in the perfume fixative eudesmol. Eudesmol is readily converted into an ester, eudesmyl acetate, which was used during World War II as a substitute for oil of bergamot. It blends well with lavender oil (Penfold and Morrison 1950). The E-methyl cinnamate-rich leaf oil of a newly discovered species from the New England Tableland in New South Wales (*E. olida*) is being produced on a very small scale as a flavour additive (Lassak 1988; Curtis *et al.* 1990).

2.3 Principal oil species

Of the more than 600 species of *Eucalyptus* probably less than 20 have ever been exploited for oil commercially, many of them outside Australia. Table 2.1 gives 19 species and their variants that constitute the principal commercial *Eucalyptus* oil species in use in the industry today.

Table 2.1 Commercial *Eucalyptus* oil species. Source: Lassak (1988)

Species	Principal leaf oil constituent and %	Oil yield (%) on fresh wt. basis
Medicinal oils		
E. camaldulensis	cineole, 10–90	0.3–2.8
E. cneorifolia	cineole, 40–90	ca. 2.0
E. dives (cineole variant)*	cineole, 60–75	3.0–6.0
E. dumosa	cineole, 33–70	1.0–2.0
E. elaeophora (= *E. goniocalyx*)	cineole, 60–80	1.5–2.5
E. globulus†	cineole, 60–85	0.7–2.4
E. leucoxylon	cineole, 65–75	0.8–2.5
E. oleosa	cineole, 45–52	1.0–2.1
*E. polybractea**	cineole, 60–93	0.7–5.0
E. radiata subsp. radiata (cineole variant)*	cineole, 65–75	2.5–3.5
E. sideroxylon	cineole, 60–75	0.5–2.5
E. smithii†	cineole, 70–80	1.0–2.2
E. tereticornis	cineole, 45	0.9–1.0
*E. viridis**	cineole, 70–80	1.0–1.5
Industrial oils		
E. dives (phellandrene variant)	phellandrene, 60–80	1.5–5.0
E. dives (piperitone variant)*	piperitone, 40–56	3.0–6.5
E. elata (piperitone variant)	piperitone, 40–55	2.5–5.0
E. radiata subsp. radiata (phellandrene variant)	phellandrene, 35-40	3.0-4.5
Perfumery and flavouring oils		
E. citriodora (citronellal variant)†	citronellal, 65–80	0.5–2.0
E. macarthurii (leaf oil)	geranyl acetate, 60–70	0.2–1.0
E. macarthurii (bark oil)	geranyl acetate, 60–68	0.1–0.4
E. staigeriana	citral (a + b), 16–40	1.2–1.5
E. olida (syn. E. aff. campanulata)	E–methyl cinnamate, 95	1.6–6.1

* main Australian commercial species † main commercial species grown overseas

2.4 Formation and development of oil glands in the leaf

Oil glands appear to originate from single cells within the epidermis and/or in the mesophyll layers of the developing leaf blade. These are also associated with the epidermis of the midrib, veins and margins of the leaf, and undergo a complex sequence of intracellular divisions to form new cells that play an essential role in the formation and development of the gland. The gland or globular cavity itself is established as an intercellular space (schizogenously) between the new cluster of central cells, after their walls have thickened to support the gland. These supporting cells are thought to be responsible for the biosynthesis of the oil that fills the cavity. A detailed account of the development and structure of oil glands is given by Carr and Carr (1970).

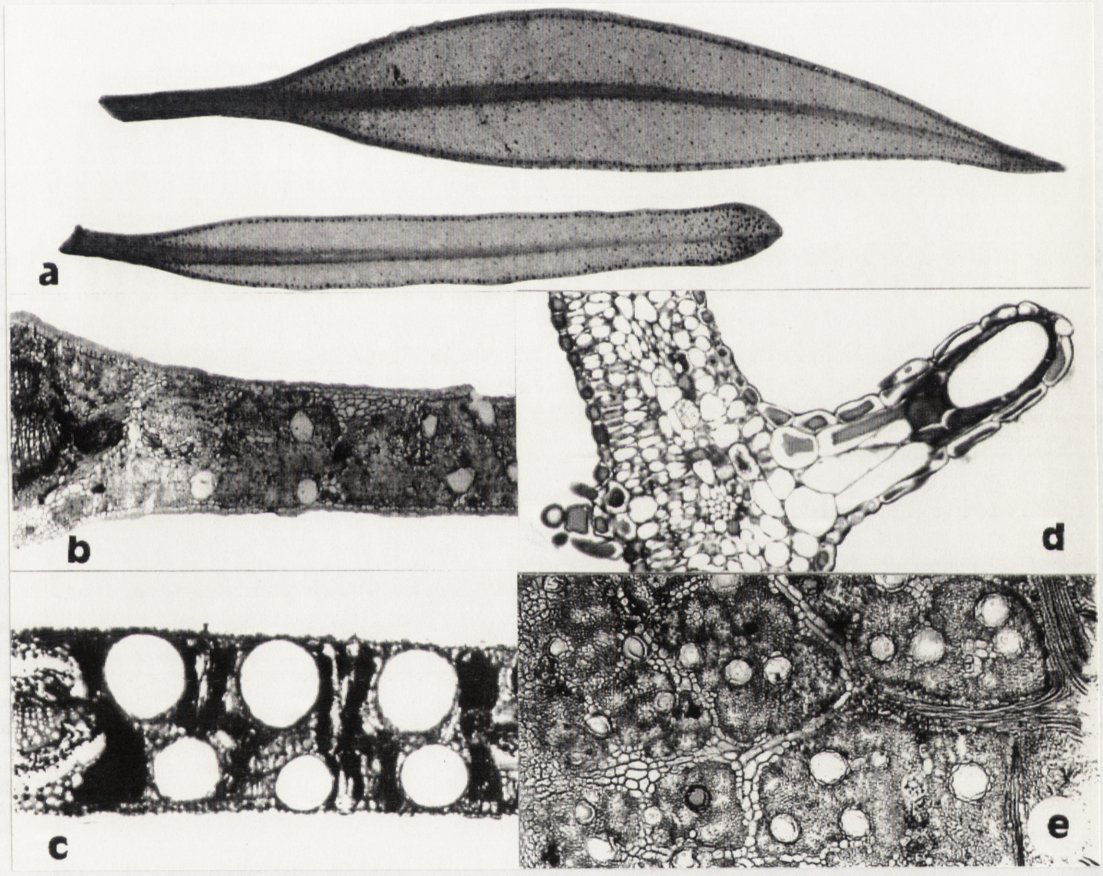


Figure 2.2 Oil glands in eucalypt leaves. a - 'Cleared' young (10 days) leaf of *E. camaldulensis* showing distribution of oil glands at edge of blade and along midrib. b - TS leaf of *E. camaldulensis* showing random arrangement of oil glands in leaf parenchyma. c - TS leaf of *E. kochii* showing large oil glands. d - TS of developing bristle hair of *E. citriodora* showing emergent oil gland. e - Horizontal section through leaf of *E. camaldulensis* showing venation associated with oil glands.

Glands close to the leaf surface (superficial glands) are characterised by cap cells, usually 2–8 in number, also created in the sequence of cell divisions, and covered by a cuticle distinctively thin and lacking in ornamentation. Oil glands in seedling leaves are entirely superficial and their distribution sometimes shows a relationship with the distribution of stomata. For example, leaves with their stomata on the lower surface of the leaf (hypostomatous) also bear their superficial glands on the lower side. In leaves with stomata on both surfaces (amphi-stomatous), superficial oil glands may be as frequent on the upper side of the leaf as on the lower, but often they are more frequent on, or even confined to, the lower surface. In juvenile leaves and especially in adult leaves some or all of the oil glands may be initiated from mesophyll cells. These internal oil glands lack evident cap cells (Carr and Carr 1985). Figure 2.2 provides examples of the distribution of oil glands in three species.

Some eucalypt species display glands that partially emerge from or are elevated above the leaf surface. Some also bear microscopic hairs from the outer surface of their cap cells, either on seedling leaves alone or carrying through to the adult state (Figure 2.1; g,h). Ladiges (1984) found that emergent oil glands occur in all *Eucalyptus* subgenera and are most conspicuous in Blakella and Corymbia which always have four cap cells on which the hairs develop but occur at low frequency or are absent in most species of subgenus Symphyomyrtus. Their presence or absence and association with ontogeny, the degree of emergence of the glands and their ornamentation are useful taxonomic traits.

An example of the pattern of oil gland formation and development in eucalypt leaves is provided by Carr and Carr (1976) for *E. baudiniana* :

'The first oil glands in the leaf appear in association with the midrib, when the leaf primordium is only a few mm long. Oil glands are also initiated in association with the marginal veins which are the next element of the venation to be blocked in. These early oil glands continue to enlarge for a time during the further expansion of the leaf and they therefore eventually constitute some of the largest oil glands of the mature leaf. As the main costal venation is blocked in, oil glands appear between the lateral veins. By the time the leaf is about half its mature width there are more than a dozen developed oil glands - usually those which will attain the intermediate sizes - in each of the panels bounded by the main lateral veins. As the vein islets are blocked in they also acquire oil glands, the smallest of which occupy the smallest territories within the venation pattern. All of these glands continue to expand after initiation, at first rapidly then very slowly until the maximum size (which is partly determined by the number of epithelial cell layers initiated) is reached. Oil glands cease to be initiated and expand before the leaf reaches its maximum size. The population of oil glands consists, therefore, of a number of sets each corresponding roughly to a stage of blocking in of the venation and consequently of diminishing initial and maximum size. A frequency distribution of diameters of oil glands is therefore not normal but consists perhaps of several overlapping distributions.'

Oil glands, therefore, if visible in the leaf, are characterised by size, shape, position, density and apparent association or lack of association with the venation. M.I.H. Brooker (pers. comm.) believes that the oil glands together with leaf venation make useful patterns for analysis and recognition of species groups in *Eucalyptus*.

The job of identifying high-yielding oil-producing species would, of course, be easier if there existed a correlation between a species average oil gland size and density and yield of essential oil. In fact, work to date suggests that the correlation is poor with several

species possessing conspicuously large and numerous glands ranking relatively poorly in terms of oil yield (M.I.H. Brooker pers. comm.).

2.5 Chemistry, biosynthesis and function

Eucalyptus leaf oils are composed of complex mixtures of volatile organic compounds, often involving 50 or even 100 or more separate compounds, of such chemical types as hydrocarbons, alcohols, aldehydes, ketones, acids and esters. Some popular, yet unresolved, theories about their function are discussed below. The following section on chemistry and biosynthesis outlines only the basic facts underlying oil metabolism. The references given should offer a useful source for those wanting more information.

2.5.1 Chemistry and biosynthesis

Essential oils of eucalypts are composed predominantly of mono- and sesquiterpenes. Terpenes, a generic term harking back to the first isolation of this class of natural product from turpentine oil, can be recognised as compounds which contain a sequence of two or more isoprene units (C_5H_8) joined either head to tail or head to head or compounds which are derived from these initial condensations due to a range of secondary chemical transformations. They are all biosynthesised from mevalonic acid which is itself formed from photosynthates following the general scheme as outlined in Figure 2.3. The resultant product of the coupling of two isoprene units, geranyl pyrophosphate, serves as a precursor to the several hundred naturally occurring monoterpenes (C_{10}). Head-to-tail coupling of three isoprene units provides farnesyl pyrophosphate which is the progenitor of the 15 carbon-containing sesquiterpenes (C_{15}) and so on (Erman 1985).

While the details of the biosynthetic pathways involved in the synthesis of particular compounds are still to be elucidated, the levels of precursor and activity of enzymes appear to be the governing factors (Manitto 1981; Beale 1990). For example, in the synthesis of the monoterpenes the activity of various cyclases, enzymes that catalyse the crucial cyclisation reactions from the acyclic precursor, geranyl pyrophosphate, determines which parent monoterpene carbon skeletons are produced. These primary end-products frequently undergo secondary transformations involving a further complex and dedicated set of enzymes. Some 20 cyclases have been thus far identified with the expectation that the number found in nature may approach 50 (Croteau 1987, 1988a).

Monoterpenes (C_{10}) are the major constituents of the lower boiling point and less polar fraction of *Eucalyptus* oils and may be classified into three groups - acylic (open chain), monocyclic (one ring) and bicyclic (two rings). Myrcene, 1,8-cineole and the pinenes are well-known representatives of these three groups. The sesquiterpenes (C_{15}), the other major class of compounds found in the oils, fall into four groups - acyclic, monocyclic, bicyclic and tricyclic. Farnesol, humulene, the eudesmols and globulol are representatives of these. Figure 2.4 gives a listing of a selection of terpenes commonly found in eucalypt leaf oils by their type and structural arrangement.

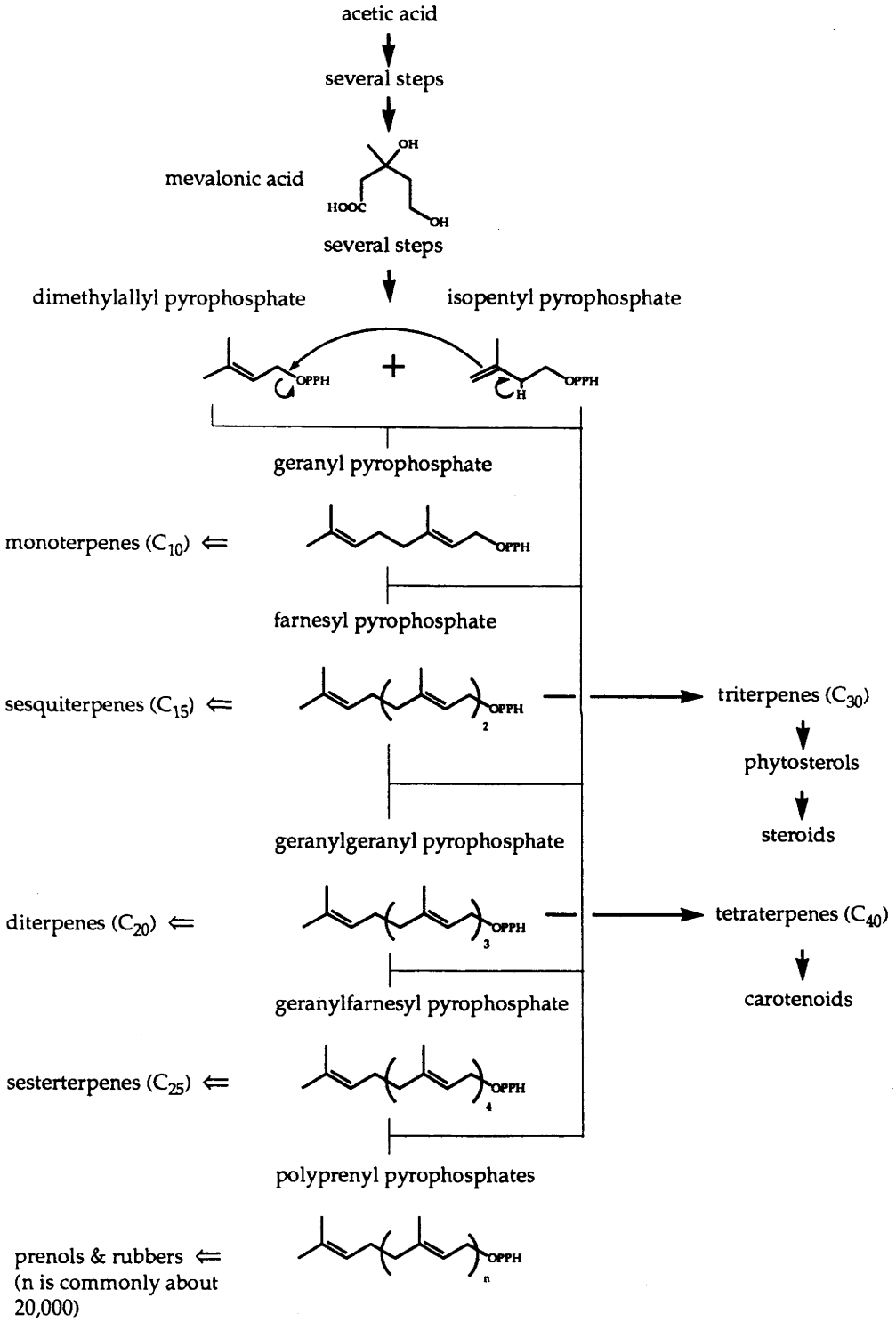


Figure 2.3 General scheme for the biosynthesis of the terpenes (derived from Erman 1985).

The exact place of formation of essential oils is not known with any clarity. The weight of evidence points to the formation of oil in the region of photosynthetic activity in the cells surrounding the oil gland followed by its passage through the cell wall into the interior of the gland (Haagen-Smit 1949). In cells in the non-photosynthetic mesophyll, the mevalonic acid needed for oil production presumably forms at the site of synthesis from translocated photosynthate (see review in Dell and McComb 1978).

2.5.2 Function

While theories abound, there are few 'hard' facts supporting either an external (ecological) or internal (either physiological or metabolic) role for the essential oils of *Eucalyptus*. Traditionally, *Eucalyptus* oils along with the essential oils of other genera have been regarded as the relatively toxic waste products of plant metabolic processes with no practical value to the plant (Penfold and Willis 1955). The apparent lack of transport of the oils from the leaves back into the stem immediately prior to leaf fall, as with most 'reserve' materials in plants, and their storage in highly specialised secretory structures has been used in support of this hypothesis.

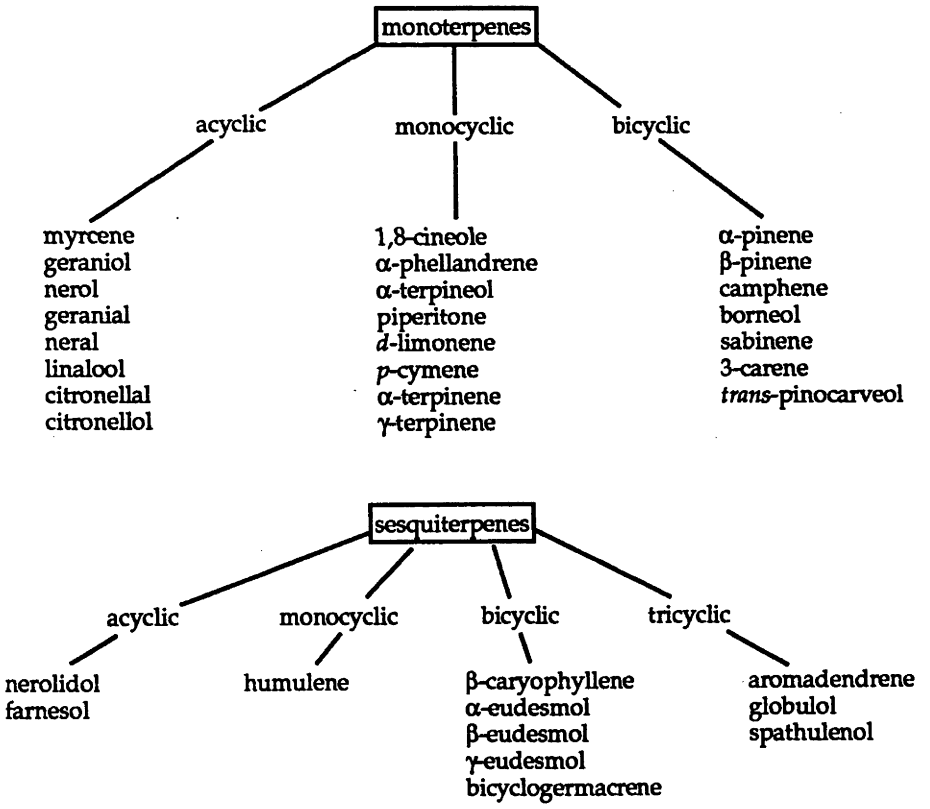


Figure 2.4 A selection of terpenes commonly found in the leaf oil of eucalypts, grouped according to their type and chemical structure.

In various other essential oil plants, this traditional view of the function of plant terpenes has been challenged by investigators whose plant feeding studies with radio-labelled carbon dioxide, mevalonic acid and terpenes have shown that essential oil may provide a 'metabolic' pool for synthesis of indispensable plant components such as pigments, sugars, amino acids and certain respiratory coenzymes (Erman 1985). For example, work on the herbaceous species, peppermint (*Mentha piperita*) and sage (*Salvia officinalis*), in the United States provides 'compelling' evidence that the monoterpenes do break down into compounds which can be re-utilised in lipid biosynthesis in the developing root or rhizome or may, conceivably, be further oxidised in energy production (Croteau 1988b).

While specialised biochemical studies of this type have yet to be attempted with eucalypt species, it is tempting to speculate that *Eucalyptus* oils also have a metabolic role to play within the plant. The often reported and complex phenomenon of seasonal variation in oil concentration might then be partially explained at least in terms of a dynamic balance between synthesis of terpenes (increase in concentration) and their chemical alteration and utilisation within the leaf or plant.

By far the most popular theories concerning the role of *Eucalyptus* oils in nature centre on their contribution to the survival potential of this genus in relatively harsh Australian environments. There has been frequent speculation that the oils possess properties that assist the plant in repelling leaf-eating insects. Indirect evidence supports this claim as eucalypts introduced to areas outside of Australia prove highly, although not exclusively, immune to the locally adapted herbivores.

Within Australia an extremely large number of insects feed on eucalypt foliage and have evolved various processes to metabolise or circumvent the presence of the oil and in some cases use them in their own defence (e.g. Australian saw fly larvae store and regurgitate *Eucalyptus* oil when threatened). There is little direct evidence to suggest that eucalypts are themselves protected by the oils. However, the oils might serve to restrict the number and kind of their enemies (Morrow *et al.* 1976). In addition, few studies have shown a relationship between foliar oil concentration and level of insect herbivory (Morrow and Fox 1980). One notable exception is the recent work undertaken by Hi-Fung Li, Davies and Madden of the University of Tasmania. Phytochemical studies of eucalypts growing in Tasmania and feeding bioassays have shown that host tree selection by paropsine chrysomelid defoliators is affected by the qualitative and quantitative mix of monoterpene hydrocarbons. Ovipositional preference and rates of larval feeding and survival are significantly influenced by 1,8-cineole, α-phellandrene and α-pinene.

Early cold resistance in seedlings of several eucalypt species grown in Florida U.S.A. has been associated with increased levels of α-phellandrene and ρ-cymene in the foliage (Shimizu 1974).

There is evidence also that terpenes leached from the leaves of eucalypts contribute to allelopathic effects on the forest floor inhibiting the germination and growth of competitors (Al-Mousawi and Al-Naib 1975; Del Moral and Muller 1969). It has been suggested, although not proven, that *Eucalyptus* oil vapours near the leaf surface may reduce water loss and that the oils in the flowers might release odours attractive to pollinating agents, as has been shown elsewhere (Penfold and Willis 1955).

The presence of volatile oils in both the litter and green leaf of most eucalypts and many of their understorey associates (e.g. bottlebrushes, paperbarks, tea trees) may be a contributing factor in the ignition and spread of wildfire in the Australian bush (Pompe and Vines 1966) assisting in the regeneration and maintenance of eucalypt forests (Mount 1964). Contemporary thinking, however, considers oils of minor significance relative to other factors operating in a wild fire situation (Simpfendorfer 1989).

2.6 Sources of variation in composition and yield

Sources of qualitative and quantitative variation in oil characteristics between and within species may be attributed to three major factors, while a fourth dimension is added through the errors which may easily arise in determination of this variation:

1. Genetics.
2. Type and age of leaf.
3. Environment.
4. Techniques of extraction and analysis.

These factors must be considered when comparing the essential-oil status (yield and composition) of species or even different individuals of the same species. Failure to apply appropriate methodological and sampling controls to take account of these major sources of variation is, unfortunately, a feature of early studies of *Eucalyptus* oils, rendering the data of dubious value.

2.6.1 Genetic variation within and between species and heritability of oil traits

Variation in the composition of *Eucalyptus* oils occurs at all levels of the taxonomic hierarchy to an extent that the value of their chemistry to the taxonomy of the genus is limited (McKern 1965). On occasions, however, leaf oils have assisted botanists in interpretation of patterns of variation within and between species (e.g. Johnstone 1984; Simmons 1974) and in detecting hybrids (e.g. Simmons 1985).

Qualitative differences are most common between species, however, the variability is such that chemical differences alone neither constitute sufficient grounds for assessing the taxonomic distinction between species nor indicate affinity towards other species. There are many examples of species that are closely related taxonomically, differing substantially in the chemical composition of their oils (Penfold and Willis 1961; Boland *et al.* 1991). However, the reverse is also true.

Within species, variability is typically, although by no means exclusively, quantitative in nature. An example of qualitative variation within a species and the importance of studying geographic variation in oil traits before attempting to categorise a species' oil type can be found in work on *E. camphora* (Coorey *et al.* in press). Although this species is named after the high camphor (eudesmol) content of trees in the 'type' population, a study of the oils of seven morphologically similar populations throughout the species' range could only find camphorous compounds in two populations.

An extreme form of quantitative variation within species, commonly found in *Eucalyptus* and in a wide variety of other plant families (Hellyer *et al.* 1969), is the occurrence of

chemical forms. Chemical forms are 'plants in naturally occurring populations which cannot be separated on morphological evidence, but which are readily distinguished by marked differences in the chemical composition of their essential oils' (Penfold and Willis 1953). Chemical forms are generally well defined and readily distinguishable from each other, since, even though there is some overlap in percentage of the major oil component, any given tree can be assigned to a chemical form if several major oil components are considered (Simmons 1974). They do not appear to be a result of site differences, seasonal variation, leaf ageing effects or hybridisation.

Penfold and Morrison (1927) first reported such forms in *E. dives* and called the variants 'physiological forms'; to distinguish four separate and distinct oil forms they called them 'Type', 'Variety A', 'Variety B' and 'Variety C' in order of discovery. The use of this terminology has now been discontinued in favour of simply referring to the biochemical variants as variants, forms, chemoforms, chemovars or chemotypes, and highlighting the major oil component, for example '*E. dives* (cineole variant)'. Subsequent work has identified 5 chemical forms in *E. dives*, 6 in *E. radiata* (Johnstone 1984), 4 in *E. citriodora*, 4 in *E. racemosa* (syn. *E. micrantha*), 3 in *E. elata* (syn. *E. andreana*), and 2 in *E. piperita* (Penfold and Willis 1961) as well as distinct variants in *E. camphora* and *E. ovata* (Simmons 1974). Each form may occur in separate distinct populations, however, they often mingle on the one site with individual trees (or chemotypes) locking crowns with other chemical forms suggesting that oil traits are under strong genetic control. The potential importance of chemical forms either positively as a rich source of required chemicals or negatively as adversely affecting the quality of oil has been noted by Hillis (1986).

Less complex than the case of chemical forms, but also an important factor when considering a species for commercial production, is the often reported phenomenon of population to population and tree to tree variability in yield of chemical compounds. Highly significant differences between populations and trees in yield of often commercially important compounds such as 1,8-cineole have been reported for *E. kochii* (Brooker *et al.* 1988), *E. camphora* and *E. ovata* (Simmons 1974; Simmons and Parsons 1987; Coorey *et al.* in press), *E. punctata* (Southwell 1973) and for several other species. Oil characteristics in *Eucalyptus* appear to be strongly inherited (Pryor and Bryant 1958), hence, significant improvement in the economics of oil production by means of selection and breeding for these traits seems possible. However, there is little appropriate genetic information to gauge the costs and benefits of such a program. The genetic control of essential oil composition is now well-established for a large number of other taxa (e.g. *Mentha* (Murray and Hefendehl 1972), *Pinus* (White and Nilsson 1984), *Pseudotsuga* (von Rudloff and Rehfeldt 1980)). However, there has been little work on the genetics of *Eucalyptus* oil. The only documented estimates of genetic parameters for oils in a eucalypt species are the heritabilities for 1,8-cineole yield for 50 open-pollinated families of *E. kochii* as 6-month-old seedlings, at 18 months and 3.8 years in an unreplicated progeny test (Barton *et al.* 1991). These authors reported family heritabilities up to 0.83 and estimated increases in cineole yield (w/w %, fresh leaf) from around 2% in native populations to 2.8% in improved populations from progeny test selection at an intensity of one parent in every 10. Although total oil production per unit land area depends both on the amount of leaf produced as well as leaf oil content, there is no information published on genetic associations between oil yields and growth traits in eucalypts.

2.6.2 Variation due to type and age of leaf

Eucalypts typically develop up to five distinct morphological types of leaf during their life time, each type corresponding to a certain ontogenetic stage in their development. The types are:

1. Cotyledons.
2. 'Seedling leaves' (for about 5-10 leaf pairs above the cotyledon).
3. 'Juvenile leaves' - often conspicuously different from adult leaves in several morphological characters and may persist for a number of years.
4. 'Intermediate leaves' - occur in the transitional zone between (3) and (5) and are usually larger and coarser (the transition may take place abruptly or gradually and at different ages).
5. 'Adult leaves'.

These phases are quite separate from and should not be confused with physiological leaf age commonly denoted by the terms 'young leaf' (mean age of about one month or less - these are the growing tips or flush growth of the tree), 'mature leaf' (mean age of about 6 months) and 'aged leaf' (about 12–18 months old) (Penfold and Willis 1961). The average life of leaves of naturally occurring eucalypts is about 18 months. This is, of course, subject to wide variation with some leaves lasting only a few months in the case of quick-growing species and some persisting for 3-4 years in slow growing species. Any of the three arbitrarily selected physiological age groups, therefore, can exist in any one of three ontogenetic stages - juvenile, intermediate and adult. As Bryant (1950) points out the 'juvenile' leaves of coppice growth may often be 'aged' and usually stay on the tree much longer than the 'adult' type leaves on trees in virgin stands.

There are few definitive studies on the effects of ontogeny on leaf oils and in a number of studies it is not always obvious whether comparisons are clear of the confounding influence of physiological leaf age. Some reports suggest there is no (e.g. in *E. dives*, Penfold and Willis 1961) or only limited (e.g. in *E. delegatensis*, Weston 1984) qualitative difference between juvenile and adult phases, while others show a more complex picture. Coorey *et al.* (in press), for example, showed geographic variation in the comparison of the constituents of juvenile and adult leaves of *E. camphora*. The composition of adult and juvenile oils were mostly similar within provenances and different between provenances with the exception of two Victorian populations which showed marked differences between the adult and juvenile phases.

The yield of oil from seedling leaves is invariably much lower than that of other phases (e.g. *E. delegatensis*, Boland *et al.* 1982), while the comparison between the juvenile and intermediate phases and the adult are inconsistent and appear to depend largely on species. For example, reports of the juvenile-intermediate leaf of coppice growth being lower (e.g. in *E. nitens*, Franich 1986), equivalent (e.g. in *E. radiata*, Donald 1980) or higher in yield (e.g. in *E. polybractea*, Brooker *et al.* 1988) with no qualitative difference to the adult foliage of the same tree are quite common. Mature leaves of juvenile *E. dives* gave similar yields to mature leaf from adult trees (Penfold and Willis 1961). Tjandra (1986) and Barton *et al.* (1991) in their work on *E. kochii* found no qualitative variation between parents and progeny, however, they did report significant quantitative variation with age of plant. In comparison to their parents, juvenile leaf from 6 month old seedlings grown in the glasshouse was lower in cineole and higher in terpene hydrocarbon yields. The same seedlings planted in a field trial, were sampled at 18

months and three years. Cineole yield increased steadily and at 3 years closely resembled parent values. A similar result was reported for *E. citriodora* where it took 3 years of growth for oil and citronellal contents of progeny to attain stable levels (Mascarenhas *et al.* 1987).

It appears that progeny trials for chemosystematic and genetic studies may be reliably assessed for oil production within the relatively short period of 3 years.

The contrast in oil yield between young and mature leaves is often marked. However, there appears to be a high level of variability in the direction and extent of these differences not only between species but also between individuals of the one species in the same population. A note of warning in drawing inferences from studies of this phenomenon was sounded by Bryant (1950) who drew attention to the largely unavoidable but potentially compounding influence of the position of the leaf on the tree and the influence of light intensity. Aged leaves generally yield less oil than recently matured leaves as reported in *E. cneorifolia* (Berry 1947), *E. dives* and *E. radiata* (Bryant 1950; Penfold and Willis 1961), *E. macarthurii* (Penfold and Morrison 1950) and *E. camphora* and *E. ovata* (Simmons 1974), but no significant difference was observed in the case of *E. smithii* (Bryant 1950). The yield of oil at various ages appears to be determined by a complex pattern of quantitative change in individual or groups of compounds, some remaining constant, some increasing and some decreasing with age. Patterns can vary even between individuals of the one species on the one site and, in fact, appear dependent on the genetic constitution of individual trees (Simmons and Parsons 1987). Published work on other taxa shows that oil composition can change with leaf maturation, e.g. *Angophora* (Leach and Whiffin 1989) and *Melaleuca* (Southwell 1988; Brophy *et al.* 1989) and, potentially, could be a serious source of error in oil studies if not considered in sampling strategies. There are few reports of qualitative changes with leaf age in eucalypts. Work on *E. camphora* and *E. ovata* suggests that while substantial quantitative changes may be taking place with age, the actual mix of compounds present in the oil of any one individual is relatively stable (e.g. Simmons 1974). Bryant (1950), however, found qualitative variation in some samples of older leaves of *E. radiata* where phellandrene was absent in contrast to high levels in young leaves.

2.6.3 Environmental variation

Where comparisons must be made between trees, between stands growing under different conditions, within the one tree at different times or between trees in different physiological conditions it is important to realise that many environmental factors may affect the quantity and the chemical composition of the oils produced (Bryant 1950). From a commercial view point also it is important to establish those influences that might have a significant impact on oil production and, therefore, on the economics of a harvesting operation. The effects of environmental factors are likely to involve a highly complex network of variables, and very little information is available concerning them. Most studies to date have aimed to ascertain the extent of seasonal and diurnal influences on volatile oils.

Detailed studies on seasonal fluctuations in oil yield and proportions of constituents have been carried out for many taxa but the results are rather contradictory. Thus several workers have reported no significant seasonal variation of volatile oils in a number of widely separated plant genera, e.g. *Picea* (von Rudloff 1972), *Mentha* (von Rudloff and Hefendehl 1966) and six genera of Australian rainforest trees (Whiffin and Hyland

1989). However, other reports indicate that seasonal variation in volatile oil composition does occur in some taxa, e.g. *Juniperus* (Adams 1970) and *Pseudotsuga* (Maarse and Kepner 1970). The few reports considering diurnal variation indicate that diurnal changes in volatile oils can also occur (see review in Leach and Whiffin 1989).

Recent studies on these sources of variation in two myrtaceous genera closely related to *Eucalyptus* are of interest. They are *Angophora* and *Melaleuca*. In *A. costata*, Leach and Whiffin (1989) detected changes in the proportions of constituents in the oil over seasonal and diurnal periods but these were shown to be much less than genotypic variation between individuals. While seasonal changes were weakly linked to monthly or seasonal temperatures and diurnal changes appeared to be associated with humidity, the authors concluded that seasonal and diurnal patterns in oil composition could not be conclusively related to any environmental parameter. Moreover, because of the overriding effect of genotype on changes in the oil composition with time, they highlighted the inappropriateness, at least for *A. costata*, of the common practice of pooling data or bulking foliage from several trees for studies of this type. In *M. alternifolia* (terpinen-4-ol type) only minor variation has been found in the proportion of the major components in the oil with time. Oil yields, however, reveal a significant annual cycle of change being at their lowest in late winter and early spring and highest during summer and autumn (Williams and Home 1988). Murtagh (1988) found that oil concentration in twigs of *M. alternifolia* varied markedly from site to site and on a monthly and daily basis. The monthly pattern of variation suggested a strong positive correlation between water availability and oil concentration, while the pattern between days showed oil concentration to be negatively correlated with daily minimum temperatures. An association between oil concentration and the metabolic activity of the plant is suggested.

Seasonal fluctuations in oil yield of various eucalypt species had been noted by bush cutters who believed oil yield to be lower in the winter months than in summer (Penfold and Willis 1961). Early attempts to study this phenomenon suffered from lack of refinement of experimental procedure and led to confusing and contradictory results. Unfortunately only very limited recent work using modern analytical techniques and adequate sampling controls is available for review. Simmons and Parsons (1987) in a study of seasonal variation in the oils of *E. ovata* and *E. camphora* found that the yield of oil in mature leaves of *E. ovata* remained relatively constant throughout the study period while in *E. camphora* the highest yields occur in the winter months with slightly lower yields over the summer months. Compositionally, individual trees of both species differed markedly in the patterns of variation in several major oil components including cineole, some showing a linear increase, some a linear decrease and some giving no clear pattern. Variation in oil composition did not appear to be closely correlated with climatic variables and was best explained by a leaf ageing effect rather than a strictly seasonal effect. They concluded that there are likely to be considerable differences in patterns of variation both within and between species particularly in those taxa with high levels of infraspecific variation.

Seasonal variation in cineole production was studied by Brooker *et al.* (1988) for *E. kochii* and *E. plenissima*. Ten sites in the field were used in the investigations, and monthly samplings were made of single trees (3 to 11 trees per site), for 2 years. There was a small seasonal effect with the highest production of 1,8-cineole occurring in January and February and the lowest in August. There was inconsistency in pattern between years and the authors concluded that seasonal effects should have little influence on harvest time. The variation between individual trees within the one site far outweighed

the modest seasonal effect again pointing to the overriding importance of individual genetic makeup on volatile oils.

Most previous studies on environmental variation in *Eucalyptus* oils have been undertaken on long-lived, adult trees of relatively slow growth rate in their natural habitat. If, as several workers suggest, there is a strong relationship between variation in oil traits and the metabolic state of plants, it is highly likely that seasonal and diurnal changes in oil characteristics may be of much greater significance amongst young, physiologically active plants in fast growing plantations. For example, Donald (1991) reported that harvesting for oils in young plantations of *E. radiata* in South Africa was restricted to the summer months because of dramatic falls (up to 45%) in oil yields in the other seasons. He believes that the poor yields in winter are due to rapid growth during this period and a consequent reduction in the amount of photosynthate available for the production of oil. A sampling strategy to assess changes in oil traits with time in this situation must recognise and allow for the possible confounding influences of key factors such as genotype, ontogeny and physiological age of leaf as well as features of the site and its environment.

2.7 Variation due to sampling, extraction and analysis techniques.

Some of the many factors involved in determining oil composition have been discussed above. For the successful study of these effects it is imperative not to add further complications such as those caused by various sampling, extraction and analytical procedures.

Although the characterisation of the terpenoids of *Eucalyptus* oils by gas liquid chromatography is relatively simple for a chemist expert in this field, the inexperienced investigator can encounter many problems and pitfalls from sampling techniques to analysis and interpretation of data. Methods are best learnt by personal instruction from an experienced operator and through training courses. Useful sources of information are books such as Masada (1975) and Sandra and Bicchi (1987) and publications that deal specifically with chemical evaluation of eucalypt leaf oils such as Ammon *et al.* (1985) and Barton *et al.* (1989).

2.8 Discussion

As a result of a world-wide resurgence of interest in naturally occurring chemicals, the international market for *Eucalyptus* oil, which has been stable for many years, may again expand. There is certainly renewed interest both nationally and internationally in supplies of seed of appropriate oil-producing species for trial in temperate and tropical environments. A problem faced by newcomers to the industry is that much of the key background literature on *Eucalyptus* oils and especially covering aspects of their formation and biology, is fragmented, contradictory and of dubious value because of questionable sampling and analytical practices. Much basic research on *Eucalyptus* oils remains to be done. The future of the industry may hinge not only on the identification and commercial development of new uses for established oils, but also on a much expanded screening of a diverse genetic resource for new and interesting chemicals suitable for specialty users (Boland *et al.* 1991). The lack of progress over the past 30 years or more towards better definition of the role of essential oil in *Eucalyptus* is

disappointing. This knowledge is basic to an understanding of the regulation of synthesis within the plant and environmental interactions which may have a detrimental economic impact on production. Clearly, this is an area that deserves more attention.

This review highlights some of the complexity of qualitative and quantitative variation in oil characteristics in *Eucalyptus* and draws attention to some of the principal sources of this variation. Many studies, even recent ones, do not apply rigorous enough sampling controls. Much of the available information on aspects like seasonal variation is confounded by other influences such as changes in leaf age and genotypic differences. The industry would benefit from more carefully controlled experiments aimed at pinpointing the key factors affecting oil production.

CHAPTER 3. ANALYTICAL METHODS FOR EVALUATION OF RED GUM LEAF OILS

3.1 Introduction

From the preceding chapter it is clear that 1,8-cineole, the principal therapeutic agent and solvent of medicinal grade oils, is the compound most sought after in present world markets. Rules set by the various pharmacopoeias dictate that all oils for medicinal use be at least 70% 1,8-cineole. This quality target can be manipulated by producers by the universal process of rectification and the common practice of blending. Therefore, yield of 1,8-cineole in the leaves measured in units of weight of oil per unit fresh- or dry-weight of leaves, is the oil trait of most commercial interest. Potential to increase yield of this compound through tree breeding is the major focus of this thesis.

As will be seen in later experiments, four other monoterpenoid compounds are prominent in the oils of the northern red gums. In order of their frequency of occurrence and contribution to total oil yield they are α-pinene, limonene, β-pinene and ρ-cymene although in the case of the latter it was often present in trace amounts only and was not universally quantified. In total these compounds with 1,8-cineole comprise 80% (SD ± 8%) of the oil of the regular Petford chemotype as determined by GLC on samples steam distilled with cohobation for 8h. Their combined yield in individuals and provenances, where the proportion of 1,8-cineole in the oil is 70% or greater, may be considered an approximation of the total yield of monoterpenes and a crude estimate of the total oil yield achievable in a bush extraction operation without cohobation and with relatively short distillation times. Cohobation is the practice of recycling the distillation water back into the distillation pot during oil extraction to minimise loss of constituents that are partly water soluble.

For the reasons outlined above, it was decided to concentrate in this study on the quantitative determination of 1,8-cineole, α-pinene, limonene, β-pinene and ρ-cymene (when present in quantifiable amounts). Some aspects required a more detailed analysis (this Chapter and no. 6) where steam distillation-gas liquid chromatography (GLC) combined with GLC-mass spectrometry (MS) procedures were utilised. Although several laboratory methods for the quantitative determination of 1,8-cineole are well established (e.g. the o-cresol method see Langenau 1949), Ammon *et al.* (1985) found that all suffered from substantial defects including lengthy application times and inaccuracies. These authors promoted a rapid, simple and sensitive solvent extraction method involving ethanol followed by use of GLC for the simultaneous quantitative determination of terpenes including 1,8-cineole directly on the leaf extracts without a prior separation process. This chapter is largely concerned with the testing and modification of the same solvent extraction technique and GLC analysis to suit the particular conditions applying to this study.

A description is also provided of the steam distillation-GLC and combined GLC-MS procedure used for detailed analysis of the oils of the northern red gums, determination of leaf moisture contents by oven drying and the testing of various field methods thought to have potential for ranking trees as to their oil yielding capacity.

3.2 Steam distillation-GLC combined with GLC-MS procedure

3.2.1 Introduction

There may be more than 90 different compounds in an oil sample of the northern red gums. Considerable expertise is required by the chromatographer in making a detailed analysis of these compounds. GLC equipment and columns capable of high resolution and preferably combined with a mass spectrometer are needed.

Identification of the full suite of compounds present in the oils of individual trees and bulked samples was required in two experiments. Initially as the standard against which to test the solvent extraction-GLC method (this chapter) and in Experiment 1, Chapter 6. In this endeavour the collaboration of an essential oils expert at the University of NSW, Dr J.J.Brophy, was sought. The following methods which are used routinely by Dr Brophy for eucalypt oil analysis (Boland *et al.* 1991) were adopted for the quantitative and qualitative analysis of the red gum oils.

3.2.2 Extraction by steam distillation

The leaves, when they arrived at the Sydney laboratory from northern Queensland, were weighed and, if they could not be steam distilled immediately, were allowed to air dry. The isolation of the oil from the leaves was achieved by steam distillation with water cohobation of the leaf material (approximately 100g) in a Dean and Stark apparatus (Figure 3.1a). This was modified to give lower phase return of water. The flask contained approximately 0.5 litres of water. The length of distillation was a standard 8h to ensure capture of the oxygenated monoterpenes and sesquiterpene hydrocarbons. In all cases no more oil was seen to be produced after this period.

Once distillation was complete a small amount of pentane was added to the oil to increase its volume and the oil and water in the side arm of the apparatus were separated. The pentane/oil solution was dried over anhydrous sodium sulphate, the solution was transferred to another container and pentane allowed to evaporate over night. The oil yield was calculated on the weight of oil remaining and the fresh weight of leaf material as it arrived in the laboratory.

3.2.3 GLC and combined GLC-MS

Analytical gas chromatography (GLC) was carried out on a Shimadzu GC6 AMP gas chromatograph. A SCOT (support coated open tubular) column of SP 1000 (85m x 0.5mm) which was programmed to increase in temperature from 65°C to 225°C at 3°C min^{-1} was used with helium carrier gas. For combined GLC-MS the gas chromatograph was connected to an AE1 MS12 mass spectrometer through an all glass straight split interface (Figure 3.2a). The mass spectrometer was operated at 70 eV ionising voltage and 8000V accelerating voltage with the ion source at 200°C. GLC conditions for combined GLC-MS were the same as for the analytical GLC. Spectra were acquired every six seconds and processed by a VG Display Digispec data system. GLC integrations were performed on a Milton Roy CI-10 electronic integrator.

Compounds were identified by GLC retention times identical to known compounds and by comparison of their mass spectra with either known compounds or published spectra (Stenhagen *et al.* 1974; Heller and Milne 1978, 1980, 1983).

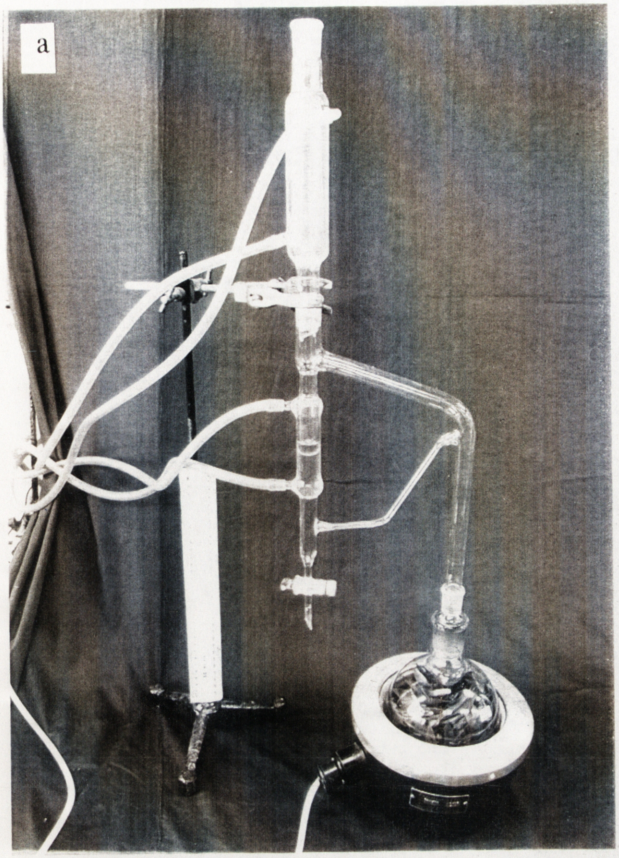
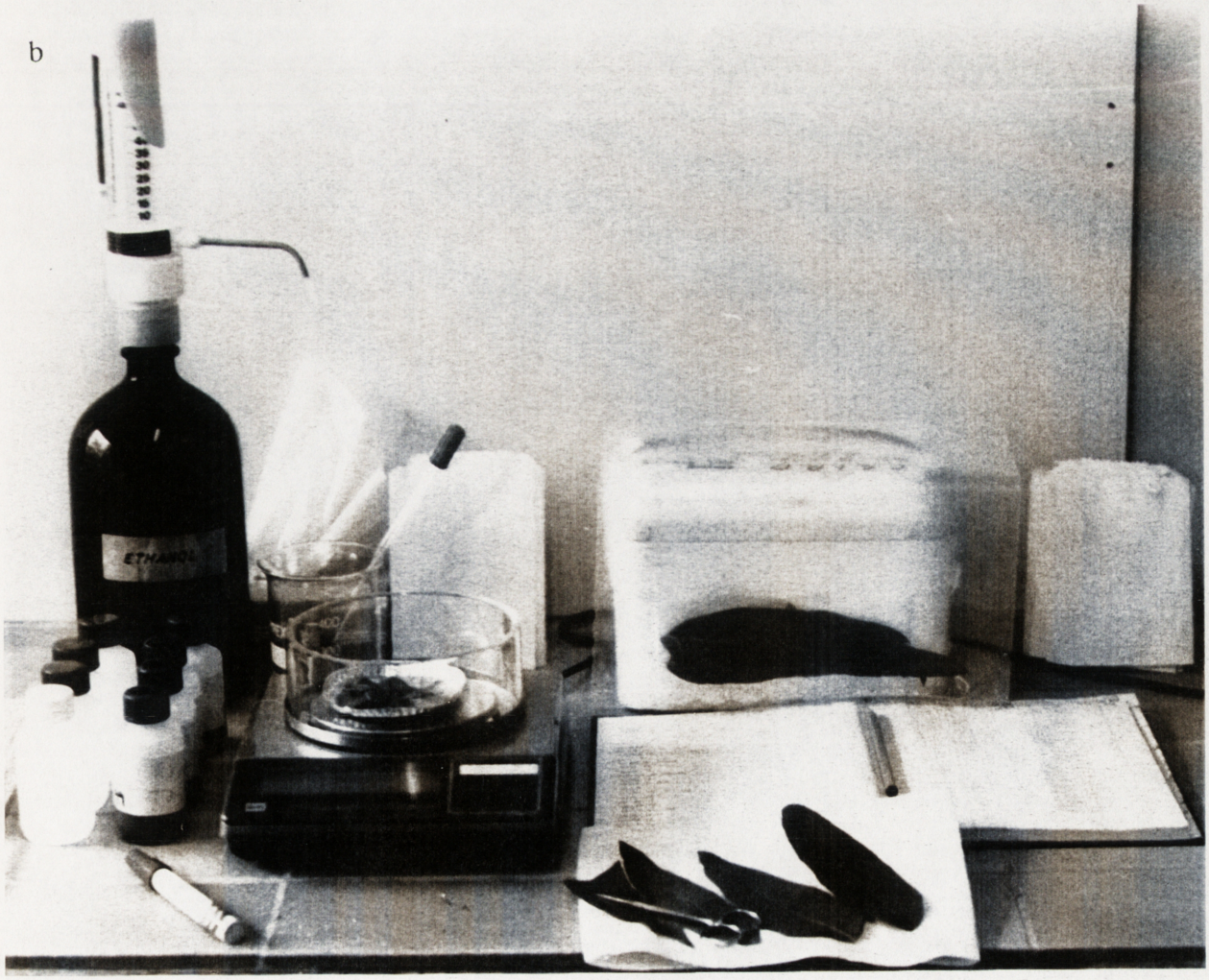


Figure 3.1 Extraction methods
a - Dean and Stark still used in the steam distillation of *Eucalyptus* leaves;
b - Materials used in the ethanol extraction method.



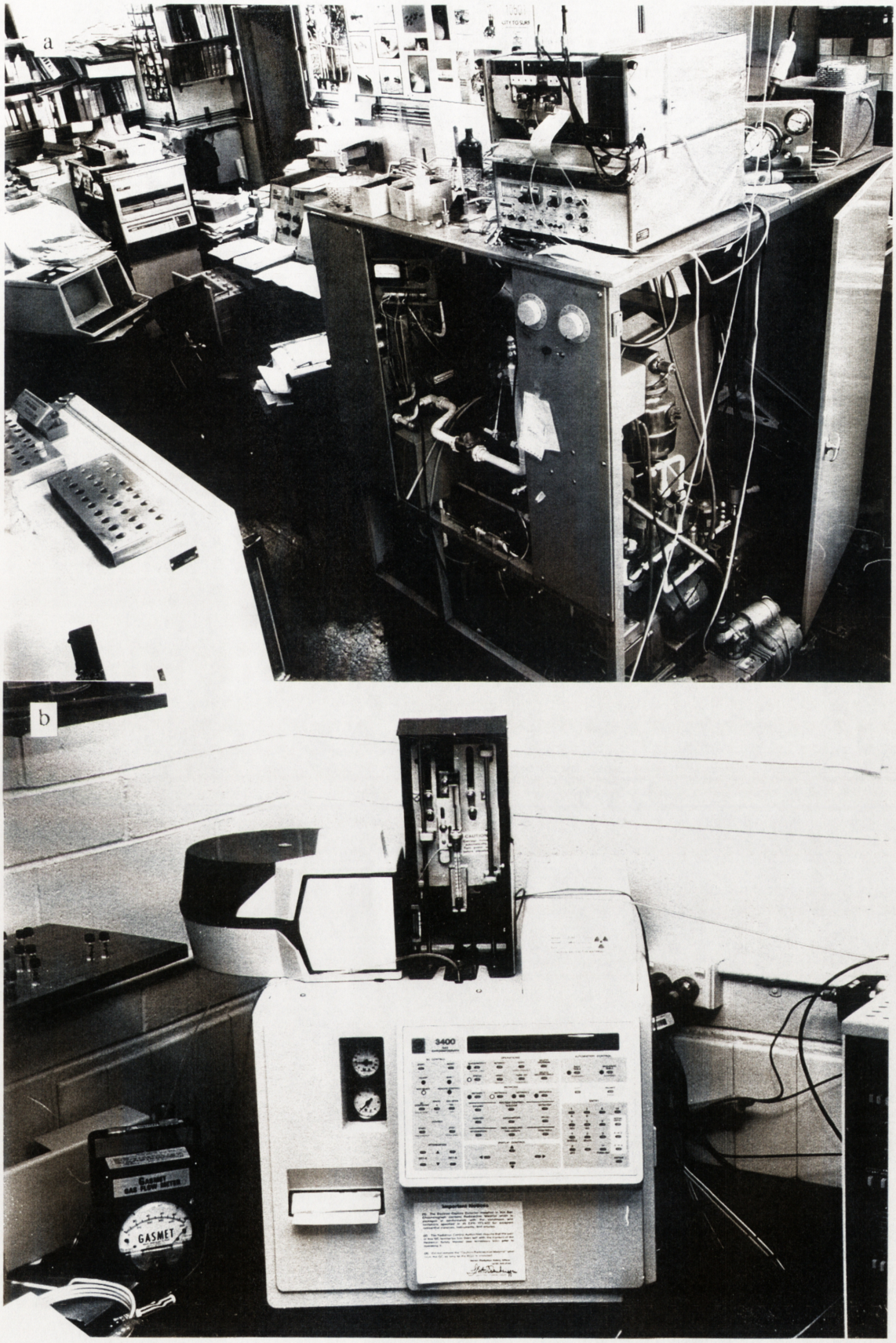


Figure 3.2 Equipment used for evaluation of red gum leaf oils. a - Gas chromatography (GLC) - mass spectrometry at Department of Organic Chemistry, University of NSW. b - A Varian 3400 gas chromatograph at ANU's Department of Forestry.

3.3 Solvent extraction-GLC procedure

3.3.1 Extraction by ethanol

3.3.1.1 Introduction

In this study, the search for higher yielding provenances and individuals required that literally thousands of eucalypt leaf samples had to be extracted of their oil and the oil analysed by GLC. It was clear from the outset that the standard steam distillation method for oil extraction, although needed for some specialised parts of the project (e.g. GLC-MS), would be totally impractical as a general method because of time and equipment constraints. The alternative was to look for a suitable solvent extraction procedure that could efficiently and accurately cater for the large number of samples.

The important features of both steam distillation and solvent extraction methods have been reviewed by Koedam (1987) including discussion of several papers showing that chemical alteration of essential oil components may occur due to either extraction process. Ammon *et al.* (1985) compared the solvent extraction-GLC method with the steam distillation-GLC procedure on various *Eucalyptus* species and found several discrepancies, all occuring during steam distillation. Their studies showed a significant loss of about 50% in yield of α-pinene in at least two species and suggested that other structural rearrangements could be occuring in heat-labile components such as unsaturated compounds due to steam distillation. They also confirmed the accuracy of the solvent extraction-GLC methodology for determination of terpene content and showed the suitability of ethanol as a solvent in which all the terpenes in eucalypt leaves are miscible, problems with oxidation and reduction are minimal, penetration of the leaves is excellent and boiling point is relatively high.

The rapid, simple and sensitive ethanol extraction method described by Ammon *et al.* (1985), and used by Brooker *et al.* (1988), appeared appropriate for this study. In this method, weighed leak-proof sample bottles containing 50ml of ethanol (of a grade suitable for direct on-column injection in GLC analysis) are taken in the field. A branch is removed from the upper part of the crown and 3g (4g in some cases) per tree of mature leaf with petioles removed are weighed by accurate spring balance and placed in these bottles. Large leaves are broken up so they will immerse in the solvent. Blanks, bottles containing 50ml of ethanol only, are opened for a short period at each collection site to simulate the time the bottle is open to the atmosphere during sampling. Sample bottles and blanks are weighed accurately on return to the lab and leaf weight calculated accurately allowing for loss of ethanol as indicated by the blanks. Before adoption a small trial was undertaken to test the technique on the red gums and compare the results with the standard steam distillation-GLC procedure.

3.3.1.2 Materials and methods

In conjunction with Experiment 1, Chapter 6, samples were collected for steam distillation-GLC-MS and for ethanol extraction-GLC. This work was based on the three northern Queensland red gum sites of Einasleigh River, Walkamin, and Petford.

Five widely-spaced trees were individually sampled at each site and a bulk sample harvested from another 5 trees at each of the three sites making a total of 18 samples which could be used for comparison. Care was taken in this and subsequent trials to take

only leaves estimated to be of similar age and stage of maturity in each tree. These were obtained from within the crown, below the immature leaves but above leaves showing signs of senescence. In the WA method described in 3.3.1.1, the ethanol is transported to the field. It was not feasible to do this in the present project because of restrictions on the air transportation of volatiles like ethanol. Instead, samples were placed in plastic bags and stored over ice for a few days prior to air dispatch to Canberra. As this trial will show, if leaves are kept cool and do not sweat and discolour there does not appear to be any problem with storing the leaves for some weeks before immersion in the solvent. Also by handling the bottles and leaves in the laboratory there are other efficiences. Blanks are not needed, fewer weighings are required and loss of ethanol can be reduced to an insignificant amount.

In the laboratory, 100ml of ethanol was weighed accurately into sample bottles, 5g of leaves with their petioles removed were sampled to represent each collection and placed in the ethanol. The materials used in ethanol extraction are illustrated in Figure 3.1b. At least two weeks were allowed for full extraction (Ammon *et al.* 1985). Chemical analysis was by GLC directly from the ethanol extracts using method 1 in Table 3.1, where the four principal monoterpenes of 1,8-cineole, α-pinene, limonene and β-pinene were quantified on a fresh leaf basis (w/100g, fresh leaf).

3.3.1.3 Results and discussion

The results are given in Table 3.2. When the yield estimates were compared by paired t-test there was no significant difference between methods for any of the four compounds tested. The non-conformity of tree JD 1643 suggests a sampling or labelling error, particularly as β-pinene was not detected in the ethanol extract when it was shown to be present in reasonable quantities in steam distilled oil. Other differences can be readily explained in terms of sample differences as a different set of leaves was taken from each tree for application of each method. There was no hint of a consistent high loss of α-pinene through steam distillation as reported by Ammon *et al.* (1985).

The trial indicated that the ethanol extraction method closely matched a standard steam distillation extraction procedure in quantifying four common monoterpenes in the oil of the northern red gums and could be adopted with confidence in this project.

3.3.2 GLC analysis of ethanol extracts

3.3.2.1 Introduction

The use of GLC has been exploited for *Eucalyptus* oil determinations since the 1960's. It has greatly increased the speed and accuracy of chemical analysis of essential oils and permitted the use of small samples. Packed column-GLC has now given way to capillary column-GLC. Combined with ancillary instrumentation such as electronic integrators and plotters, this method now provides sensitivity and resolution and a more accurate quantitative determination of the concentrations of the compounds of interest than earlier methods.

Table 3.1 A description of the methods used in the GLC analysis of ethanol extracts in this project

Method No.	Machinery /detector	Inj. Temp °C	Det. Temp °C	Column	Carrier gas and flow rate	Sample size ul (spit ratio)	Conditions T.1 °C	H.T. min	R.1 °C min^{-1}	T.2 °C	H.T. min	R.2 °C min^{-1}	F.T. °C	H.T. min	Tot. run time min
1	Shimadzu GC 9 A /FID	270	360	6 m x 3.0 mm i.d. glass tube packed with 13% OV 330 coated on 80-100 mesh Chromosorb W - HP	N_2 at 30 ml min^{-1}	6 (-)	60	2	10	220	0	20	260	0	20
2	Varian 3700 /FID	180	300	1.8 m x 3.0 mm i.d. glass tube packed with 2% OV 17 coated on 80-100 mesh Chromosorb Q	N_2 at 14 ml min^{-1}	1 (-)	75	3	10	-	-	-	220	3	21
3	Varian 3400 /FID	220	280	20 m x 0.32 mm bonded FSOT coated with Superox FA	N_2 at 1.5 ml min^{-1}	1 (1:20)	80	0	20	-	-	-	220	3	10
4	'as for 3'	"	"	"	"	0.5 (1:20)	70	3	5	90	-	10	220	5	25
5	'as for 3'	300	230	20 m x 0.25 mm bonded FSOT coated with Superox FA with 1 m DB 1 joined to 'front-end'	N_2 at 0.4 ml min^{-1}	1 (1:100)	80	3	5	90	-	20	200	2.5	13

FID - flame ionisation detector; Inj. - injector; Det. - detector; FSOT - fused silica open tubular; T.1 - initial temperature
H.T. - hold time; R.1 - initial rate of temperature increase; T.2 - second temperature; R.2 - second rate of temperature increase; F.T. - final temperature.

Table 3.2 Comparison of methods (steam distillation - GLC v's ethanol extraction - GLC) in the quantification of four monoterpenes in the leaf oils of the northern red gums. (Yield g/100 g, fresh leaf.)

1. Einasleigh River												
Tree No:	**JD 1643**		**JD1645**		**JD 1647**		**JD 1649**		**JD 1651**		**Bulk**	
Method:	**Steam**	**Ethanol**	**Steam**	**Ethanol**	**Steam**	**Ethanol**	**Steam**	**Ethanol**	**Steam**	**Ethanol**	**Steam**	**Ethanol**
α-pinene	0.12	0.02	0.11	0.12	0.02	0.02	0.02	0.03	0.30	0.41	0.03	0.06
β-pinene	0.17	0	trace	0	0	0	trace	0	0.02	0.07	trace	0
limonene	0.12	0.13	0.05	0.06	0.05	0.05	0.08	0.09	0.03	0.05	0.09	0.07
cineole	0.42	0.81	0.47	0.40	0.12	0.14	0.61	0.75	0.60	0.66	1.26	1.06
2. Emu Creek												
Tree No:	**JD 1678**		**JD1680**		**JD 1682**		**JD 1684**		**JD 1687**		**Bulk**	
α-pinene	0.11	0.13	0.07	0.08	0.08	0.08	0.05	0.06	0.02	0.04	0.11	0.15
β-pinene	0.10	0.19	0.11	0.12	0.07	0.08	0.24	0.25	trace	trace	0.08	0.09
limonene	0.19	0.16	0.16	0.08	0.16	0.08	0.05	0.04	0.04	0.08	0.20	0.18
cineole	0.65	0.63	1.02	0.86	0.95	0.90	0.11	0.09	1.00	0.98	0.95	1.04
3. Walkamin												
Tree No:	**JD 1633**		**JD 1635**		**JD 1637**		**JD 1639**		**JD 1641**		**Bulk**	
α-pinene	0.22	0.20	0.01	0.02	0.22	0.16	0.06	0.07	0.10	0.11	0.17	0.14
β-pinene	0.14	0.13	trace	0	0.18	0.17	0.31	0.30	0.14	0.19	0.19	0.16
limonene	0.07	0.05	0.28	0.21	0.04	0.03	0.07	0.07	0.11	0.09	0.03	0.03
cineole	0.06	0.05	0.14	0.15	0.26	0.18	trace	0	0.36	0.33	0.09	0.07

During the course of this project, which has involved the chemical analysis of thousands of ethanol-oil extracts, three gas chromatographs were used and four columns. The methods employed followed an evolutionary process depending on the equipment available at the time. Packed columns were used at first on Shimadzu GC9A and Varian 3700 machines. These were replaced by capillary GLC once a suitable gas chromatograph (i.e. a Varian 3400) was made available (Figure 3.2b).

The principal aim of the chemical analysis of the ethanol extracts was to quantify a maximum of five compounds, 1,8-cineole, α-pinene, limonene, β-pinene and, when present in quantifiable amounts, ρ-cymene. Columns had to be selected and optimum operating conditions determined to achieve separation of the compounds of interest in the minimum time possible for accurate and reliable quantification. Parameters affecting this separation include liquid phase type and thickness, column length and diameter, gas type and flow rate, oven temperature and gradient and split ratio (Jennings 1987).

3.3.2.2 Materials and methods

Packed column-GLC

Two columns of different stationary phase were used. OV 330, a polar phase, was used by Ammon *et al.*(1985) and gave good separation of the compounds of interest. Once this column was exhausted OV 17, a semi-polar phase, was employed. This column had the disadvantage of ρ-cymene co-eluting with 1,8-cineole which resulted in the over estimation of 1,8-cineole in some samples. All samples evaluated on the OV 17 column were later run again on capillary-GLC and an adjustment made to the initial quantification of 1,8-cineole if a significant proportion (>0.01g/100g fresh leaf) of ρ-cymene was indicated.

The temperature programming and other operating conditions are given in Table 3.1. Identification of compounds was by retention time to those of known standards which was later confirmed by GLC-MS. Quantitative determinations were on a 'tissue basis' (Squillace 1976) where the weight of compounds of interest were determined per unit weight of fresh and dry leaf (i.e. w/100g of fresh or dry leaf) using an external standard. In this method, a 'cocktail' in ethanol, containing known amounts of each of the compounds of interest, was chromatographed several times during the course of each day. The calibration factor for each compound was calculated as the ratio of the amount of that compound in the 'cocktail' to its peak area determined by the integrator. When analysing the ethanol extract, the amount of each compound was obtained by multiplying the peak area for that compound by its calibration factor and by multiplying by a common factor (e.g. 33.33 if 3g of leaves were sampled) to convert to w/100g of fresh leaf. A further conversion was made once leaf moisture content of each sample was determined to give w/100g of dry leaf.

The simplicity of this method conceals certain experimental difficulties, the foremost of which is the need to know the volume of sample injected very accurately as results are directly dependent on this volume. To ensure reproducibilities of the order of 1-5% (typically 2%) each sample was run twice and repeated a third time if outside this range. The mean of the peak areas for a particular compound was then used for quantification.

Capillary column-GLC

On arrival of the Varian 3400 with automatic injection, a decision had to made concerning the choice of column and operating conditions to meet project objectives. Matters influencing the selection of capillary columns for essential oil analysis have been extensively reviewed by Sandra and Bicchi (1987). A wide range of polar (e.g. Superox-FA, Carbowax 20M, BP 20, BP 21) and semi-polar (e.g. RSL 300) capillary columns is commonly used for the chemical evaluation of *Eucalyptus* oils although β-phellandrene may remain unresolved in some cases (I.A. Southwell and J.J. Brophy pers. comm.). Fortunately, this is hardly a consideration in this project as β-phellandrene has not been detected to any extent in the oils of the northern red gums (see Chapter 6). It was decided to adopt the Superox-FA stationary phase for the present work as this gives good separation of the five compounds of interest and other components of the oil. Commonly 40 separate compounds were resolved under the operating conditions adopted. In addition, elution order is similar to that of the SP 1000 column used in the steam distillation-GLC work so comparisons could be made in developing the methodologies.

The temperature programming and other details of operating conditions are given in Table 3.1. Identification of compounds was by retention times compared to those of known standards. Usually fast run times (10 and 13 minutes/sample) were adopted when large numbers of samples were to be examined in experiments often requiring some weeks of machine time. The slower run time of 25 minutes was used when small batches were involved and could be examined within a working week. The first Superox-FA column was exhausted after some 6000 injections and was replaced by a second of smaller internal diameter (ID = 0.25mm). The small ID column gave similar separating efficiency but over a slightly narrower range of peaks. This resulted in α-pinene eluting with the tail of the solvent. Retention time for α-pinene was increased by addition of 1m of DB1 to the front of the column at the expense of only a slight reduction in separation between limonene and 1,8-cineole.

Quantitative determinations were on a 'tissue basis' as described earlier but making use of an internal standard which is recognised as generally the most accurate method of quantification in GLC work. There are several advantages of this procedure over use of an external standard including independence of the sample size injected. The first step in this procedure is to find a suitable internal standard; a compound that acts chemically like the compounds of interest, elutes in an empty area of the chromatogram preferably near the target compounds and is well-resolved both in the oil samples and in the calibration mixture. After trial of several candidate compounds, the hydrocarbon, n-tetradecane (C_{14}), was adopted as the internal standard. Complications were that it was not immediately miscible in ethanol and did not store well in the oil extracts. The first problem was overcome by dissolving the n-tetradecane in chloroform at a predetermined and standard concentration (0.4g/ml)and carefully adding by Eppendorf pipette a constant volume of this mixture (50ul) to each 50ml ethanol extract such that each sample received 0.02g of n-tetradecane (0.4mg/ml). The same procedure was followed in adding the internal standard to the calibration mixtures. The problem of deterioration was circumvented by ensuring that extracts were analysed within one week of addition of the internal standard.

The calibration mixtures were blends in ethanol of known amounts of all the compounds of interest (5) plus the internal standard such that their peak areas were approximately

equal. From the chromatograms of these mixtures the response factors were calculated according to the equation-

$$RF_a = \frac{M_a \times A_i}{M_i \times A_a}$$

where RF_a=response factor of analyte (a) versus internal standard (i); A_i =peak area of internal standard; A_a=peak area of analyte; M_i =weight of internal standard in grams; M_a =weight of analyte in grams.

Several such mixtures were prepared during the course of the study and were assayed frequently. Over time, slight shifts in RF's were recorded and were attributed to slow deterioration of the various columns and injection ports. The integrator was recalibrated at regular intervals to compensate for these shifts and regular machine maintenance kept changes to a minimum.

Once the response factors were determined, the quantification of the compounds of interest in the ethanol extracts could proceed using the equation-

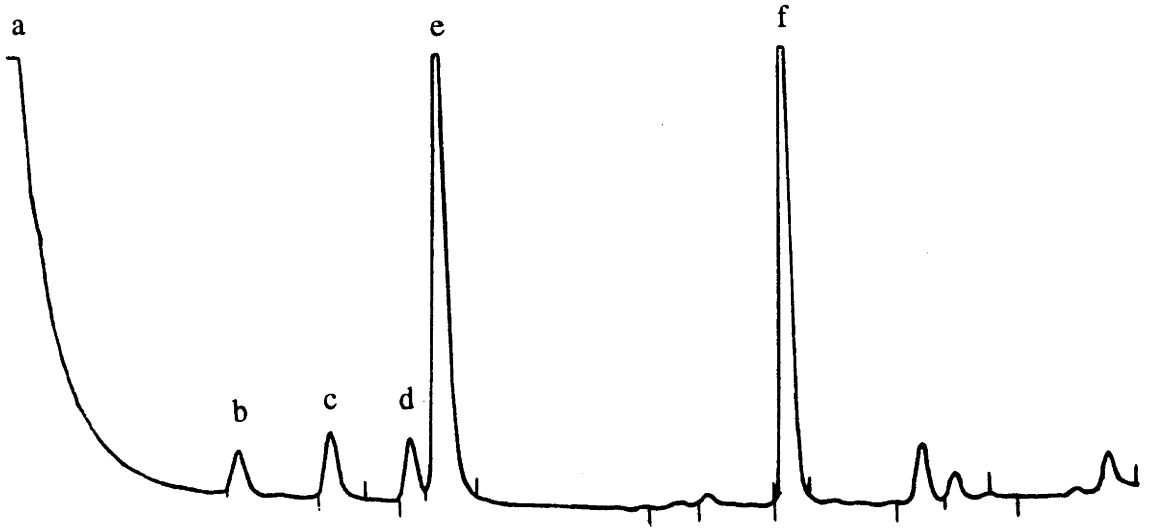
$$M_a = RF_a \times \frac{A_a \cdot M_i}{A_i} \times \frac{100}{M_{fl}}$$

M_a =weight of analyte(g)/100g of fresh leaves; RF_a =response factor of analyte versus internal standard; A_a=peak area of analyte; A_i =peak area of internal standard; M_i =weight of internal standard in 50ml of extract (kept at a constant 0.02g); M_{fl} = weight of fresh leaves in 50ml extract.

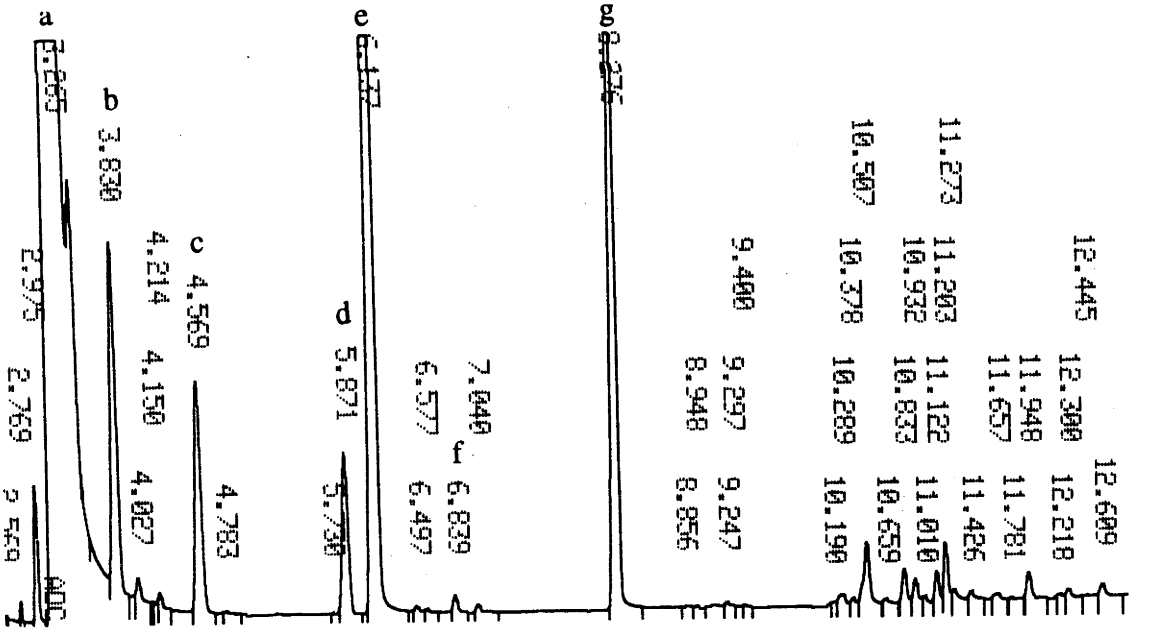
The on-board integrator to the Varian 3400 could be programmed to undertake these calculations and include them in the report on each chromatogram thus saving on the time of manual calculation. To convert the figures to a dry weight basis, the fresh weight mass of each sample was muliplied by another factor determined from replicate samples for which leaf moisture content was known.

3.3.2.3 Results and discussion

An example of a chromatogram obtained from the column packed with OV 17 and run under the conditions described in Table 3.1 is given in Figure 3.3a. The methods described provided a useful start to the project while awaiting the availability of capillary column-GLC equipment. However, the need for some adjustments to the OV 17 determinations of 1,8-cineole when significant amounts of ρ-cymene were present was an inconvenience. Later checks of packed column v's capillary column quantifications showed a close match and confirmed the accuracy of the older technology.



A. a - solvent peak, b - α-pinene, c - β-pinene, d - limonene, e - 1,8-cineole, f - n-tetradecane.



B. a - solvent peak, b - α-pinene, c - β-pinene, d - limonene, e - 1,8-cineole, f - ρ-cymene, g - n-tetradecane.

Figure 3.3 Examples of chromatograms of ethanol extracts of *E. camaldulensis* leaves. A - on a packed, OV17 column. B - on a capillary, Superox-FA column.

The main problem with use of the packed columns in the context of this project was the lack of automatic injection. It was only possible to analyse about a dozen samples in an extended working day due to the careful work required to use an external standard and manual injection. As the determination of many thousands of samples was a requirement of the project, these methods were superceded by capillary column-GLC with automatic injection as soon as practicable. An example of a capillary column-GLC trace is given in Figure 3.3b.

3.4 Water determination of foliage samples

3.4.1 Introduction

The yields of components in *Eucalyptus* oil are usually expressed per unit of weight of fresh leaves. It must be recognised, however, that yields expressed in this manner become dependent on fresh weight density of the particular leaf samples which may be highly reflective of the water content of those samples.

Leaf moisture content is affected by many factors including leaf age, species, vitality of the individual tree, position of the leaf in the crown, time of day, site, season, weather and soil moisture (Hakkila 1989). Oil yields should, therefore, be calculated on a dry weight basis to allow for the large differences in moisture contents (Penfold and Willis 1961). This is especially so when making critical comparisons between oil yields of individual trees, such as in the calculation of genetic parameters or studies of seasonal variation.

Many methods have been described for the determination of water in fresh plant material including Karl Fischer titration of ethanol extracts (Ammon *et al.* 1985) and oven-drying. The titration procedure applied to the same field-derived ethanol extracts used in oil determinations is rapid and undoubtedly the most accurate of the two methods. However, the equipment is expensive and was not available for use in this project. The oven-dry method based on duplicate samples to those taken for ethanol extraction in the laboratory was the most convenient alternative. It is subject to some inaccuracies due to loss of some volatiles including oil from the leaves during storage and transport of leaves and during oven-drying commonly carried out in a convection oven at 70°C for >12h. The losses due to oven-drying which lead to a slight over-estimation of moisture content and, therefore, of yields of oil on a dry weight basis are minor and according to Murtagh (1988) can be safely ignored in calculations of this type.

To check this conclusion, a small trial was undertaken to test the level of inaccuracy likely because of the loss of volatiles during oven-drying. The results are reported below. Any losses due to storage and transport from northern Australia, Zimbabwe and elsewhere could not be avoided. However, care was taken to avoid leaves sweating by keeping them cool (over ice or refrigerated) and expediting their dispatch from the field to the laboratory.

3.4.2 Materials and methods

Four leaf samples, recently received from the field, were available in sufficient quantity for the trial. Each was divided into three 3g samples. One was placed in 50ml of ethanol for oil determination in the regular manner. One sample was dried slowly at room

temperature over silica gel in a desiccator and one was oven dried at 70°C for 48h which is three times the period the leaves are normally subjected to heat. Once the samples were dry, they too were placed in ethanol and the oil allowed to extract over a two week period. The yields of 1,8-cineole, α-pinene, limonene and β-pinene were determined by method 4 in Table 3.1.

3.4.3 Results and discussion

The sum of the yields on a fresh weight basis of the four major monoterpenes by treatment is given in Table 3.3.

Table 3.3 Loss of monoterpenes from duplicate samples of leaves from 4 trees subjected to mild and severe drying procedures.

Tree No.	Total yield of the four major monoterpenes (w/100g, fresh leaf)		
	Control	Room Temp. over silica gel	Oven dry at 70°C for 48h
1	2.04	2.19	2.03
2	0.77	0.55	0.53
3	1.85	2.04	1.73
4	1.84	1.72	1.21
Means	**1.63**	**1.63**	**1.38**

The results show that the mild drying treatment appeared to cause some loss of oil in two samples but on average was equivalent to the control. Application of the relatively harsh oven-dry method of 70°C for 48h caused an average loss in oil of 0.25g per 100g of fresh leaf or 15%, although one sample was identical to control suggesting that leaf samples may differ in their ability to retain oil when desiccated by heat.

In this project, oil yields averaged about 1g/100g of fresh leaf while leaf moisture contents averaged 55%. The over-estimation of moisture content by less than half a percentage point is insignificant and confirmed the appropriateness of the oven-dry method for water determinations in this project.

The oven-dry procedure adopted as standard for leaf moisture content (MC) determinations was:- weigh the sample as soon as it was removed from cold storage (W_1), place in an oven at 70°C overnight (approx. 15h), cool over silica gel in a desiccator and reweigh (W_2), calculate MC% by the equation:-

$$MC\% = \frac{W_1 - W_2}{W_1} \times 100$$

3.5 Alternative methods of selecting high oil-yielding phenotypes

3.5.1 Introduction

The chemical analysis of *Eucalyptus* oil by the procedures described above is accurate but specialised, time-consuming and largely laboratory based. The screening of a large number of trees for 1,8-cineole yield, as would be necessary in selecting superior trees for this trait in a tree breeding program is, therefore, a costly exercise. Clearly, a rapid method for assessing the potential of individual phenotypes as 1,8-cineole yielders, and especially one that could be applied in the field, would be highly advantageous in limiting the cost of extraction and analysis and for speeding up the selection process.

Subjective methods such as olfactory and taste testing have been used successfully by some as an initial step in selecting high 1,8-cineole producing phenotypes (C.Davis pers. comm.). The olfactory test proved to be highly unreliable when applied to *E. camaldulensis*, so possible objective tests were sought that were both reliable and could be applied in a field laboratory close to base tree populations. The results of tests of three such methods, oil gland density, stomatal frequency and multiple internal reflectance infrared spectroscopy (MIR) are given below.

3.5.2 Materials and methods

3.5.2.1 Oil gland density

Leaves from 100 trees along Emu Creek near Petford were collected for chemical analysis in January, 1988. The leaves were held on ice and air freighted to Canberra (see Chapter 6 for details).

On arrival, 5 leaves from each tree were selected at random and used for oil gland density determination. This was done by removing a rectangular section at the widest part of each leaf from the mid-rib to the margin and counting, from the abaxial side, the number of glands in three microscopic grids; near the mid-rib, in the centre and near the intramarginal vein. These figures were then averaged (3 density counts x 5 leaves) to give an average oil gland density per tree for comparison with oil yield data for the same tree.

3.5.2.2 Stomatal frequency

For 15 of the same 100 trees, chosen to represent the range of 1,8-cineole yields found in the population, a further 5 leaves were taken and a similar section removed at the widest part of each leaf. The leaf cuticles on the upper (abaxial) and lower (adaxial) surfaces of the leaves were separated and mounted on slides according to the methods of Carr *et al.* (1971). Three points were then photographed at a standard magnification; near the mid-rib, in the centre and near the intramarginal vein. Stomatal counts were then made (3

abaxial counts + 3 adaxial counts x 5 leaves) and averaged for comparison with oil yield data.

3.5.2.3 MIR infrared spectroscopy

A full description of materials and methods is given in our publication (Gibson *et al.* 1991). In brief, about a dozen leaves from each of 10 mature *E.camaldulensis* trees known to be poor (4), medium (3) and high (3) in 1,8-cineole yield were collected at Petford on the 17 June, 1989. Three of the trees chosen for their poor yield of 1,8-cineole were of the distinctive low-cineole-high-sesquiterpene chemotype described in Chapter 6. The leaves were kept cool and air freighted to Canberra where the leaf oils were assayed by standard solvent extraction-GLC analysis (method 4, Table 3.1) and by MIR.

MIR spectra were obtained from the adaxial sides of two leaves per sample using a Perkin Elmer 1800 Spectrophotometer with a DTGS detector and a Specac #11000 MIR accessory with a 25 reflection KRS-5 MIR element. The range of wave numbers used was 800-2000 cm^{-1}. One hundred and twenty eight scans were collected in single ratio mode at 4 cm^{-1} resolution against the un-clamped element, held in a special holder.

A 50 x 20 mm rectangle along the lamina of the leaf was clamped to one side of the MIR element at a reproducible torque of 0.075 Nm, while the other side was covered with aluminium foil to avoid interference from the material covering the clamp. The element was cleaned with dichloromethane-moistened tissue after each run to remove oil and wax, and a fresh background was collected after five runs.

Each spectrum was converted to absorbance, flattened using a 3-point algorithm (the points being computer selected), smoothed using a Gavitzky-Golay algorithm with a 13-point window, and expanded so that the strongest band spanned the range of 0-1.5 absorbance. Computer-selected bands and computer-calculated absorbances were used throughout this study. The computer used was a Perkin Elmer 7500 running under idris o/s and using Perkin Elmer CDS-3 Applications Software.

3.5.3 Results and discussion

3.5.3.1 Oil gland density

It is important to appreciate that oil gland density as assessed by transmission light microscopy, is likely to be open to large inaccuracies caused by the distribution of the glands in mature leaves. In transverse section the glands in *E. camaldulensis* leaves are dispersed seemingly at random through the blade some occurring near the upper and lower surfaces and some in the centre of the leaf (Figure 2.2b). Therefore, when looking longitudinally through the microscope, glands often overlap or are obscure. In this situation it is impossible to make accurate counts. Nevertheless, as the method can be easily applied, an attempt was made to see if there was some correlation between oil gland density and oil yields.

Average oil gland density of the 100 trees screened was 11 mm^{-2} (S.D. ± 2) with a range of means from 6 to 15 mm^{-2} (see Table 3.4).

Table 3.4 Mean values for 1,8-cineole yield, oil gland density and stomatal frequency for 9 trees of Petford *E. camaldulensis* selected at random from 100 to represent the high, medium and low cineole-yielding classes.

Tree No.	Yield+ class	Yield of 1,8-cineole w/100g, dry leaf	Oil gland density no. #mm^{-2} (range)	Stomatal frequency no. mm^{-2} (S.D.)
24	High	3.11	15 (11-22)	155 (29)
9	High	2.85	10 (7-12)	191 (39)
30	High	2.52	11 (8-14)	221 (60)
1	Med	2.04	10 (5-14)	176 (50)
8	Med	1.96	13 (8-17)	209 (59)
16	Med	1.87	11 (9-14)	153 (38)
3	Low	1.52	14 (8-24)	180 (54)
45	Low	1.42	9 (7-12)	159 (56)
27	Low	1.31	6 (4-9)	248 (44)

+ an approximate classification into high, medium and low cineole-yielding types

After exclusion of the low-cineole-high-sesquiterpene chemotypes from the data set, correlation coefficients were determined between phenotypic oil gland densities and 1,8-cineole yield (r=0.03) and total yield of the four major monoterpenes (r=0.08). No relationship was indicated so further oil gland density determinations, as an aid to selecting high oil-yielding phenotypes, were discontinued.

3.5.3.2 Stomatal frequency

Mean stomatal frequencies and oil yields for a range of trees are given in Table 3.4. On average, there were slightly more stomata per unit area of leaf on the adaxial leaf surface (189 cf 185) but there was considerable variation between trees. Correlation coefficients between average stomatal frequency and 1,8-cineole yield (r=0.04) and total yield of the major monoterpenes (r=0.08) were very low and at odds with the paper of Bisen *et al.* (1984) where an inverse relationship between stomatal frequency and cineole-rich phenotypes of *E. tereticornis* was reported.

From the findings of this trial the use of stomata counts per unit area as an indicator of a phenotypes oil-yielding capacity cannot be justified.

3.5.3.3 MIR infrared spectroscopy

The MIR spectra from leaves of the regular chemotype showed peaks at 1050 and 1078cm^{-1} that, with peaks at two other wave numbers, characterise pure 1,8-cineole in infrared spectroscopy. The range of 1,8-cineole yields was well correlated with the range of absorbances at wave number 1078cm^{-1} (r=0.95) and at wave number 1050cm^{-1} (r=0.93). In addition, the peaks in the 1000 to 1100cm^{-1} region gave a characteristic appearance to the spectra from the higher and lower 1,8-cineole content leaves of regular chemotype and distiguished them from the sesquiterpene chemotype (Fig 3.4). Thus allowing the broad ranking of trees as to their 1,8-cineole producing capacity simply by subjective assessment of their spectra trace.

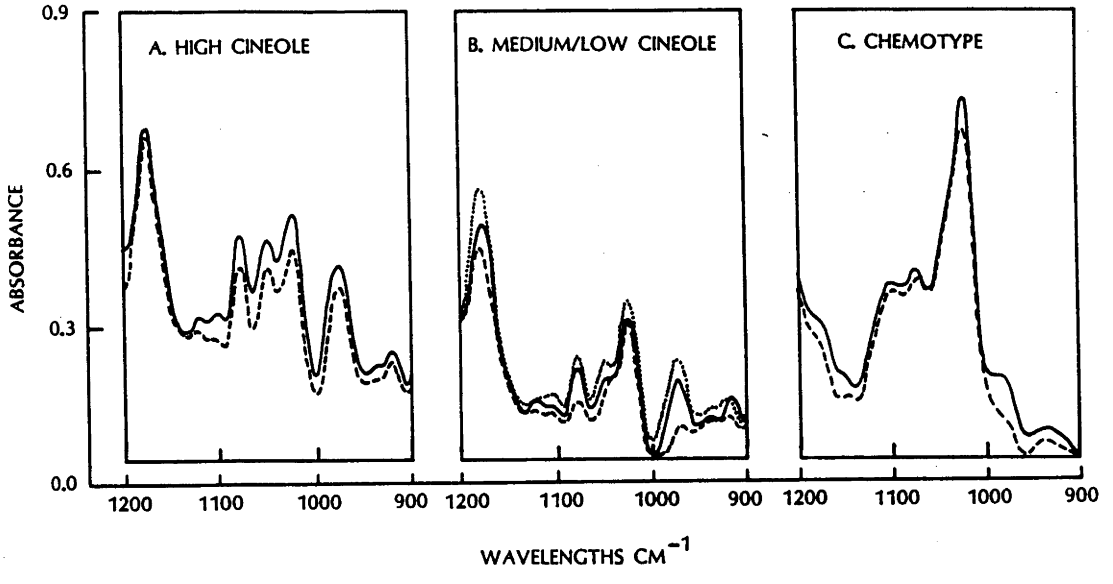


Figure 3.4 Examples of MIR spectra obtained from the surfaces of *Eucalyptus camaldulensis* leaves from trees of the regular chemotype (A and B) and of the sesquiterpene chemotype (C). 1,8-cineole yield (g per 100 g fresh leaf): A, 1.908 g (——) and 1.831 g (---); B, 1.240 g (...), 1.178 g (——) and 0.407 g (---); C, 0.299 g (——) and 0.018 g (---)

A spectrum is obtained directly from the leaf surface and a time period of only 10 to 15 minutes is required to obtain each, inclusive of the time needed to clean the element between determinations. The time required compares favourably with the period needed for a GLC determination of ethanol extract or steam distilled oil but without the considerable lag time for oil extraction inherent in the conventional methods. Determinations in the field allow the user to map and label only those trees of further interest and to collect seed, scions or cuttings from selected trees immediately they are identified thus saving on time and on the number of visits to the field site. This results in considerable savings in research costs.

These data suggest that MIR infrared spectroscopy could be a valid and practical technique for the initial screening of large numbers of trees of *E. camaldulensis* for oil type and 1,8-cineole yield, either in the field using a robust, transportable instrument, or from leaves kept in cold storage, although the chemical techniques must still be used when an accurate oil analysis is required.

Unfortunately, as a suitable spectrometer was not readily available, this promising technique could not be tested further on this project.

3.6 Conclusions concerning analytical methods

Ethanolic extraction of mature leaves gave solutions suitable for direct injection into a gas chromatograph for the quantitative determination of the major components of red gum oil. Comparison with conventional steam distillations confirmed that ethanol extraction was appropriate for analysis of red gum oil yield, and because of convenience when handling large numbers of samples, it was the preferred method employed throughout this study.

Potential alternative indirect (oil gland and stomatal frequencies) and direct (MIR spectroscopy) methods of assessing a trees' cineole-yielding capacity were tested. While the indirect methods failed, MIR infrared spectroscopy was shown to have considerable potential for further development for field-testing eucalypts for 1,8-cineole yields.

Any error associated with the oven-dry method of determining leaf moisture contents for converting fresh-weight oil yields to a dry-weight basis were shown to be small and insignificant. The oven-dry method for determining leaf moisture contents was used throughout this study.

CHAPTER 4. VALIDATION OF FIELD SAMPLING TECHNIQUES FOR AND INFLUENCE OF ONTOGENTICAL CHANGES ON STUDIES OF GENETIC VARIATION IN OIL YIELDS OF RED GUMS

4.1 Introduction

Techniques developed for sampling the leaves of the Western Australian mallees, *E. kochii* and *E. plenissima*, to determine 1,8-cineole content were the basis for methods adopted in this project. These methods were outlined by Brooker *et al.* (1988), Barton and O'Reilly (1986) and Tjandra (1986).

The Western Australian studies, based largely on adult trees growing in their natural habitats, showed that as long as similarly-aged mature foliage was collected, oil production did not change with position, i.e. aspect, in the crown or with time of sampling within a single day. A small seasonal effect on oil production was found but the effect was not consistent over the two years studied and appeared to be of little economic consequence. Tree to tree variability in monthly patterns of oil occurrence was high but the ranking of high and low oil producing trees did not change. This work also indicated that plant yield of 1,8-cineole was reasonably consistent after about 18 months-of-age. However, it was preferable to delay a final ranking of progeny until about 3 years-of-age when yields closely resembled parent values.

In the present project samples were to be taken from both mature trees in natural stands and from young, 1 to 4-year-old, progenies established in fast-growing plantations. *E. camaldulensis* belongs to section Exsertaria of the subgenus Symphyomyrtus (Pryor and Johnson 1971) and so is not closely related to either Western Australian species that occupy section Bisectaria of the same subgenus. It was thought prudent, therefore, to test the validity of some of the above findings before deciding on a field sampling strategy for this project. The leaf oils of eucalypts appear to be strongly genetically controlled, but they are influenced by other factors such as ontogeny and age of leaf and environmental effects (see Chapter 2). The importance of these non-genetic factors needs to be considered where comparisons are to be made between trees of different ages, growing on different sites and from leaves collected at different times of the year.

Barton and O'Reilly (1986) found that leaves from basal coppice shoots of *E. polybractea* gave consistently higher yields of 1,8-cineole than leaves from the crowns of plants in unharvested bushland. The timely harvesting of coppice could, therefore, result in peak production of 1,8-cineole. It was decided to test the significance of harvesting coppice leaves versus mature leaves from the crown prior to felling.

A detailed description of the oils of *E. camaldulensis* is given in Chapter 6. In brief, oil from trees at Petford (regular chemotypes) consists largely of 1,8-cineole (70%). This and three other monoterpenoids, α-pinene, β-pinene and limonene, make up 80% (SD ± 8%) of the oil. However, yields of these compounds vary greatly between trees. In addition there is a distinctive chemotype present in the population, at a frequency of 1 in 10 trees, which is characterised by low 1,8-cineole content (ca 10% of the oil) and a high proportion of sesquiterpenoids (ca 60%).

It was noted in various experiments that the distinctive low-cineole-high-sesquiterpene chemotype did not appear in the oil evaluations of young plants. The youngest plant to exhibit the sesquiterpene chemotype pattern was a single 2-year-old tree amongst the 28 trees sampled in the coppice trial described in this Chapter (Exp. 3). The coppice leaves from shoots from the stump of this tree, sampled frequently from age 3 months to 13 months, produced 1,8-cineole dominated oils in contrast to the sesquiterpene dominated leaf oils of the original crown. The inference was that the 'genetic switch' to activate production of large quantities of sesquiterpenes, seemingly at the expense of 1,8-cineole, was age dependant.

If reliable selections of individual plants for 1,8-cineole yield and quality (% cineole) can be made very early in their life span, it may then be possible to shorten the generation time in a breeding program. As the commercially undesirable sesquiterpene chemotype should be avoided in a selection and breeding program to improve oil yield and quality in this species, it is important to know when the change to sesquiterpene dominated oils takes place. It is also important to know when rankings and yields of 1,8-cineole become stable, thus allowing reliable selections to be made.

Trials were undertaken, a) in natural stands of *E. camaldulensis* near Petford in northern Queensland including trees of advanced age (Exp. 1), and b) in experimental plantings of young age (1 to 4 years) near Gympie in southeastern Queensland (Exps 2 to 5). The objectives of these trials were to determine:

- if season of sampling or aspect on the tree had any control over oil yields of mature leaves on adult trees in the field;
- the influence of height and aspect on the tree of sample collection on estimation of oil yields of mature leaves on young plantation grown trees (2 yrs-old);
- if oil yields of coppice leaves at various ages vary significantly from parent values as represented by young fast-growing saplings;
- at what age (ontogenetic phase) yield and quality of oils of young trees reflect adult characteristics for these traits; and
- the extent of variation in oil yields in young trees attributable to non-genetic sources such as season, site, leaf age and stress.

Experiments 1 to 4 are discussed here while Exp. 5 is dealt with in Chapter 5.

4.2 Experiments, objectives and methods

A brief description of the trial sites at Wongi, Tuan and Toolara near Gympie in southeastern Queensland and some details of the topography and rainfall in the natural stands near Petford in northern Queensland are given in Table 4.1. The area near Gympie has a subtropical climate of mainly warm wet summers and cool dry winters. However,

during the two years when leaves were harvested for oil analysis, the normal climatic pattern was disturbed by erratic rainfall leading to unseasonal droughts and flooding (Fig. 4.1). Petford is located in the wet/dry tropics with pronounced winter drought, with only 50mm of rain falling in the 5 months, May to September.

Table 4.1 A brief description of the location, soils and rainfall at the trial sites

	Wongi	Tuan	Toolara	Petford
Latitude (S)	25° 26'	25° 47'	26° 00'	17° 20'
Longitude (E)	152° 26'	152° 50'	152° 47'	149° 57'
Altitude (asl)	60m	50m	60m	490m
Slope	<1°	<1°	<1°	variable
Aspect	north east	north	south east	north west
Soil type	nodular grey earth*	lateritic podzolic*	red and gleyed podzolics*	sandy soils over rock
Average rainfall (mm)	1004	1388	1369	856

* according to Stace *et al.* (1972)

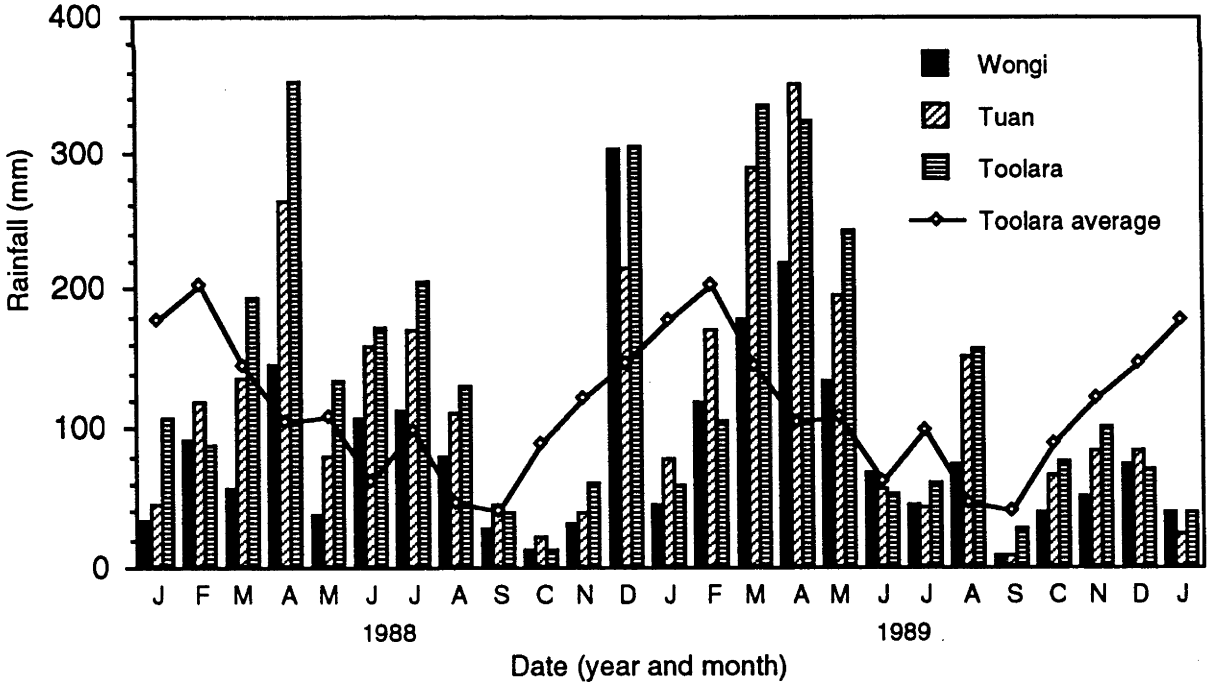


Figure 4.1 Monthly rainfall at each of the Gympie trial sites over the study period compared to the long-term mean monthly rainfall at Toolara.

4.2.1 Experiment 1: Seasonal and crown variation, Petford

The aim of this trial was to determine the effect of season of leaf harvest and position of sampling in the crown on oil yields of adult trees in natural stands.

Ten trees were selected to cover the range of oil yields found in the 110 individual adult trees sampled along Emu Creek and its tributaries in January, 1988 (see Exp. 4, Chapter 6). These trees were sampled again in two follow-up field trips. The first was in July 1988 when one bulked leaf sample per tree was obtained. In December, 1988, samples were obtained from three points around the crown; north, southeast and southwest. In January and July, at least two branches were removed from each tree at about 2/3 of tree height and equal numbers of mature leaves were plucked from each to make up a sample of 12 leaves per tree. In December, three branches, closest to the aspects sought, were removed and the leaves of these were kept separate. All leaves were judged to be of similar mature age and were handled in a similar manner, being first placed in plastic bags and refrigerated for periods up to a maximum of 14 days prior to dispatch to the laboratory. In the laboratory, 50 ml of ethanol were weighed accurately into sample bottles, 3g of leaf (petioles removed) were sampled from each collection and placed in the same bottles and a duplicate sample taken for moisture determination. At least two weeks were allowed for full extraction. Chemical analysis was by method 2 in Table 3.1.

4.2.2 Experiment 2: Validation of sampling methods, Gympie

This experiment had the objective of studying the influence of height of sample collection and aspect on oil yields in young plantation trees.

Three trees, selected at random from 20 in a line plot of 2-year-old *E. camaldulensis* at Tuan, were sampled in this experiment. Each plot was made up of the progeny of parent trees from Petford. The plot was in a buffer area adjacent to the main plantings described in Chapter 5. The trees sampled were 5 to 6m in height in January 1988. They were felled and at 2m and 4m from the base of each stem, leaves were harvested from branches that had pointed closest to the four aspects; north, south, east and west. There was one missing value at 2m in tree 1 which had no branch on the easterly side at this level. Leaf handling and oil analysis followed the methods described for Exp. 1.

4.2.3 Experiment 3: Coppice versus adult comparison, Gympie

The aim of this experiment was to compare the oil yields of coppice leaves at various ages with those of the parent, as represented by mature leaves from 2-year-old fast-growing saplings.

This experiment used the same 20 tree line plot at Tuan, as described above, and a duplicate planting at Wongi. At January 1988, all trees in each plot were felled and mature leaves collected at 2/3 of tree height. Leaf collection and handling and oil analysis of these and the coppice leaves followed the methods described for Exp. 1.

Most of the stumps at Tuan coppiced vigorously and the coppice leaves were sampled at 3, 6, 10 and 13 months from felling. The results for six trees were excluded as some samples were missed because of lack of vigour or heavy insect predation.

Coppice growth at Wongi was far slower than at Tuan and a 3 month sampling was not undertaken because the leaves were still expanding. A full sampling was undertaken at Wongi at 6, 10 and 13 months from felling. Six trees were also excluded from these data sets because of missing values. Leaf collection and handling followed routine practice and oil analyses were according to method 2 in Table 3.1.

4.2.4 Experiment 4: Changes with age, Gympie

The age (ontogenetic phase) at which oil characteristics (especially yield of 1,8-cineole and constitution) stabilise in young trees and reflect adult oil traits was the key question addressed in this experiment. Of special interest was the timing of the production of substantial amounts of sesquiterpenoids in crosses expected to carry oils of this type.

A progeny trial of control-pollinated families established in 1989 near Gympie (see Chapter 7 for details) was the source of material for this study. Two plants were selected at random in the third replicate of each of 15 families (one family, 101 x I8, was represented by one tree only). Of the 15 families, 13 were control-pollinated crosses amongst parents of known oil composition and two were open-pollinated families of trees 10 and 51. Sampled families fell into two groups based on parental oil type. Eight families originated from crosses amongst parents with regular cineole-dominated oils and 7 families had one or both parents (self of tree 10) representative of the sesquiterpene chemotype.

These plants were sampled for oil analysis at 4, 8, 12, 18 and 25 months from planting. Collection and handling of leaves followed routine methods. Fully expanded leaves, estimated to be of similar age and maturity at each successive sampling, were collected at two thirds of tree height, placed in individual plastic bags over ice and air freighted to Canberra. Parental oils were analysed in Sydney by GLC-MS on steam distilled extracts (see Chapter 3) while subsequent tests were by GLC on ethanol extracts of oil from their Gympie progeny (see method 5 in Table 3.1).

4.3 Results and discussion

4.3.1 Experiment 1: Seasonal and crown variation, Petford

The results are summarised in Figure 4.2. The yields (w/100g, dry leaf) of α-pinene, β-pinene, limonene, 1,8-cineole and their total in the leaves of 10 adult trees by month of harvest and by position in the crown (i.e. aspect) were compared by paired t-test. There were no significant differences due to these factors. Individual tree rankings for yields of these compounds were consistent throughout indicating that oil yields of adult trees in the natural stands at Petford are very stable.

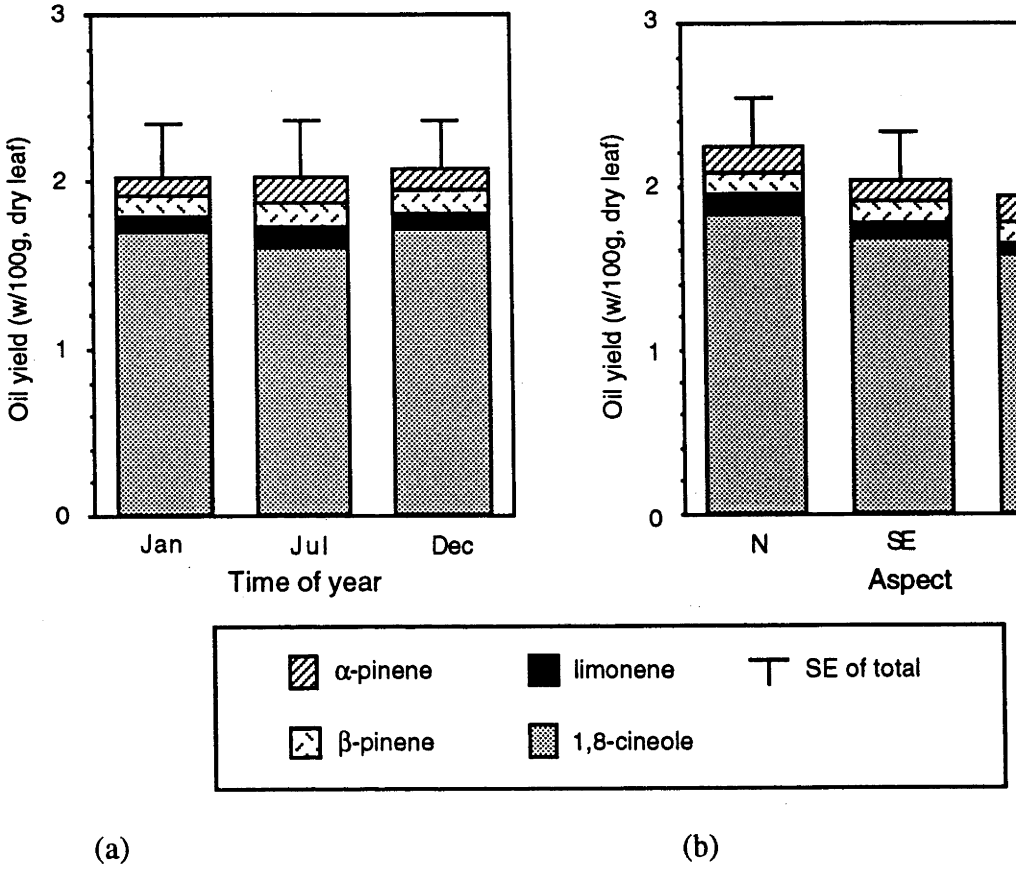


Figure 4.2 Average yields of 1,8-cineole, α-pinene, β-pinene and limonene in the leaves of 10 adult trees at Petford, (a) by month of harvest and (b) by position in the crown (i.e. aspect).

The Western Australian sampling technique was, therefore, adequate for estimating individual tree oil yields in the natural stands of the red gums. Also, harvests of leaves for ranking trees or provenances as to their oil yielding capacity may be undertaken throughout the year with little risk that seasonal differences might mask genetic variability.

4.3.2 Experiment 2: Validation of sampling methods, Gympie

The results are summarised in Figure 4.3. The influence of height of sample collection and the direction faced by the branch bearing each sample (i.e. aspect) on oil yields of the four monoterpenes was compared by t-test. There were no significant differences attributable to these factors.

This small trial and other experiments (e.g. Exp. 1 above and Brooker *et al.* 1988) indicate that, as long as the harvesting of immature foliage is avoided, a reliable estimate of oil yields in young and old trees alike can be gained from leaves taken from any part of the crown. Despite this result, a standard sampling method was adopted for this project in the interests of uniformity.

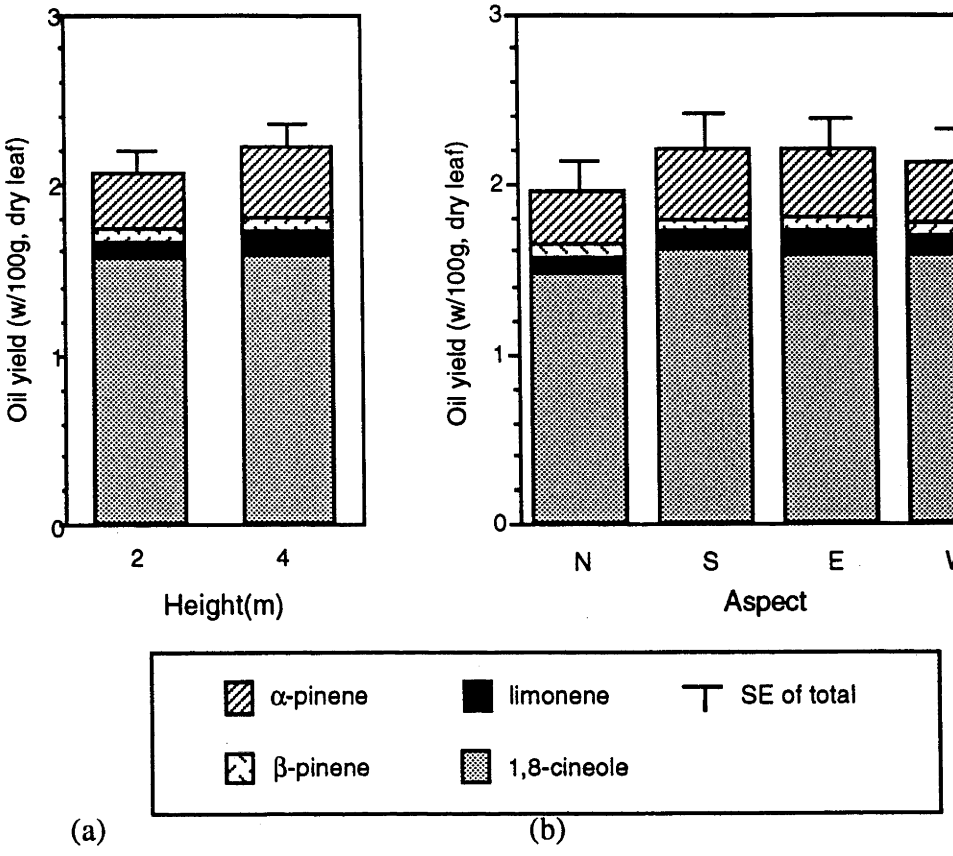


Figure 4.3 Average yields of 1,8-cineole, α-pinene, β-pinene, and limonene in the leaves of three 2-year-old trees, (a) by height of sample collection and (b) by aspect.

Wherever possible leaves to represent a tree were taken from two branches randomly selected at approximately two-thirds of tree height. For between-tree comparisons, care was taken to sample only leaves that were estimated to be of similar age and stage of maturity in each tree. These were obtained from within the crown, below the immature leaves but above leaves showing signs of senescence. Adopting this strategy was beneficial in reducing the variation attributable to leaf maturation in the sampling of any one trial at a particular point in time. However, it was not always reliable in accommodating this source of variation when sequential sampling of the same set of trees was involved (e.g. see Chapter 5).

4.3.3 Experiment 3: Coppice versus adult comparison, Gympie

Coppice oil yields by time from felling were compared with parent values and amongst themselves by paired t-test. The patterns of variation amongst oil components were somewhat different between sites with Tuan proving to be the least variable (see Fig. 4.4). At Tuan, 1,8-cineole and β-pinene yields of coppice shoots mimiced that of the crowns of the parent trees. Yields of α-pinene and limonene were significantly greater in coppice leaves at 3 and 10 months but not at 6 and 13 months. Combined as an estimate of total monoterpenes, coppice yields were significantly greater than those of the parents only at three months when the yields of all four compounds peaked.

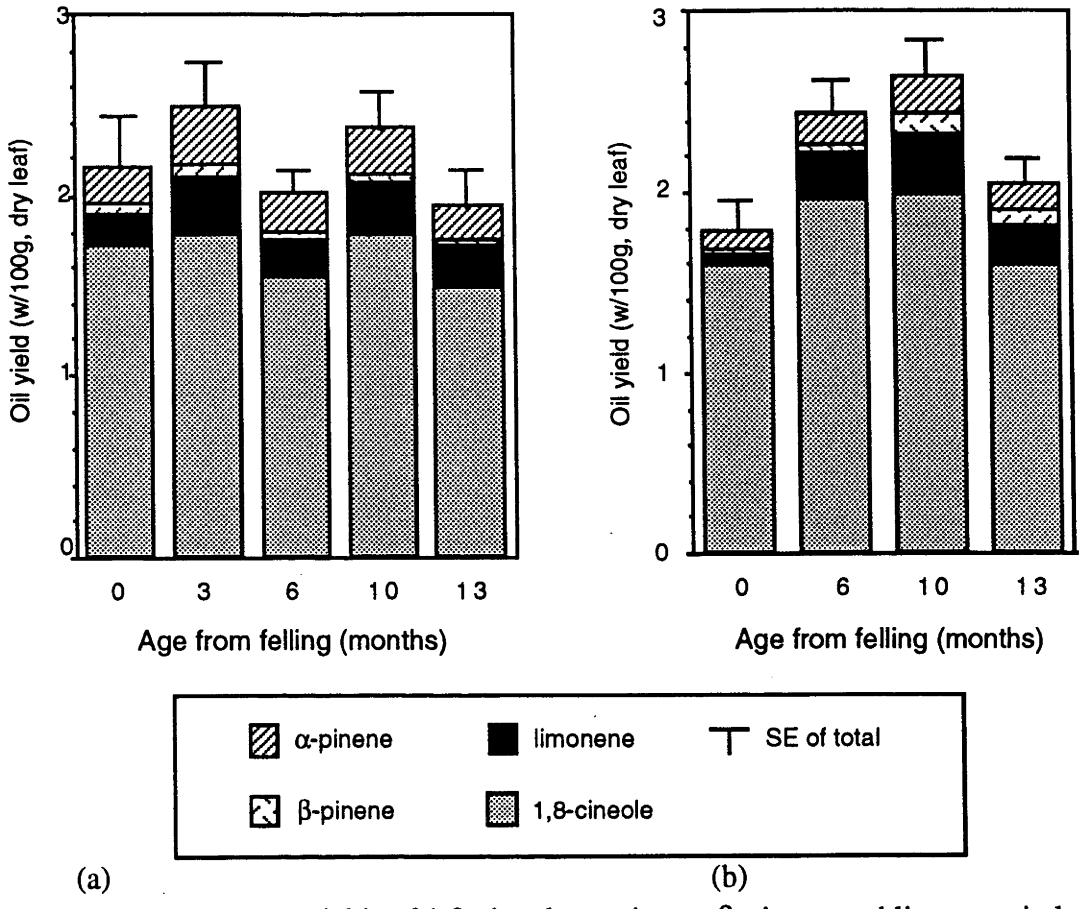


Figure 4.4 Average yields of 1,8-cineole, α-pinene, β-pinene and limonene in leaves collected from coppice shoots at various ages up to 13 months compared to yields from original crowns when felled at 2 years-of-age on two sites, (a) at Tuan and (b) at Wongi.

At Wongi, oil yields of the coppice shoots were significantly higher than in the original crowns. The yields of all four compounds peaked at 10 months representing a 49% increase in total monoterpenes and a 25% increase in 1,8-cineole. Between 10 and 13 months, yields of all leaf oil compounds dropped substantially such that 1,8-cineole yields of the coppice leaves were no longer significantly different from the original crowns.

It appears that the timely sampling of coppice leaves in *E. camaldulensis* may have the potential to boost 1,8-cineole production over mature crowns. The extent of the improvement will depend on site. However, there is a negative side with respect to quality. While cineole production is increased so too is the production of other constituents such that the percentage of cineole in the oil may fall. This may not be a problem if efficient rectification facilities are available.

4.3.4 Experiment 4: Changes with age, Gympie

A summary of the detailed analysis of parental oils is given in Table 4.2. The sesquiterpene chemotypes, trees 10 and 51, are clearly defined by the low ratio of

monoterpenes to sesquiterpenes, dominated in the case of these two trees by the sesquiterpene alcohol, spathulenol. Spathulenol was apparently absent from the oils of the other parents used in the crossing program as was another sesquiterpene, bicyclogermacrene, found in trees 10 and 51.

Variation with tree age in mean 1,8-cineole yield and the total yield of the three other prominent monoterpenes, α-pinene, β-pinene and limonene, in the leaf oils of crosses amongst the regular cineole chemotypes and comparison with mid-parent values is given in Figure 4.5. On an individual-tree-basis, four patterns of change in 1,8-cineole yield with age were displayed; 3 trees (20%) followed the average with a rapid increase between 4 and 8 months and then a gradual increase thereafter, 6 trees (40%) showed a step-wise increase in yield with age as represented by cross 82 x I8 (tree 2) in Fig. 4.6, 5 trees (33%) increased rapidly at first then declined only to recover to the original 8-month-peak at 25 months as in 101 x 58 (T3) and 1 tree (7%) (i.e. 82 x 87 (T2)) showed no change with time (Figs. 4.5 & 4.6). The mid-parent values, as an estimate of the cineole yields that should be attained by offspring of the various crosses, had still to be reached by the majority of trees (70%) at 25 months (Fig. 4.5).

Table 4.2 A summary of the detailed analysis of the leaf oils of the low-cineole-high-sesquiterpene chemotypes, trees 10 and 51, compared to the oils of the other eight parent trees of regular oil chemotype in the crosses studied. Families involving the sesquiterpene chemotypes were 48 x 51, 49 x 51, 88 x 10, 10 x 87, 10 x 10 and open-pollinated progeny from trees 51 and 10. Families representative of the regular cineole chemotype were 101 x 58, 58 x 82, 101 x I8, 82 x 82, 82 x 87, 49 x 82, and 82 x I8.

Yield of oil (w/100g, dry leaf)

Compound	Parent Trees		
	10	51	Mean of 8 other parent trees
α-pinene	0.12	0.08	0.09
β-pinene	0.53	0.34	0.01
limonene	0.06	0.05	0.02
1,8-cineole	0.45 (16%)	0.32 (11%)	2.09 (73%)
Other monoterpenoids	0.10	0.18	0.22
Total monoterpenoids	1.26	0.97	2.43
spathulenol	0.66 (23%)	0.75 (26%)	0 (0%)
bicyclogermacrene	0.01	0.10	0
Other sesquiterpenoids	0.92	1.09	0.45
Total sesquiterpenoids	1.59	1.94	0.45
Grand total	2.85	2.91	2.88

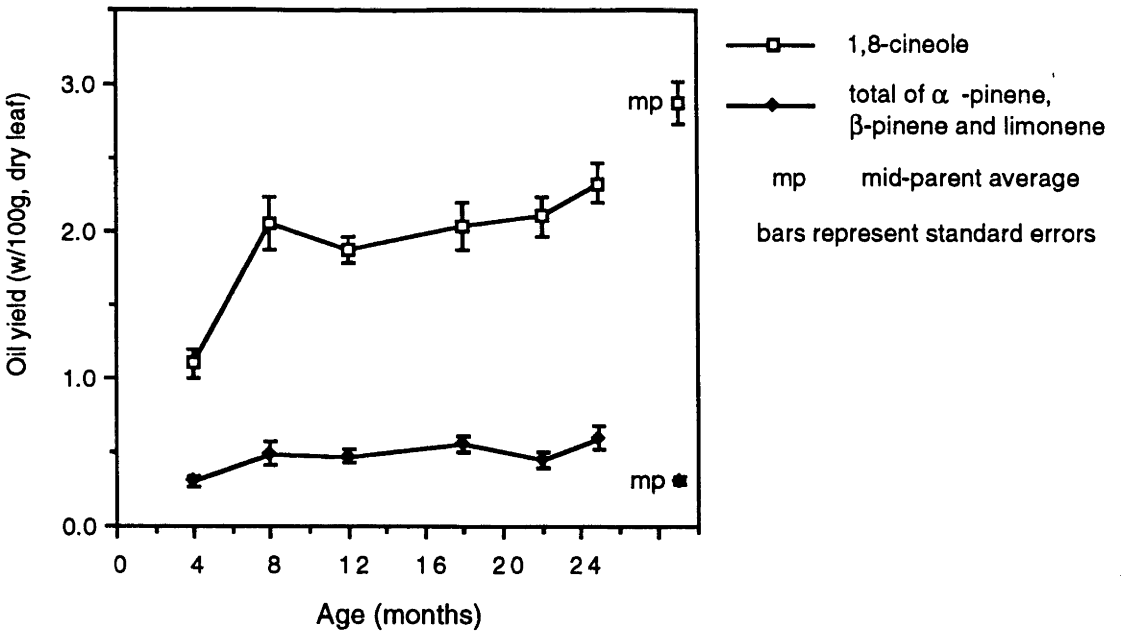


Figure 4.5 Variation in average foliar monoterpene yields of crosses amongst regular cineole-producing chemotypes with age compared to average mid-parent values for the same crosses.

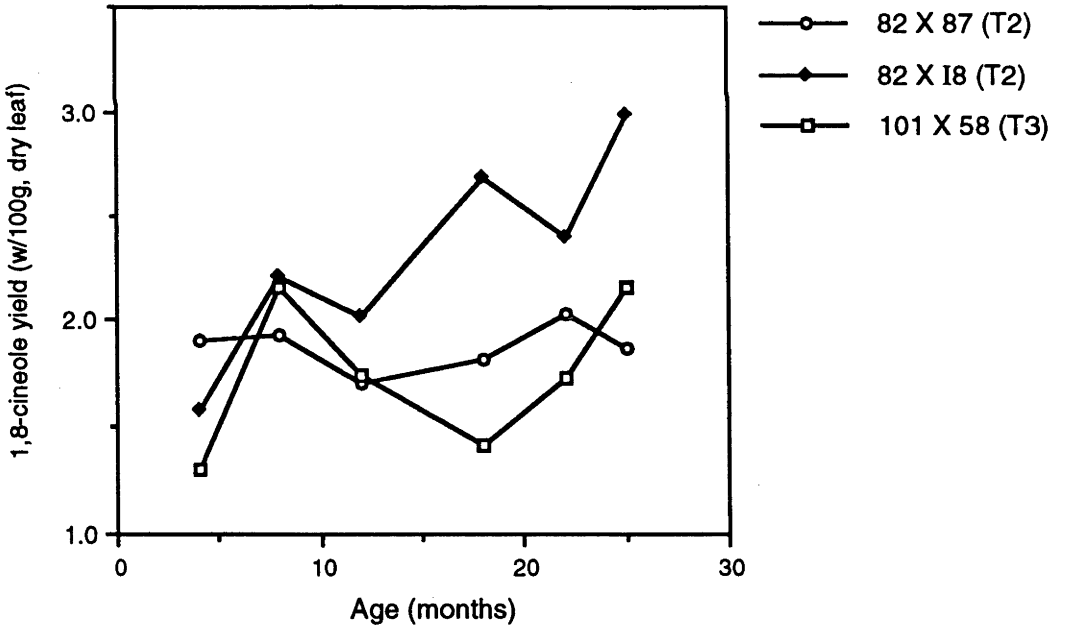


Figure 4.6 Patterns of variation in 1,8-cineole yield with age in contrast to average trends (Fig. 4.5) amongst individual trees of high cineole-yielding parentage.

Figure 4.7 gives a summary of age related changes in oil traits amongst families related to the sesquiterpene chemotypes, trees 10 and 51. There was more consistency amongst individuals in patterns of change in this group with only four trees (29%) deviating from average trends. Up to 18 months-of-age the pattern mimiced those of offspring of crosses amongst regular cineole chemotypes with sesquiterpenoids in low proportions. At 18 months, the first quantifiable traces of bicyclogermacrene and spathulenol (sesquiterpenoids present in trees 10 and 51 but not detectable in the other parents in the crossing program) were recorded in some progeny (14%). As their occurrence amongst the progeny (100% at 25 months) and yields grew with advancing age, yields of 1,8-cineole declined (Fig. 4.7). Yields of the other prominent monoterpenes at first declined past 18 months like 1,8-cineole but had recovered to near previous highs at 25 months.

The occurrence of bicyclogermacrene and spathulenol in all crosses involving trees 10 and 51 indicates the high heritability of these two compounds. At 25 months the concentration of bicyclogermacrene in the leaves of these crosses was far greater than predicted from mid-parent values while levels of spathulenol were significantly lower than might be expected at maturity (Fig. 4.8). Tressl *et al.* (1983), in their studies of the volatile constituents of hops (*Humulus lupulus;* var. Hersbrucker Spät), showed bicyclogermacrene to be the most likely precursor to the formation of the tricyclic sesquiterpenes which includes spathulenol. It appears highly likely that, with further aging of the sampled trees of *E. camaldulensis*, yields of spathulenol will increase at the expense of bicyclogermacrene.

The Kendall Coefficient of Concordance (Siegel 1956) showed the rankings for 1,8-cineole yield at the 5 ages to be unrelated. Significant changes in ranking for this trait took place between all evaluations (Fig. 4.9). Correlations between offspring and mid-parent values were poor (r= 0.23 to 0.55) until the 22nd month when r = 0.76. R further improved to 0.86 at 25 months. A contributing factor to the poor early offspring - mid-parent correlations was the time lag in the expression of the low-cineole-high-sesquiterpene characteristic of the oils of the chemotypes. Even at 25 months-of-age, 4 trees of sesquiterpene chemotype origin have still to develop high sesquiterpenes at the expense of cineole. All 4 have exhibited traces of bicyclogermacrene and spathulenol and further evaluations are required to see if these compounds develop with further ageing.

At 25 months-of-age when the last evaluation was made for this thesis, 1,8-cineole yield was still unstable with further increases expected in several progeny accompanied by the possibility of further changes in ranking with age. This result was somewhat surprising as indications in other trials were that rankings for this trait were stable from about 18 months onwards (see Chapter 5). The difference in this trial might be attributed to the fact that all crosses of regular cineole chemotype included at least one parent of high-cineole yielding capacity. These may take longer to reach their full potential than average lots. The high correlation coefficient between 25-month-yields and mid-parent values suggests that the progeny may have largely sorted themselves out into a relatively stable ranking.

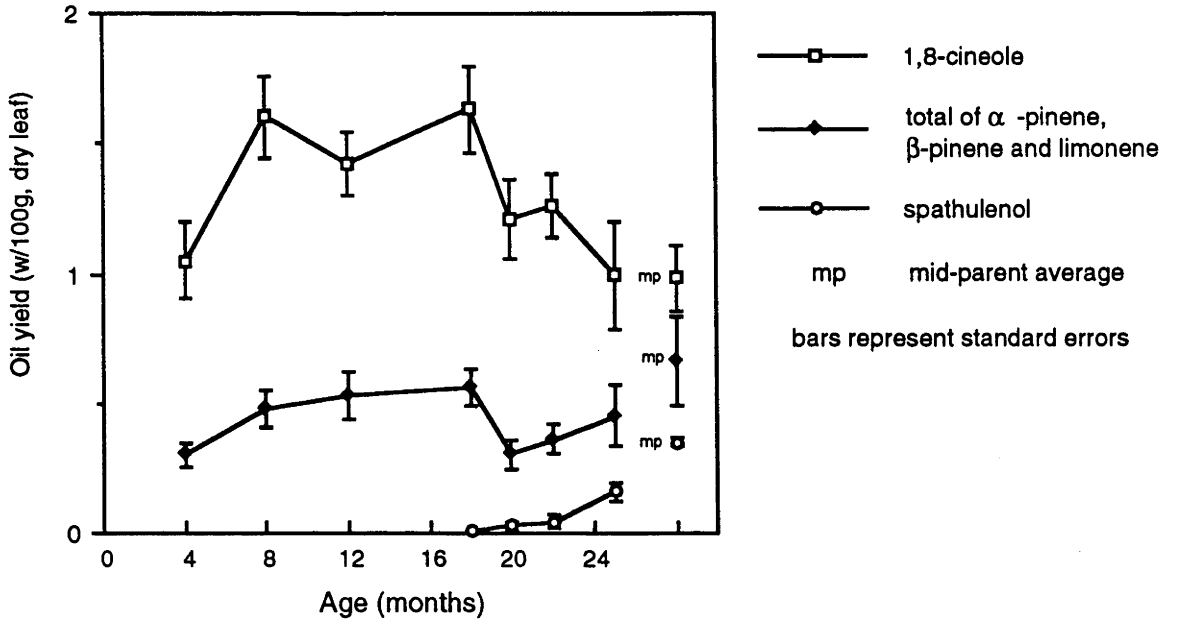


Figure 4.7 Variation in average foliar oil yields of selected compounds of families with one or both parents of the low-cineole-high-sesquiterpene chemotype with age. Mid-parent values for the crosses are given for comparison.

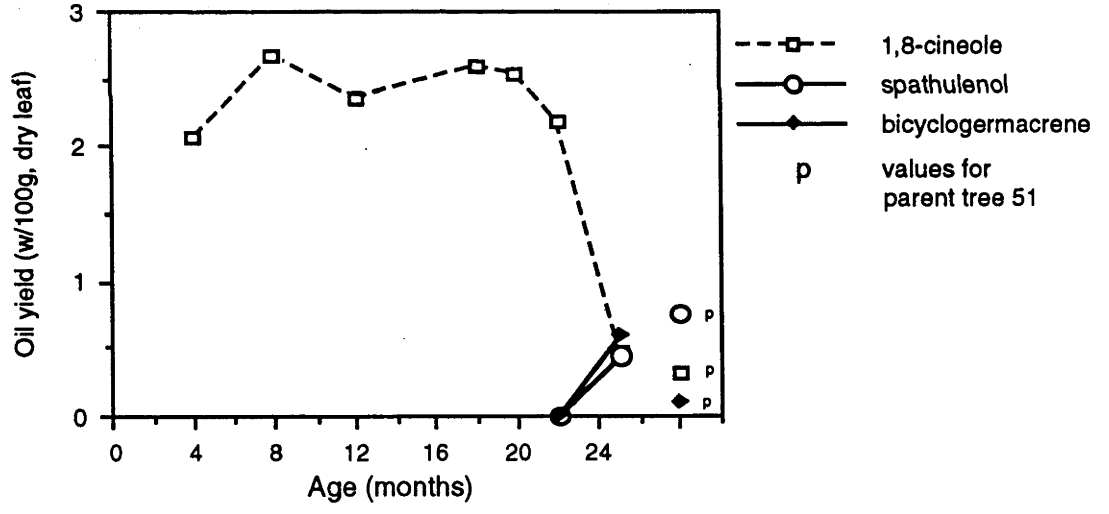


Figure 4.8 Changes in foliar 1,8-cineole yields compared to changes in yields of two sesquiterpenoids, bicyclogermacrene and spathulenol, with age in one representative tree of sesquiterpene chemotype origins (i.e. 51 op, tree 1).

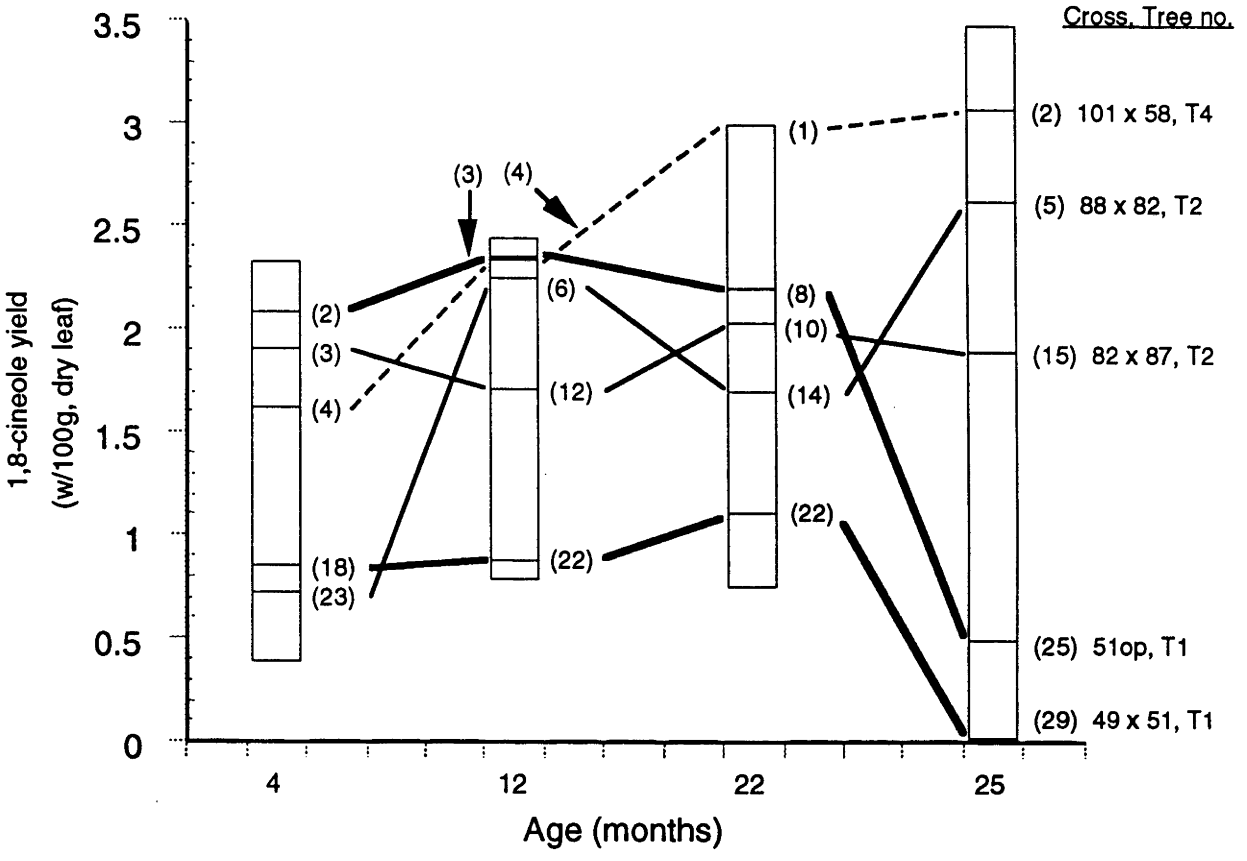


Figure 4.9 The overall range of foliar 1,8-cineole yields in all progeny evaluated with advancing age is given (see the vertical box). Changes in ranking ( ) with age of a selection of high, medium and low ranking individuals out of the total of 29 studied are illustrated. The most dramatic change in ranking occurs in the two sesquiterpene chemotypes (51 op T1 and 49 x 51 T1).

The majority of progeny expected to carry the undesirable genes for high concentrations of sesquiterpenoids did so and were easily identifiable at 25 months. In conclusion, it appears that reasonably reliable selection for 1,8-cineole yield might be undertaken as early as, but preferably not before, 2 years-of-age in this species. Further evaluations of the same set of trees are needed to test this hypothesis.

4.4 Conclusions

Time of year of sampling amongst adult trees and position in the crown (i.e. height and aspect) amongst both adult and young trees were shown not to effect estimates of oil yields of key compounds including 1,8-cineole in mature leaves of *E. camaldulensis*. Despite this consistency it was considered prudent to adopt a standard sampling strategy for this project. In all experiments, unless otherwise stated, oil yields were estimated

from fully-expanded leaves, considered to be of similar age and maturity, harvested from two branches selected at random at approximately two thirds of tree height.

The comparison in yields of 1,8-cineole, α-pinene, β-pinene, and limonene between coppice leaves and those taken from young (2 yr) crowns before felling was inconclusive. However, there does appear to be potential of lifting production of 1,8-cineole by the timely harvesting of leaves on coppice shoots at least on some sites. However, oil derived from coppice may be of lower quality (i.e. % cineole) as a consequence of an associated increase in production of all monoterpenoids at the time of peak cineole yield.

Key findings were that

- the low-cineole-high-sesquiterpene oil of the sesquiterpene chemotype takes at least two years to express itself in young plants, and
- this undesirable trait is highly heritable.

In the ageing experiment, 1,8-cineole yields had still to reach their full potential in a high proportion of progeny at 25 months-of-age. However, there were strong indications that at this age ranking was approaching some stability. These findings have important implications for determining the earliest age at which progeny can be reliably assessed for 1,8-cineole yield and oil quality. Indications were that, although absolute values might be subject to some further increases with time, a reasonably reliable assessment of individual rankings might be obtained as early as two years.

We can only speculate upon the cause of the relatively sudden development of high levels of sesquiterpenes in association with diminishing levels of 1,8-cineole in the sesquiterpene chemotypes between age 18 and 25 months. It appears to be linked to maturation processes in the tree and the activation of specific enzymes. Bicyclogermacrene appears to function as a precursor to the production of spathulenol. No other similar reports of such major age-related changes in oil composition in the genus *Eucalyptus* are known.

CHAPTER 5. INFLUENCE OF SEASON, SITE AND WATER-STRESS ON YIELD OF MONOTERPENES IN LEAF OILS OF YOUNG PETFORD *EUCALYPTUS CAMALDULENSIS* AND IMPLICATIONS FOR TREE BREEDING

5.1 Introduction

A limited assessment of the effect of time of year of sample collection on yields of major oil components, including 1,8-cineole, in the natural stands of *E. camaldulensis* at Petford showed no significant difference between summer and winter periods (see Chapter 4). However, selection for high oil yields in this provenance will be undertaken primarily in young plantations in a variety of environments. If there is significant environmentally-induced variation in oil characteristics early selections will be less reliable.

The studies reported here aimed to determine the extent of non-genetic sources of variation on oil yields (as a fraction of leaf weight) amongst young trees of Petford provenance.

5.2 Experiments, objectives and methods

5.2.1 Field Trials

The objective of the field experiments was to determine the effects of seasonal variation on oil yields among young progeny over a range of sites.

Four trials were established in January 1988 in 1- and 2-year-old experimental plantings of the same bulked seedlot of Petford *E. camaldulensis*. The seedlot was obtained from 129 parent trees. The trials were located on three sites, at Tuan, Toolara and Wongi State Forests near Gympie in southeastern Queensland (Table 4.1 and Fig. 4.1).

The older plantings at Wongi and Tuan were established in the summer of 1986 as part of other, larger species trials (Ryan and Bell 1989). Randomised complete block designs were employed with two replications per site and 36-tree plots. Five trees were selected at random in each plot (10 trees per site) and leaves harvested monthly from January 1988 to January 1990, commencing when the trees were 2 years old (Fig. 5.1). The 1-year-old species trials at Wongi and Toolara employed an incomplete block design with 10-tree row plots and four replications. Three trees were selected at random in each plot (12 trees per site) and leaves were harvested at 3-monthly intervals over the same period.

Collection of plant material and measurements

Leaves were collected at about the same time of day in the third week of each month in each trial. Twelve mature leaves were collected from each tree from two branches removed at approximately two-thirds of tree height. Leaves were obtained from the region of branch below that carrying the 'soft' immature leaves but above that bearing leaves showing obvious signs of senescence. They were placed in plastic bags, kept refrigerated and airfreighted to Canberra for analysis.

(a) (b)

Figure 5.1 Study of monthly variation in oil yields, (a) collecting leaves with long-handled pruner in a 3-year-old plot of Petford *E. camaldulensis* at Wongi and (b) heavy defoliation by insects (*Chrysomelid* sp.) during the summer months sometimes caused problems for collectors.

Height of trees in the experiment was recorded at various intervals during the 2-year period. Meteorological records were obtained from forest stations close to the trial sites.

Extraction and analysis of volatile oils

Three grams of leaf material from each sample (tree) were placed in bottles containing 50ml of ethanol (ca. 99.8%). A second sample from each tree was oven-dried at 70°C to determine water content. Chemical analysis and quantification of the major monoterpenes present in the leaf oils was undertaken by capillary gas chromatography on the ethanol extracts according to method 5 in Table 3.1.

Statistical analysis

Individual tree data for 1,8-cineole yield and total yield of monoterpenes in the older trials at Wongi and Tuan were first analysed using Fourier decomposition for each tree (Bloomfield 1976). This approach proved unsuccessful because of the very large and inconsistent trends in tree to tree variation in oil yields over time on each site. To assist in circumventing this problem and at the same time countering the effects of observations correlated in time, the data were combined to represent the nine seasons covered by the

experiment (summer 1987/88 - a short season-excluding Dec. 87 to summer 1989/90 - again a short season excluding Feb. 90). The seasonal data were then subjected to an analysis of variance to examine the effects of the main factors (site and season) and interactions on oil yields.

Statistical tests were carried out for correlations between the available meteorological data and oil yields. Data from individual trees were pooled by site and plotted against the climatic data for a visual check for relationships. Step-wise regression analysis was then used to test for correlations and levels of significance.

5.2.2 Nursery Trial

This trial was to determine: (a) if drought and waterlogging stress significantly affect yields of the major oil fractions, as was suggested by early trends in the field trials; (b) the effects of physiological age of leaves on oil yields; and (c) the presence of any significant interactions between the factors being studied.

Three ramets and the ortet of each of 6 clones were used. The clones had been produced 21 months earlier by striking cuttings from 7-month-old ortets raised from Petford seed. The clones were established in large plastic tubs, containing 3:1 pine bark and perlite, under drip irrigation in a shadehouse. Slow release fertilizer (Osmocote) was applied regularly to each plant.

The clones were kept in a glasshouse in the winter months prior to establishing this experiment. Glasshouse oil yields were assessed before the plants were hardened-off in a shadehouse for 2 weeks. They were then moved outside and allowed to acclimatise for one week before trial establishment. The height of the clones was about 2m. Physiological age of both ortets and ramets was over two years which was consistent with plants in the field trials.

The 6 clones were positioned at random in a 6x4 rectangular array of tubs at 1x1m spacing (Fig. 5.2). Two treatments in the form of factors were incorporated in the trial: clone at 6 levels and water regime at 4 levels. Water regimes were:

(a) control- well-watered with drip tray providing ready availability of water;
(b) waterlogged- tub submerged within a larger barrel of water to within 2cm of the surface with the water topped up daily and replaced every few weeks; and
(c) droughted (2 replicates)- polythene sheeting placed to exclude rain water, one substantial watering applied each week.

Collection of plant material, measurements and analysis of volatile oils

At the beginning of the experiment, the position of the newly emerged leaf pairs (leaves about 3mm long) was marked permanently on most available erect branches. Growth of these shoots under the various treatments was monitored over 5 months when the slower growing leaves under the drought stress treatments appeared to have reached maturity. At this point all leaves on the marked shoots of 4 clones (2 clones were unsuitable) were harvested by age class (5mo, 4mo, 3mo, soft tips). These will be referred to subsequently as 'new leaf' having developed under the water regimes imposed. The length of an average 5-month-old shoot was recorded for each plant. Leaf areas were measured and leaves of the 4 and 5mo age class were assessed for oil gland density and maximum gland diameter under a light microscope.

Figure 5.2 Clones of Petford *E. camaldulensis* in the nursery trial

The original mature leaf class (i.e. of full size and hardened), estimated to be six months of age on initiation of the experiment, was monitored for oil yields at monthly intervals up to 10 months of age. These will be referred to subsequently as 'old leaf' being well-matured before application of the various watering regimes. Quantification of the yields of 1,8 cineole, α-pinene, β-pinene and limonene in ethanol extracts followed the methods described earlier.

Statistical analysis

The two variates, yield of 1,8-cineole and total yield of the four major monoterpenes (as a fraction of leaf weight), for both the 'new leaf' and 'old leaf' were analysed statistically. Randomised complete block design analyses were carried out to examine the main treatment factors and their interactions, having first confirmed there were no row or column effects due to site variation. Both sets of data were then analysed as a split-plot design with time as the split-plot, following validation of the method through a test of sphericity of the variance-covariance matrix (Box 1950), to determine if leaf age was a significant factor.

5.3 Results

5.3.1 Field Trials

Patterns of growth and leaf development

The trees grew 5m in height on average during the 25 months of sampling despite droughts, floods and periodic heavy predation by leaf-eating insects. The sampled trees were more than 6m high at 3 years of age and more than 10m high at 4 years of age when the experiment terminated.

(i) Effect of growth rate on oil yields

There was no evidence of a relationship between individual tree vigour and oil yields. For example, the slowest-growing tree at Tuan gave an average oil yield close to the average for that site while the fastest growing tree had one of the lowest yields. The reverse occurred at Wongi.

(ii) Leaf maturation

The estimated time from initiation to maturation of leaves was 2 to 3 months during the most active growth phase, increasing to at least 4 months when shoot growth was restricted. Major flushes of new leaf were noted on all sites in October/November and again in February.

(iii) Variation in leaf moisture content

Mean monthly leaf moisture contents at Wongi and Tuan are given in Fig. 5.3. No direct relationship between leaf moisture content and oil yields was apparent.

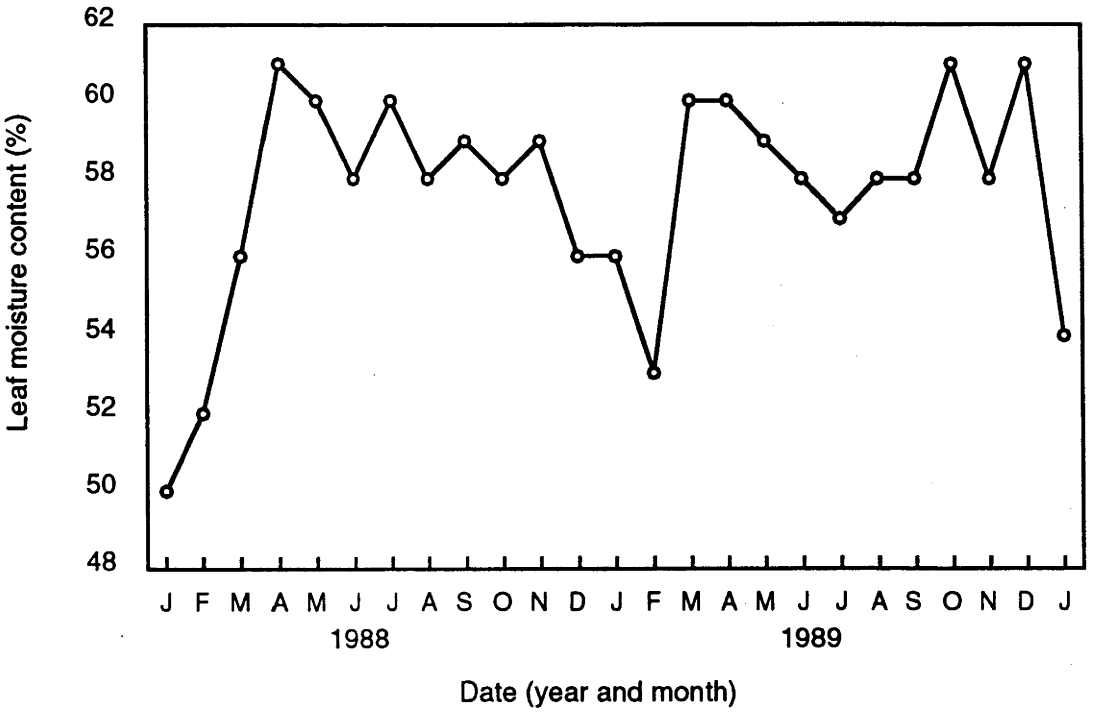


Figure 5.3 Average leaf moisture contents (%) over the study period.

Seasonal effects on oil yields

Variation in total yield of monoterpenes closely followed the trends in 1,8-cineole yield so further discussion will be limited to this compound. The proportion of 1,8-cineole of the total remained fairly constant throughout the year with a slight rise in late summer (63% to 67%).

(i) Individual tree variation

Variation in oil yield of individual trees over time could not be tested statistically but it was large. Of paramount importance in the context of this paper was the observation that the highest and lowest yielding trees on each site maintained their relative positions over the entire 25 months of the experiment (Fig. 5.4), despite some variation in ranking for oil yield amongst the other trees in the trials.

(ii) Effect of site

The lack of statistical significance in between-site differences in oil traits (Table 5.1) is possibly due to insufficient replication. The significance of interactions between site and season suggests that site does have an important influence on patterns of variation in oil yields (Table 5.1). Monthly means for yields of 1,8-cineole at Wongi and Tuan are given in Figures 5.5a and b and the overall trends between all sites compared in Figures 5.6a and b. Tuan was less variable than Wongi and there were few matching trends except for the period August 1989 to January 1990. The pattern of variation in both trials at Wongi matched-up extremely well. The pattern at Toolara, on the other hand, was in marked contrast to the other sites.

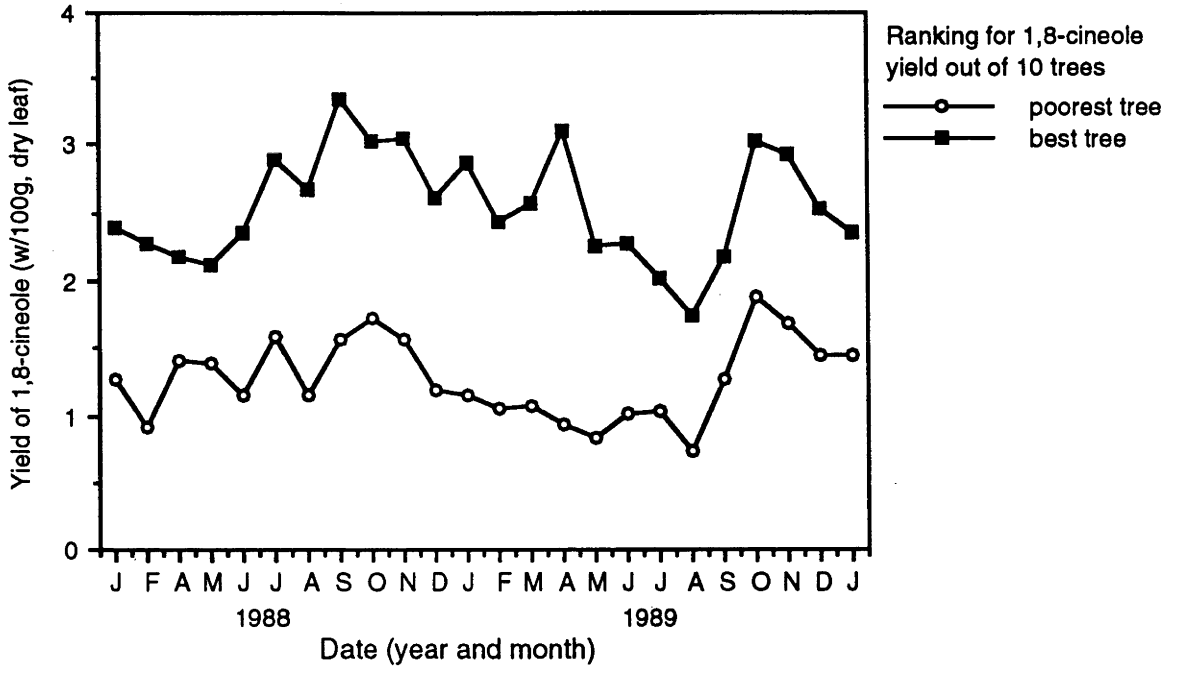


Figure 5.4 Individual tree variation in 1,8-cineole yield over time at Wongi. The highest yielding tree is compared with the poorest.

Table 5.1 Analysis of variance tables for yields (w/100g, dry leaf) of 1,8-cineole and total monoterpenes in the Gympie trials.

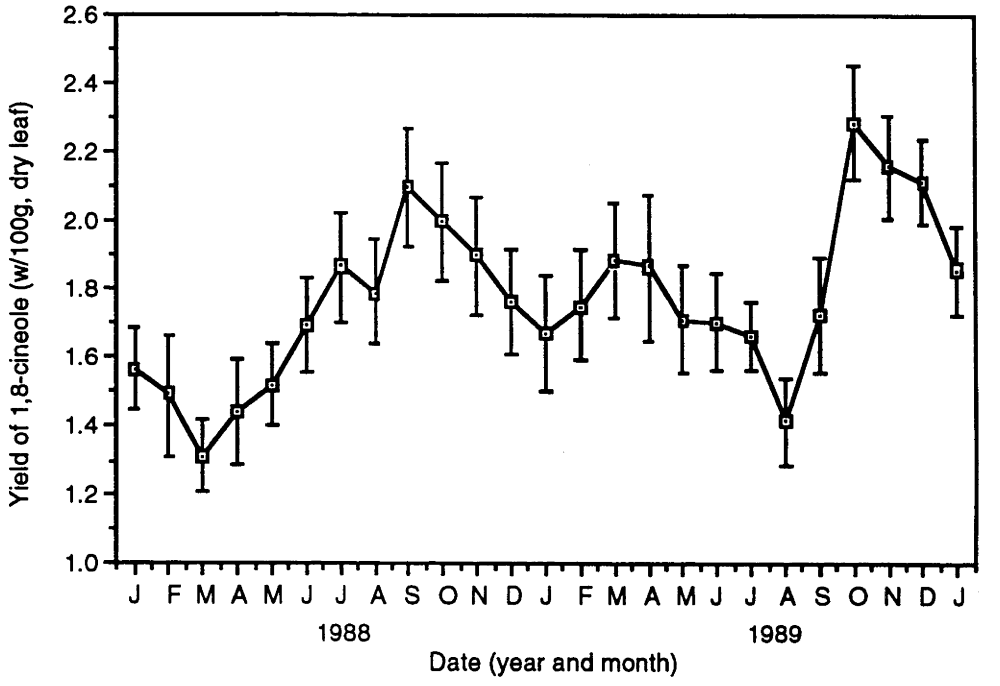
Variate: 1,8-cineole

Source of variation	d.f.	m.s.	Significance
Site.rep stratum			
Site	1	5.61	ns
Residual	2	1.89	
Site.rep.tree stratum			
(trees in plot)	16	6.00	
Site.rep.tree.season stratum			
Season	8	1.28	***
Site.season	8	0.45	***
Residual	144	0.11	
Site.rep.tree.season.month stratum	317(5)	0.06	
Total	494(5)		

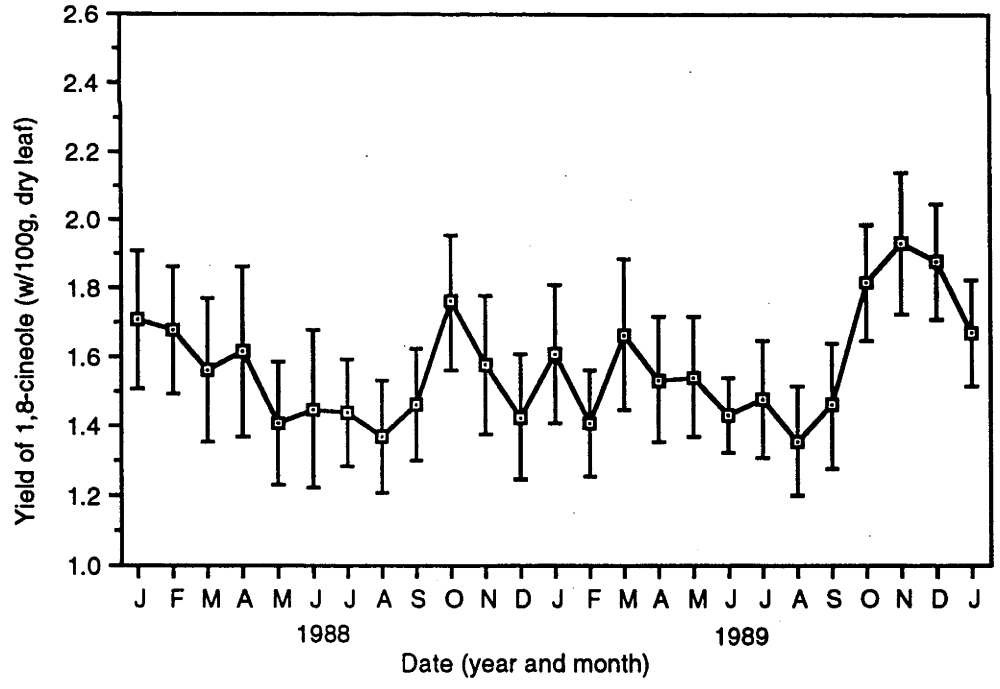
Variate: Total monoterpenes

Source of variation	d.f.	m.s.	Significance
Site.rep stratum			
Site	1	0.13	ns
Residual	2	3.76	
Site.rep.tree stratum			
(trees in plot)	16	5.71	
Site.rep.tree.season stratum			
Season	8	3.00	***
Site.season	8	0.88	***
Residual	144	0.18	
Site.rep.tree.season.month stratum	313(7)	0.12	
Total	492(7)		

***P<0.001, ns not significant



(a)



(b)

Figure 5.5 Monthly mean yields of 1,8-cineole over 25 months at Wongi (a) and Tuan (b). Bars indicate 2 x s.e.

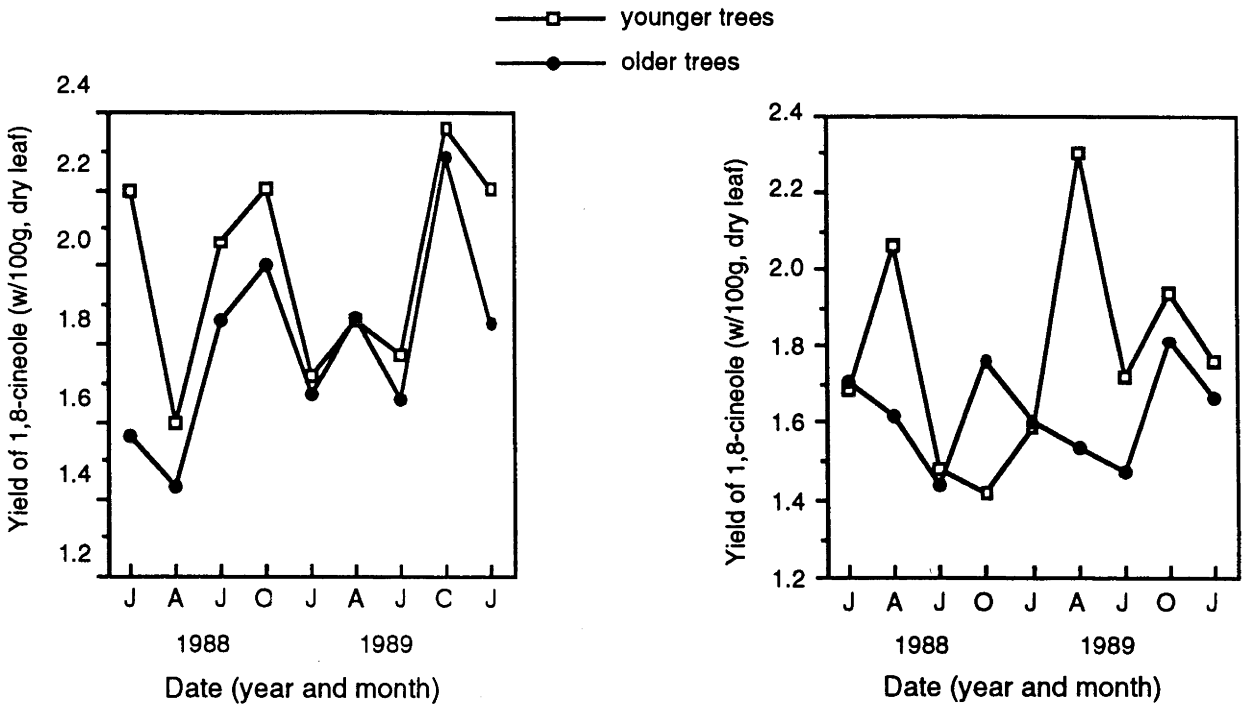


Figure 5.6 Variation in mean 1,8-cineole yields between the younger and older trees at Wongi (a) and at Tuan and Toolara (b) over time.

(iii) Variation due to season

Oil yields tended to be lowest in autumn and winter and highest during spring and early summer, although there was substantial variation between individual trees and sites. In another analysis not reported here (split-plot in time after Brooker *et al.* 1988), the year x season interaction was significant suggesting that year is also an important factor. In addition, when the patterns at Toolara, with distinct peaks in autumn and troughs in spring, are considered, it is clearly unwise to generalise about seasonal trends in oil yields in this set of experiments.

(iv) Meteorological correlations

There were no correlations between monthly yields of 1,8-cineole and meteorological data at sampling and 1 and 2 months prior to sampling. A negative correlation was significant ($p < 0.05$) for 1,8-cineole and minimum temperature 4 months prior to sampling but coefficients were very small.

5.3.2 Nursery Trial

Effect of water regime on growth of clones

Average shoot growth varied both within and between clones and by watering regime. In all six clones, reduced water supply caused large differences in growth rates and in the number and size of leaves formed (Table 5.2 and Fig. 5.7). Shoot growth and average leaf size of the droughted plants were approximately 40% of that of control plants and number of leaves was reduced by 80%. Plants in replicate 1 of the drought treatment

(drought 1) grew faster than those in replicate 2 (drought 2) indicating a difference in the severity of the stress and providing the opportunity to compare its effect on oil yields.

Table 5.2 Overall mean shoot growth of the 6 clones and standard deviations by water regime.

Treatments	Length of shoot cm (SD)
Control	57.7 ± (12.3)
Waterlogging	52.7 ± (18.0)
Drought 1	35.3 ± (10.7)
Drought 2	26.2 ± (11.0)

The waterlogging treatment gave a variable growth response depending on clone. Four clones showed little or no adverse response to this treatment while 2 clones showed a 30% and 58% reduction in shoot growth respectively. Despite this variability, oil yields of fully expanded leaves under waterlogging were greater than the controls (Fig. 5.8).

Effect of water regime on oil yields by leaf age class

Yield of 1,8-cineole and the total of the three other monoterpenes moved more-or-less in parallel with leaf age although there was variation in the proportional representation of each compound. The proportion of 1,8-cineole of the total yield of the four compounds was less at the time of peak yield (65%) and greatest in the lower-yielding, aged leaves (72%). The estimates of oil gland density and gland size, while varying between clones, did not appear to vary appreciably between treatments within clones once the leaf was at or near full expansion. This suggests that these traits are genetically fixed and are not a major influence on the observed differences due to treatment and age.

(i) 'New Leaf'

Results of oil yields in 'new leaf' showed significant effects for clone, water regime and leaf age (Table 5.3). All two-way interactions were insignificant. The drought treatments significantly reduced oil yields in leaves up to and including the peak age of 4 months (Fig. 5.8). By 5 months of age, oil yields of all treatments declined especially those of the controls. At five months the controls were similar to the droughted plants in yield of 1,8-cineole and total monoterpenes. The waterlogging treatment gave the highest oil yields except for the very young leaves at the tips of the shoots. This was most apparent in 5-month-old leaf.

Peak oil yields occurred in leaves of 3 or 4 months of age in the control and waterlogged treatments and at 4 or 5 months of age in the droughted plants. Overall the peak came at 4 months, at a time when leaves were at or near full expansion but had not fully lignified and still had a relatively high moisture content (57%). Thereafter, oil yields dropped quite dramatically in some instances (e.g. control in Fig. 5.8) to average 20% less at 5 months of age compared to leaves 4 months old.

Clone 4

Clone 5

Clone 6

a b c d

Figure 5.7 Average shoot growth over five months by treatment, a) is control; b) is waterlogged; c) is drought 1 and d) is drought 2, in three clones of Petford *E. camaldulensis*.

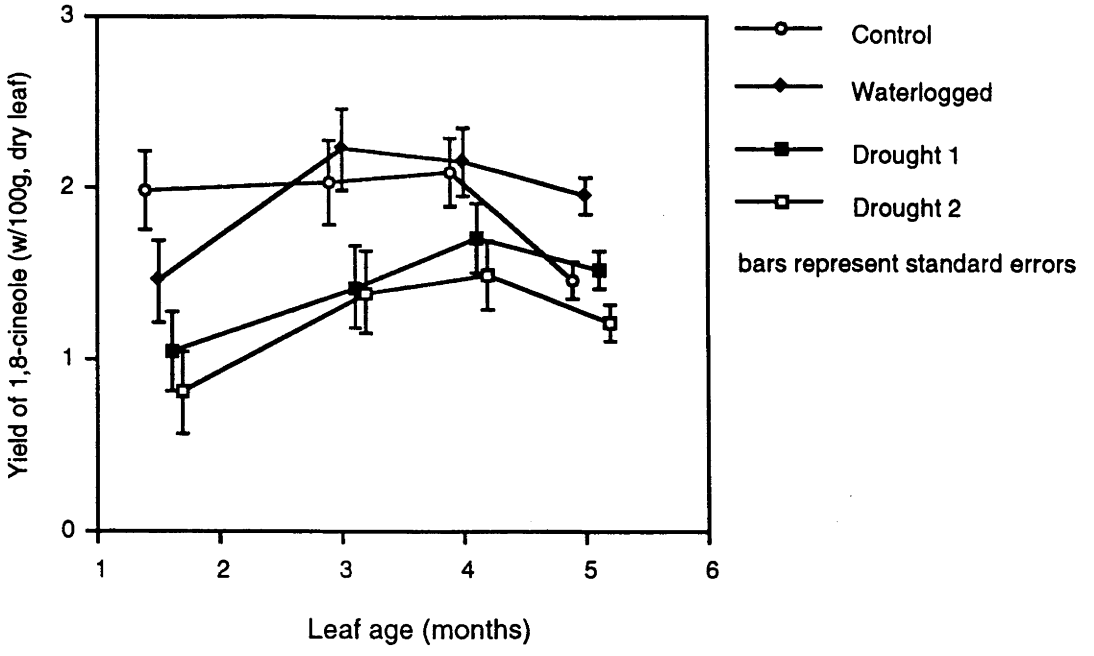


Figure 5.8 Variation in mean 1,8-cineole yield by water regime and leaf age. Plotted points at each age have been separated to avoid congestion in the figure.

(ii) 'Old Leaf'

Water regime did not influence oil yields although there were significant differences among clones. The slow downward trend in oil yields of leaves ageing on the tree (6 to 10 mths) just reached significance for total monoterpenes but not for 1,8-cineole (Fig. 5.9).

Effect of ontogeny and growing conditions on oil yields

The influence of ontogeny and growing conditions on oil yields of the ortets is given in Fig. 5.10. Seedling leaves from 7-month-old glasshouse plants were low in oils. Mature leaves from the same plants at 26 months of age collected in the glasshouse were an average of 47% greater in the total yield of the four compounds. Similar mature-aged leaves collected from the same plants two months later, after hardening-off under 50% shade and being exposed to full sunlight for one week, had increased their oil yields by an additional 62%. Thereafter oil yields of the ortets stabilised.

Table 5.3 Split-plot analysis of variance table for yields (w/100g, dry leaf) of 1,8-cineole and total monoterpenes (in brackets) in the nursery trial; (a) for 'new leaf', (b) for 'old leaf'.

(a) 'new leaf'

Source of variation	d.f.	m.s.	Significance
Row.col.stratum			
Clone	3	2.62(5.54)	** (***)
Treatment(tr)	3	1.48(2.38)	* (**)
Clone.tr	8	0.22(0.30)	
Row.col.*Units*stratum			
Time	3	0.70(1.33)	** (**)
Clone.time	9	0.19(0.24)	ns (ns)
tr.time	9	0.15(0.29)	ns (ns)
Residual	18	0.07(0.11)	
Total	53		

(b) 'old leaf'

Row.col.stratum			
Clone	5	6.63(18.82)	***(***)
Treatment(tr)	3	0.05(0.11)	ns (ns)
Clone.tr	15	0.07(0.18)	
Row.col.*Units*stratum			
Time	4	0.18(0.64)	ns (*)
Clone.time	20	0.06(0.16)	ns (ns)
tr.time	12	0.05(0.14)	ns (ns)
Residual	59	0.07(0.20)	
Total	118		

***P<0.001, **P<0.01, *P<0.05, ns not significant

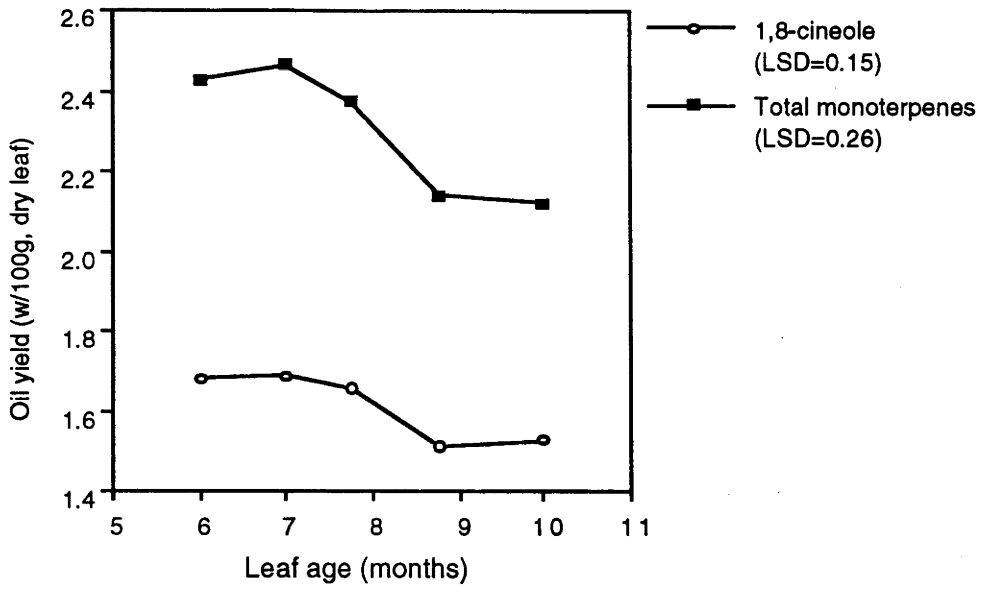


Figure 5.9 The overall effect of time (ageing) on oil yields of 'old leaf'.

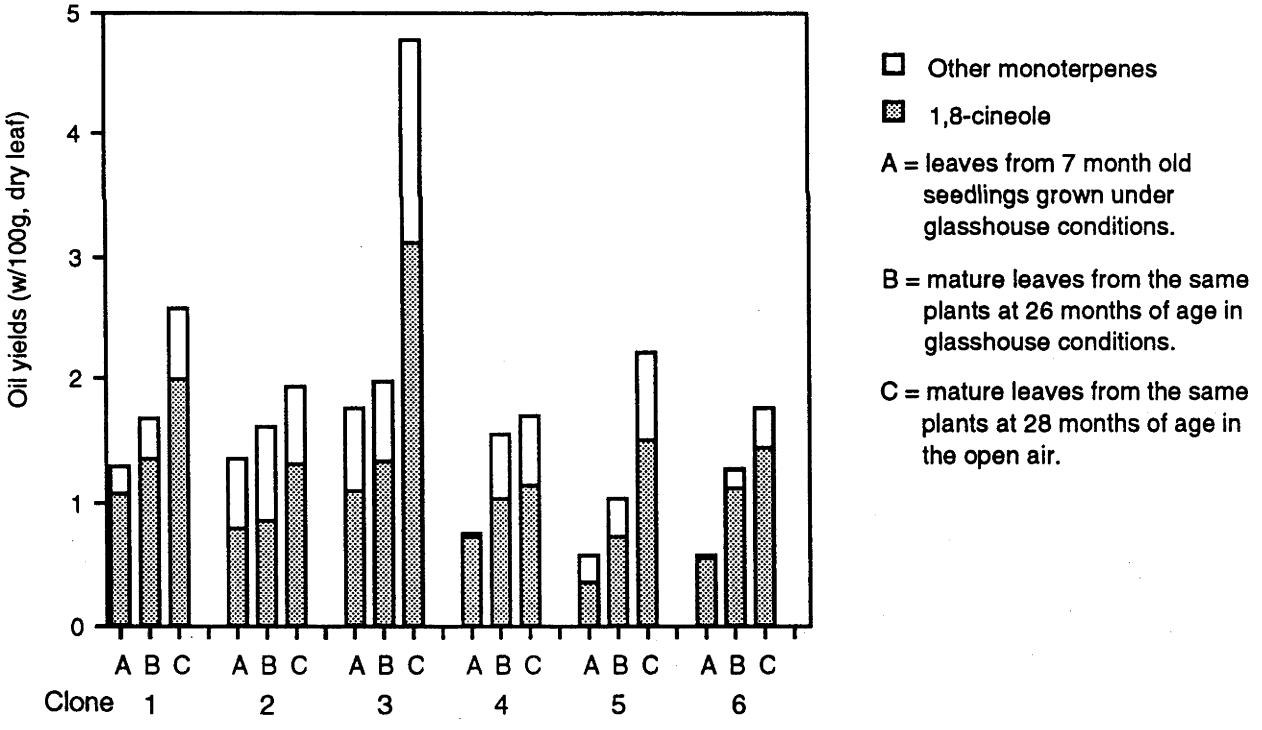


Figure 5.10 Oil yields of ortets as seedlings and 26-month-old plants in the glasshouse and at 28 months of age after hardening-off under shade for two weeks and one week exposure to full sunlight.

5.4 Discussion

Individual tree variation in oil yields with time was very large in the field trials and did not appear to be associated with growth rate. Site also had an important influence with substantially less variation at Tuan than at Wongi and Toolara. This observation is consistent with the findings of Rao *et al.* (1984) in a study of oil concentration in *E. citriodora* where seasonal influences amongst the same seedlots were strong on one site but minimal on another. Abou-Dahab and Abou-Zeid (1973) reported a significant decrease in 1,8-cineole yield from *E. camaldulensis* leaves throughout winter in Egypt followed by a slight increase in spring and then a steady rise throughout summer. In the field trials, monthly changes in yields were often large but inconsistent between trees, sites and years such that any general pattern was difficult to define. Patterns of variation could not be related conclusively to any environmental factor.

Light has been shown to be a key factor influencing oil production in various essential oil producing taxa (Haagen-Smit 1949). In the nursery trial, mature leaves estimated to be 5 months-of-age in the glasshouse showed the ability to produce oil quickly when plants were removed from the glasshouse and exposed to natural light. After this initial surge, oil yields stabilised and from six months onwards there was a slow decline. Leaves 10 months of age, showing clear signs of senescence, gave yields of oil similar to that at the start of the experiments. Water regime had no significant effect on oil yields of 'old leaf' at any age. It appears that an equilibrium is reached between synthesis and removal or loss of oil from the glands that is largely independent of environmental effects. Clones differed significantly in yields at equilibrium indicating a strong genetic influence on this trait.

In contrast, significant changes occurred in oil yields with water regime and leaf age in leaves that had developed under the influence of the various treatments. Drought significantly reduced oil production in expanding and unhardened leaves while waterlogging had the effect of extending the period of peak production to hardened, mature leaves. Estimates of oil yields also depended heavily on the stage of development of the leaves sampled. Highest yields occurred in leaves that had reached full size but were still relatively soft.

In comparing the results from the field and nursery, it is apparent that leaves were collected of differing physiological age. Every effort was made to collect leaves of similar maturity in the field but there were problems due to periods of slow and rapid turn-over of leaves in these trials and exacerbated by heavy insect (*Chrysomelid* sp.) defoliation in summer. A guide to what occurred is provided by monthly variation in average leaf moisture contents. The leaf moisture content data suggest that the bulk of the leaf sampled was equivalent to the 3-4-5 month age classes of 'new leaf' in the nursery trial. This is the period when young leaves are most vulnerable to rapid change in oil yields which are linked to maturation processes but also modified by individual genotype and external influences. For example, the lower-than-average moisture contents and oil yields in late summer suggest that ageing leaves from the October/November flush were harvested. Also, the heavy insect defoliation of young leaves during this period often left 'old' leaves as the only available samples on some trees (see Fig. 5.1b). These leaves would be past their peak in oil production. The high moisture contents and low oil yields in autumn may well be a result of harvesting immature leaves from the February flush as trees replaced foliage lost to the summer defoliators.

Total oil production depends on oil concentration in the leaves and leaf biomass. In the field trials the type of leaf collected was dictated by what was available on the branches at the time and would be representative of the class of leaf providing the major part of the leaf biomass on a particular tree when sampled. Despite the confounding of leaf age and environmental effects, the variation found in oil yields in the field trials is still of practical significance.

Although there was a high level of non-genetic influences on oil yields in 'new leaf' in both the field and nursery trials, the highest yielding plants maintained their ranking throughout although varying in absolute amounts of oil present at various assessments. As long as comparisons within site are based on leaves of the same physiological age and selections across sites are tested in a common environment, non-genetic sources of variation should be controlled and reliable selection of high oil yielding genotypes assured. If screening must be done across sites without progeny testing, then it may prove more reliable to rank trees on the basis of oil yields of the 'aged' leaves which are less subject to environmental influences.

5.6 Conclusions

Oil yields of leaves up to 5 months of age were shown to be significantly affected by physiological changes associated with leaf maturation. The extent of these changes depends in part on genotype and on external influences such as soil moisture conditions. Prolonged drought stress reduced oil production in young leaves but appeared not to affect greatly the equilibrium reached between synthesis and removal of oils at leaf maturity. Waterlogging had the beneficial effect of prolonging peak oil production.

Trends in oil yields with time differed markedly between individual trees. Although genotypic variation was great, there was detectable variation attributable to site, season and year. A relationship between seasonal patterns and climatic factors could not be established and it was deduced that part of the observed variation in oil yields with time was attributable to variability in leaf maturity.

The relative superiority in oil-yielding capacity of certain individuals in each experiment was maintained throughout despite substantial variation from non-genetic sources. This consistency in ranking augers well for tree breeders wishing to make selections amongst plantations of differing age, on different sites and at different times of the year. However, even with careful sampling controls, absolute values will vary substantially and progeny testing will be necessary to further assess the potential of the initial selections. If progeny testing is not an option then aged leaves, less susceptible to environmental variation, may provide the most reliable value for ranking purposes.

CHAPTER 6. VARIATION IN OIL CHARACTERISTICS BETWEEN AND WITHIN POPULATIONS OF THE TROPICAL RED GUMS

6.1 Introduction

In 1987 a series of investigations were begun, centred on natural stands of *E. camaldulensis* and *E. tereticornis* in northern Queensland to determine;

(1) the extent of inter- and intra-specific variation in chemical composition of the oils of both species in northern Australia,
(2) the potential of selected provenances to provide a source of cineole-rich oil, and
(3) whether chemical analysis of the oils would aid in the botanical classification of 'intermediate' populations.

Oil yields in this Chapter are expressed as the weight of oil per 100g of fresh leaves. This contrasts with other Chapters where oil yields are expressed on a dry weight basis. There are two principal reasons for expressing oil yield in this way. First, for consistency amongst these experiments as moisture content of leaves used in Experiments 1 and 2 were not determined. Second, to meet the industry convention of assessing oil-yielding potential of a species on the basis of fresh weight determinations (e.g. see Table 2.1). The variation in moisture content of leaves between and within populations in Experiments 3 and 4 was not large and an approximation of yield on a dry weight basis can be obtained by multiplying the fresh weight estimates, as given in the tables and figures, by a factor of 2.1.

6.2 Experiments, objectives and methods

6.2.1 Experiment 1: Survey, northeastern Queensland

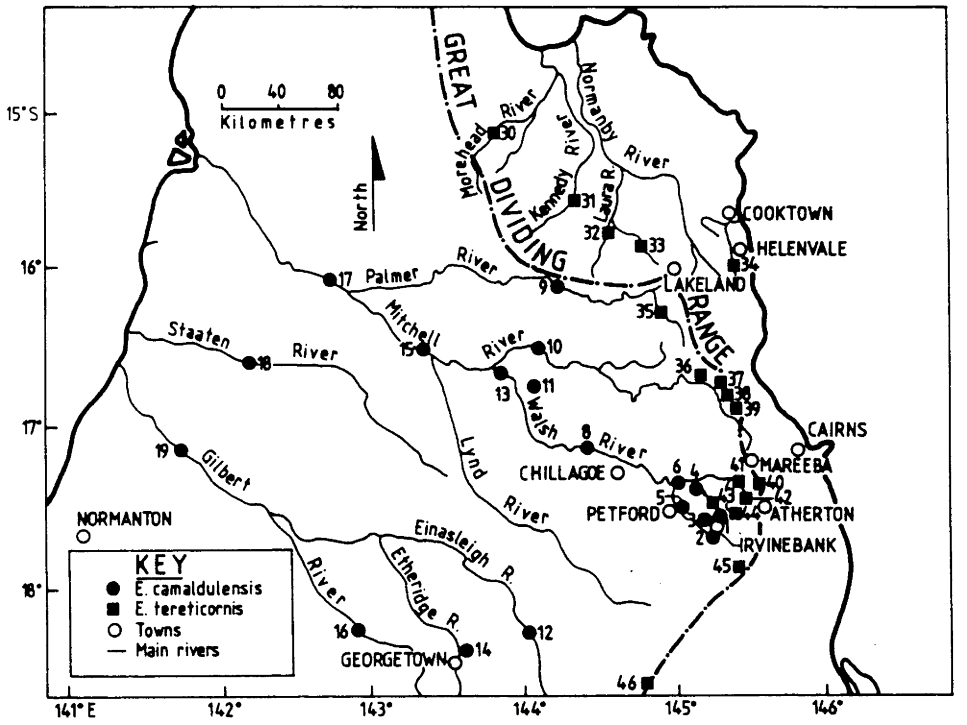
The objective of this experiment was to compare the oil composition of populations of *E. camaldulensis* and *E. tereticornis* of authentic botanical classification and Petford provenance to find;

(1) if there were qualitative differences useful for distinguishing the pure species from each other and from a population where introgression of the two species is suspected, and
(2) if any of the oils met the standards required for medicinal use.

Collection of Plant Material and Extraction and Analysis of Volatile Oils

The sites chosen were Einasleigh River, Walkamin and Emu Creek near Petford (Sites, 11, 40 and 5 in Fig. 6.1 and Table 6.1). In the field, approximately 500g of fresh leaves and terminal branchlets were sampled from each of 5 widely-spaced mature trees selected at random and a bulked sample harvested from 5 other trees at each of the three sites. Care was taken in this and subsequent trials to take only leaves estimated to be similar in age and stage of maturity in each tree. These were obtained from within the crown, below the immature leaves but above leaves showing signs of senescence. Samples were placed in calico bags and stored over ice for a few days prior to air despatch to Sydney where the leaves were steam-distilled with cohobation (Lassak 1979) for 8 hours to yield colourless to golden-yellow oils. Chemical analysis of the oils was by gas chromatography and mass spectrometry (see Chapter 3).

Map 1



Map 2

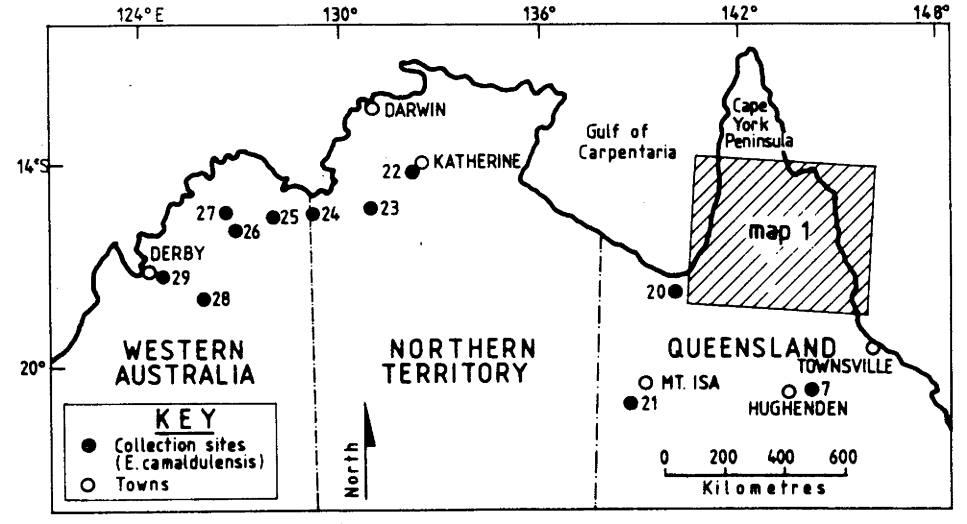


Figure 6.1 Location of provenances of *E. camaldulensis* (Maps 1 & 2) and *E. tereticornis* (Map 1) sampled in Australia and Zimbabwe for analysis of leaf oils.

Table 6.1 Origin of provenances of *E. camaldulensis* and *E. tereticornis* sampled in Australia and Zimbabwe for analysis of leaf oils.

Provenance and Map No	Provenance name	Lat (°S)	Long (°E)	Alt (m)	Leaf collection N.Aust.	Zimbabwe
E. camaldulensis (1-29)						
1	Hales Siding	17°22'	145°13'	780	X	
2	Headwaters, Emu Creek	17°29'	145°13'	860	X	
3	NW Irvinebank	17°24'	145°09'	710	X	X
4	Eureka Creek	17°11'	145°03'	460	X	
5	Emu Creek, Petford	17°20'	144°57'	490	X	X
6	Flat Rock Pool	17°10'	144°56'	420	X	
7	Bullock Creek	20°49'	144°48'	451		X
8	NW Chillagoe	16°59'	144°18'	240		X
9	Palmerville	16°00'	144°05	400	X	
10	"Mt Mulgrave"	16°23'	144°02'	160	X	
11	"Wrotham Park"	16°40'	144°02'	152		X
12	Einasleigh River	18°11'	144°01'	390	X	
13	Walsh River	16°33'	143°47'	140	X	
14	Etheridge River	18°16'	143°33'	340	X	
15	Lynd River Junction	16°28'	143°18'	170	X	
16	Gilbert River Bridge	18°12'	142°52'	150		X
17	Healeys Yard	16°01'	142°34'	150	X	
18	Staaten River	16°32'	142°03'	150	X	
19	Gilbert River	17°11'	141°45'	75	X	
20	SW Normanton	18°07'	140°20'	50		X
21	SW Mt Isa	21°26'	139°06'	410		X
22	Katherine	14°29'	132°15'	95	X	X
23	Victoria River	15°35'	131°02'	35		X
24	Cockatoo Creek	15°38'	129°01'	50		X
25	Pentecost River	15°48'	127°43'	60		X
26	Gibb River	16°08'	126°30'	430	X	
27	Drysdale River	15°41'	126°22'	400	X	
28	Fitzroy Crossing	18°06'	125°42'	110		X
29	May River	17°25'	124°07'	45		X
E. tereticornis (30-46)						
30	Morehead River	15°02'	143°40'	50	X	X
31	Kennedy River	15°26'	144°10'	80	X	X
32	Laura River	15°33'	144°27'	100		X
33	Ruth Creek	15°43'	144°37'	130	X	
34	Helenvale	15°47'	145°13'	170		X
35	Palmer River	16°07'	144°47'	400	X	X
36	Holmes Creek	16°32'	145°07'	370	X	X
37	Leichhardt Creek	16°35'	145°12'	370	X	
38	Luster Creek	16°39'	145°14'	390	X	
39	Spear Creek	16°39'	145°19'	370	X	X
40	Walkamin	17°09'	145°26'	630	X	
41	"Springmount"	17°11'	145°20'	540	X	
42	Baldy Mt	17°16'	145°26'	930	X	
43	Stannary Hills	17°19'	145°13'	750	X	
44	Watsonville	17°22'	145°19'	790	X	
45	Archer Creek	17°38'	145°25'	80	X	
46	SW Mt Garnet	18°24'	144°45'	890		X

6.2.2 Experiment 2: Survey, northern Australia

The purpose of this experiment was to survey natural populations of *E. camaldulensis* and *E. tereticornis* in northern Australia at a broad scale. The survey set out to determine;

(1) geographic variations in yield (w/100g, fresh leaf) of the major oil consititutents, the monoterpenes - 1,8-cineole, α-pinene, β-pinene and limonene, within and between species, and

(2) provenances that might merit further investigation as cineole producers in addition to those already identified at Petford.

Collection of Plant Material and Extraction and Analysis of Volatile Oils

In January, April and June, 1987, leaf samples were collected from 32 natural populations of *E. camaldulensis* and *E. tereticornis* including several provenances considered to be intermediate in character to populations of authentic botanical classification (see Fig. 6.1 and Table 6.1). At each collecting site, about twelve mature leaves were taken from the crowns of each of five widely-spaced trees. They were placed in plastic bags, kept separate by individual trees, and held on ice to prevent sweating until the field parties returned to the laboratory, often a period of three to four weeks. In the laboratory, 100 ml of ethanol were weighed accurately into sample bottles, 5g of leaf were sampled to represent each provenance (approx. one leaf (1g) per individual tree) and placed in the same bottles. At least 2 weeks were allowed for full extraction (Ammon *et al.* 1985). Chemical analysis was by gas chromatography (see method 1 in Table 3.1).

6.2.3 Experiment 3: Assessment of progeny test, Zimbabwe

This experiment had goals similar to Experiment 2 but with two important differences. First, it was based on replicated progeny/provenance trials (c.f. natural stands) which allowed detailed statistical analysis of the data. Second, the provenance trials were planted in Zimbabwe on a site representative of the areas where the tropical red gums grow best and the trees were of a young age (3.75 years) reflecting more closely the conditions under which these species might be harvested for their oils.

Collection of Plant Material and Extraction and Analysis of Volatile Oils

This experiment involved the sub-sampling of 14 provenances of *E. camaldulensis* and 8 provenances of *E. tereticornis* from two extensive progeny/provenance trials of these species (total area of 15.3 ha) established in January 1985 at Mtao, in Zimbabwe. Mtao lat. 19°20'S, long. 31°29'E is about 170km south of Harare, on deep aeolian Kalahari sands at an elevation of 1480m and with 690mm mean annual rainfall (Matheson and Mullin 1987) concentrated in 5 months of the year, November to March.

The trials used seed collected in tropical Australia and local and other African sources as controls. They were established in randomised incomplete block experimental plantings with individual family row plots of 10 trees grouped by provenance in each block (S. Bleakley pers. comm.). Four of the *E. tereticornis* provenances were represented by bulked seedlots only. There were five replicates (blocks) in each trial although only two replicates (Blocks 1 and 2) were sampled in this study.

In October 1988, when the trees were 3.75 years from planting, leaves were collected in blocks 1 and 2 of each experiment from 2 trees of each of 3 families in each of 14

provenances of *E. camaldulensis* and 4 provenances of *E. tereticornis*. Families and trees were selected at random and provenances chosen to cover a broad geographic range. Four bulked *E. tereticornis* seedlots were also sampled and included in the analysis. The height and breast height diameter of each tree sampled was recorded. A total of 264 were sampled.

About a dozen mature leaves were collected from each tree at approximately two-thirds of tree height. They were placed in plastic bags and refrigerated for about 10 days prior to being airfreighted to Australia. From each tree 3g of leaf material was carefully weighed and placed in sample bottles containing 50 mls of ethanol.

Chemical analysis was by gas chromatography (see method 3 in Table 3.1). An internal standard, n-tetradecane, was added to each sample and quantification of 1,8-cineole, α-pinene, β-pinene, limonene and ρ-cymene was based on response coefficients determined for each of these compounds relative to the internal standard. A rough approximation of total yield of monoterpene compounds was obtained by assuming that other peaks in the early part of the chromatogram had a response coefficient of 1 relative to n-tetradecane and adding their mass to the total of the major monoterpenes.

Statistical Analysis

The frequency distribution of the oil yield data among trees was highly non-normal and strongly skewed towards the lower values. To cope with this non-normality, a general linear model using a logarithmic link function relating the mean of the given observation to its linear predictor (Baker and Nelder 1978) was employed. The model was $Y_{ijkl} = u + R_i + P_j + F_{jk} + W_{ijkl}$ where u is the overall mean, R_i is the effect of the ith replicate, P_j is the effect of the jth provenance, F_{jk} is the effect of the kth family in the jth provenance and W_{ijkl} is the random error associated with the lth tree in the kth family in the jth provenance and ith replicate. The statistical significance of each term was tested by comparing the decrease in the deviance associated with the inclusion of the term in the model with the appropriate F statistic.

6.2.4 Experiment 4: Survey, Petford

The objectives of Experiment 4 were:

(1) to determine the extent and distribution of variation in cineole content throughout the Emu Creek (Petford region) catchment; and

(2) to identify both high and low oil-yielding genotypes for inclusion in later experiments to determine genetic parameters.

Collection of Plant Material and Extraction and Analysis of Volatile Oils

In the January of 1988 and 1989 and again in May and June of 1989, leaves were collected from 370 individual trees over some 40 km along Emu Creek and its tributaries. This area includes populations commonly referred to as Petford or Irvinebank provenances due to their relative proximity to these townships.

At least two branches were removed from each tree and about equal numbers of mature leaves were plucked from each to make up a sample of about 12 leaves per tree. These were placed in plastic bags and refrigerated for varying periods up to a maximum of about

14 days prior to despatch to the laboratory. The laboratory methodology was identical to that described for Experiment 3.

6.3 Results and discussion

6.3.1 Experiment 1: Survey, northeastern Queensland

The summary of results in Table 6.2 indicated very minor qualitative differences in oil compounds found between the populations chosen to represent core *E. camaldulensis* and *E. tereticornis* and one reputedly of intermediate character in northern Queensland. Under more intensive sampling these minor differences may disappear altogether. There appears to be little scope, therefore, to use oil fingerprinting as a tool in elucidating taxonomic differences between these species throughout this region or as a basis for inferences about the possibility of introgression of *E. tereticornis* in the Petford *E. camaldulensis* population. Clearly quantitative variation in the major oil components is of greater interest. Although there is considerable overlap between species there is very substantial quantitative variation within and between provenances especially in yields of cineole.

This experiment shows that, amongst the Petford and Einasleigh River populations of *E. camaldulensis*, there are individual trees that give an excellent 1,8-cineole-rich oil. In addition, the crude extract from Petford may qualify directly as a medicinal oil as it is high in cineole, up to 84%, with good odour and negligible phellandrene. These trees yield reasonable quantities of oil, about 1-2% (fresh weight). It is important to note, however, that oil yields obtained under the cohobation conditions used in these analyses may be about one-third greater than those obtained under field conditions. Oil yields of this magnitude are below the threshold for economic production in Australia but may be acceptable when oil is harvested as a secondary product to wood in countries where labour costs are not as high as in Australia.

This experiment led to the recognition of a distinct chemotype in the population at Petford (see Table 6.2), characterised by low cineole content (about 10%) and high proportions of sesquiterpenoids. This chemical form is present in the population at a frequency of about 1 in 10. Assuming it is a genetically stable form, it has the potential to affect oil quality adversely. This cineole-poor form should be avoided in any selection program aimed at improving the quality of *E. camaldulensis* oils for medicinal purposes.

Most previous assessments of the oils of *E. camaldulensis* have labelled them of little or no value for pharmaceutical purposes due to low cineole and high α-phellandrene contents and low yields (e.g. Penfold and Willis 1961; Rao *et al.* 1970; Senanayake *et al.* 1983; Ndou *et al.* 1986). The identification in this study of cineole-rich populations of *E. camaldulensis* with commercial potential highlights the importance of chemical forms in this genus and the need for extensive sampling before categorising a whole species as to its oil type(s).

Table 6.2 Total yield of oil, the main components in the oil and the range in percentage of each amongst trees and between provenances of *E. camaldulensis* and *E. tereticornis* from northern Queensland, Australia. Compounds are listed in order of their increasing retention time on a polar glc column (FFAP). The oil analysis of the low-cineole, high-sesquiterpene chemotype amongst the Petford provenance is given for comparison with regular oil types of the same provenance.

Compound	Type*	*E. camaldulensis* Einasleigh River % in the oil	*E. camaldulensis* Petford % in the oil		*E. tereticornis* Walkamin % in the oil
			Regular	Chemotype	
α-pinene	m h	2.1-23.4	1-6-6.8	2.2-4.07	0.78-27.0
β-pinene	m h	0.0-13.0	0.05-6.4	1.72-18.7	0.04-18.0
α-phellandrene	m h	0.0-7.9	0.0-0.10	0.01-0.05	0.0-0.94
limonene	m h	2.0-10.1	3.6-12.6	1.72-2.27	3.5-18.7
1,8-cineole	m	19.3-84.0	38.4-83.8	4.7-15.63	0.11-32.9
γ-terpinene	m h	0.05-6.2	0.02-17.8	0.04-0.33	0.06-1.5
ρ-cymene	m h	0.39-33.8	0.13-5.7	0.03-0.51	0.40-5.7
terpinolene	m h	0.01-0.28	0.32-1.6	0.05-0.90	0.04-0.44
campholenic aldehyde	m al				0.0-1.9
pinocarvone	m k	0.0-0.76	0.0-0.04	0.0-1.71	0.0-0.22
terpinen-4-ol	m a	0.10-2.5	0.13-2.4	0.09-0.13	0.12-0.73
β-caryophyllene	s h	0.0-0.10	0.0-0.03	0.01-0.20	0.02-1.9
aromadendrene	s h	0.72-8.0	0.79-3.5	0.20-1.38	0.23-7.9
trans-isopinocarveol	m a	0.0-4.9	0.0-0.35		0.0-1.9
alloaromadendrene	s h	0.0-0.83	0.27-0.79	0.0-0.85	0.30-1.3
α-terpineol	m a	0.81-2.1	1.3-2.6	0.72-1.43	0.30-5.8
viridiflorene	s h	0.01-0.08	0.07-0.56	0.12-1.71	0.03-0.86
germacrene	s h			0.0-1.0	
bicycologermacrene	s h			0.40-14.3	0.0-16.9
myrtenol	m a	0.0-0.43			0.32-1.0
sabinol	m a	0.0-1.1			
globulol	s a	2.6-12.9	3.1-6.7	2.0-9.7	3.2-15.5
viridiflorol	s a	0.16-1.1	0.29-0.70	1.0-4.8	0.86-8.4
spathulenol	s a	0.0-0.21		0.0-25.93	0.0-12.9
γ-eudesmol	s a	0.0-1.3			0.0-8.6
α-eudesmol	s a	0.0-2.1			0.0-9.8
β-eudesmol	s a	0.0-3.0			0.0-14.6
Other C_{10} compounds in trace amounts	m	26	17	5	25
Other C_{15} compounds in trace amounts	s	18	15	34	20
Oil yield (g/100g of fresh leaf) Mean ±SD		1.07 ± 0.35	1.65 ±0.37		1.17 ±0.25

* m is monoterpene (C_{10}); s is sesquiterpene (C_{15}); a is alcohol; al is aldehyde; h is hydrocarbon; k is ketone

6.3.2 Experiment 2: Survey, northern Australia

The results of the analyses and quantification of α-pinene, β-pinene, limonene and 1,8-cineole in the bulk ethanol extracts of 18 *E. camaldulensis* and 14 *E. tereticornis* provenances are summarised in Figures 6.2 and 6.3. To assist in interpretation, the *E. camaldulensis* provenances are plotted in order of increasing longitude starting at the Drysdale River in the northwest of Western Australia and concluding at the headwaters of the Emu Creek system in Queensland. *E. tereticornis* is graphed in order of increasing latitude in Queensland from Morehead River in the north to Archer Creek, southwest of Ravenshoe.

The sampling method, using bulked rather than individual tree analysis, and the combining of results of different field trips in the absence of a detailed knowledge of the effects of sampling time on oil patterns, constrains the inferences that can be drawn from the data. However, a number of trends are apparent. There are substantial differences in oil contents between provenances within species. Greater oil contents are found in the eastern part of the occurrence of *E. camaldulensis* in northern Australia while provenances located towards the Gulf of Carpentaria, Katherine in the Northern Territory and provenances in northwestern Western Australia have little oil. Provenances with a cineole content of 1% or more of the weight of fresh leaf were centered on the Petford region, along Emu Creek (prov. 2 & 5) and a tributary, Gibbs Creek (prov. 3) and on the Walsh River at Flat Rock Pool (prov. 6). Only the Morehead River provenance of *E. tereticornis* (prov. 30) had a cineole content that was greater than 1% of the weight of fresh leaf.

These data at first suggested that the relationship between yields of cineole and β-pinene might assist in seeking taxonomic affinities of anomalous provenances. Subsequent work, however, showed great heterogeneity in oil composition even within core populations, thus negating the value of this relationship for elucidating taxonomic problems in the region.

This survey indicated that the rapidly-growing provenances of *E. camaldulensis* from the Petford region offered most scope as a source of commercial quantities of oil. There was still a need, however, to confirm this result using young progeny growing on a site representative of those where Petford provenance is utilised on a large scale. The experimental plantings in Zimbabwe were used for this purpose.

6.3.3 Experiment 3: Assessment of progeny test, Zimbabwe

An outstanding and consistent feature of the Zimbabwe data was the very substantial within and between family heterogeneity in yield of the major monoterpenes. This observation indicates that the populations are highly variable in terms of their leaf oils (Table 6.3a & b). Despite great within-provenance variability, analysis of variance showed highly significant differences between populations in all oil traits assessed in *E. camaldulensis* and significant differences for most of them, including cineole, in *E. tereticornis*. Provenance means for yields of cineole and total monoterpenes and the standard error of the differences are given in Figures 6.4 and 6.5.

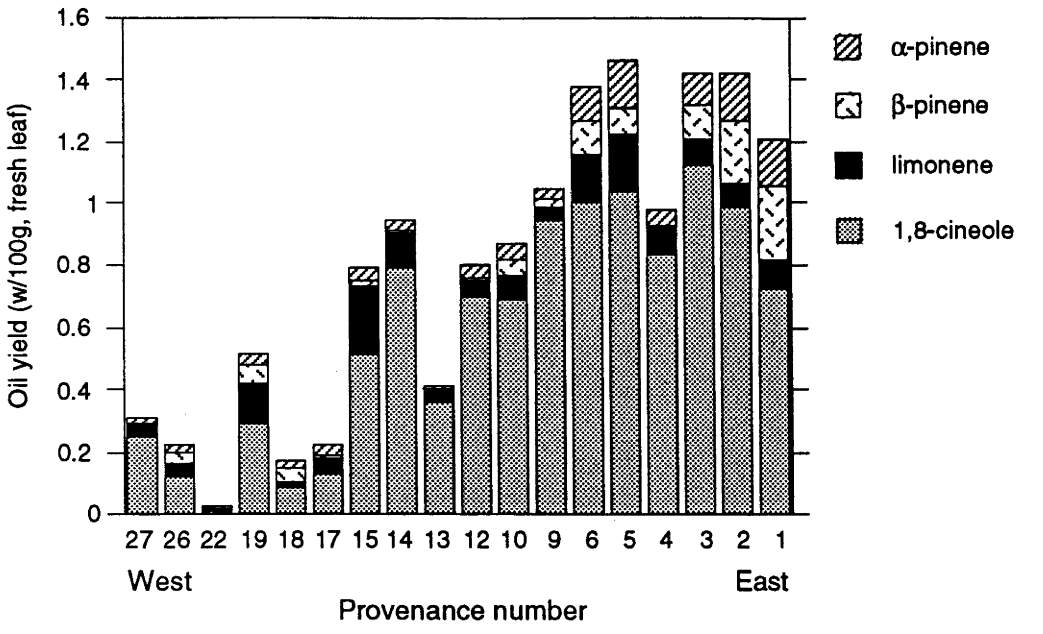


Figure 6.2 Yield of 1,8-cineole, α-pinene, β-pinene and limonene in 18 *E. camaldulensis* populations as sampled in the wild in northern Australia.

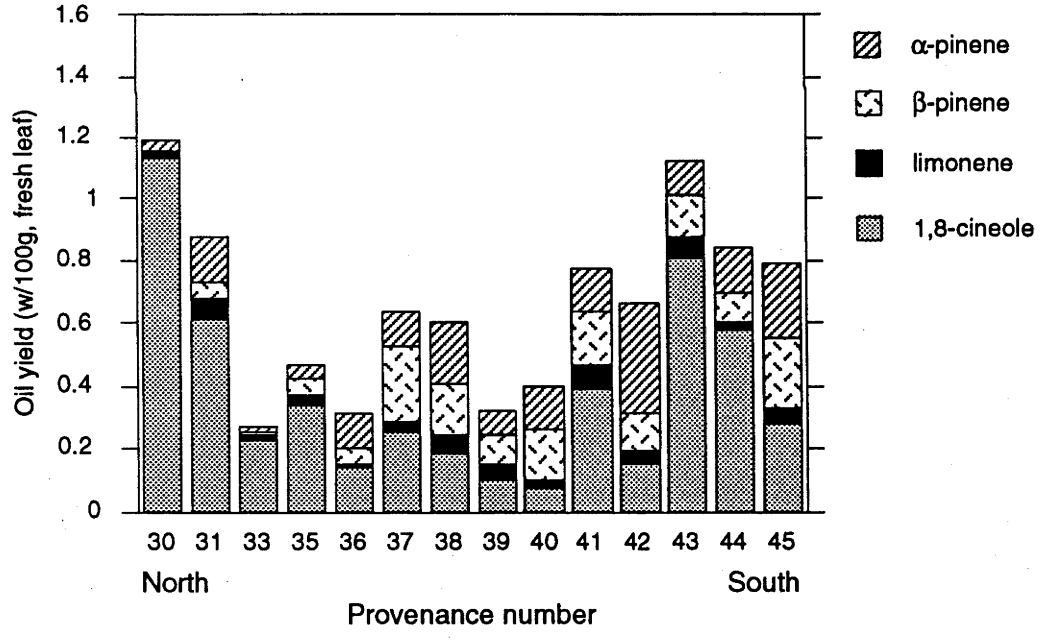


Figure 6.3 Yield of 1,8-cineole, α-pinene, β-pinene and limonene in 14 *E. tereticornis* populations as sampled in the wild in northern Queensland.

Table 6.3

(a) Change in deviance and significance levels for particular oil yields, w/100g of fresh leaf, assessed at 3.75 yrs in an open-pollinated progeny/provenance trial of *E. camaldulensis* planted in Zimbabwe.

Source	d.f.	1,8-cineole	α-pinene	β-pinene	limonene	ρ-cymene	Total mono-terpenes
Replicates	1	0.5^{ns}	0.00^{ns}	0.12**	0.09*	0.03^{ns}	0.18^{ns}
Provenances	13	3.80**	0.91**	0.38**	0.26**	0.90**	1.76**
Families	28	0.24*	0.06^{ns}	0.03**	0.06**	0.09**	0.18^{ns}
Residual	123	0.14	0.05	0.01	0.02	0.04	0.13

**P<0.01; *P<0.05; ns not significant

(b) Change in deviance and significance levels for particular oil yields, w/100g of fresh leaf, assessed at 3.75 yrs in an open-pollinated progeny/provenance trial of *E. tereticornis* planted in Zimbabwe.

Source	d.f.	1,8-cineole	α-pinene	β-pinene	limonene	ρ-cymene	Total mono-terpenes
Replicates	1	0.00^{ns}	0.36**	0.16^{ns}	0.05^{ns}	0.02^{ns}	0.16^{ns}
Provenances	7	1.98*	0.47*	0.65**	0.17^{ns}	0.19^{ns}	1.95*
Families	8	0.39**	0.13^{ns}	0.06^{ns}	0.05*	0.09^{ns}	0.39**
Residual	79	0.13	0.04	0.07	0.02	0.12	0.11

**P<0.01; *P<0.05; ns not significant

Analysis of variance showed that there were significant differences for both height and diameter amongst the *E. camaldulensis* provenances but in *E. tereticornis* only the height was significantly different. Provenance means for height are given in Figure 6.6.

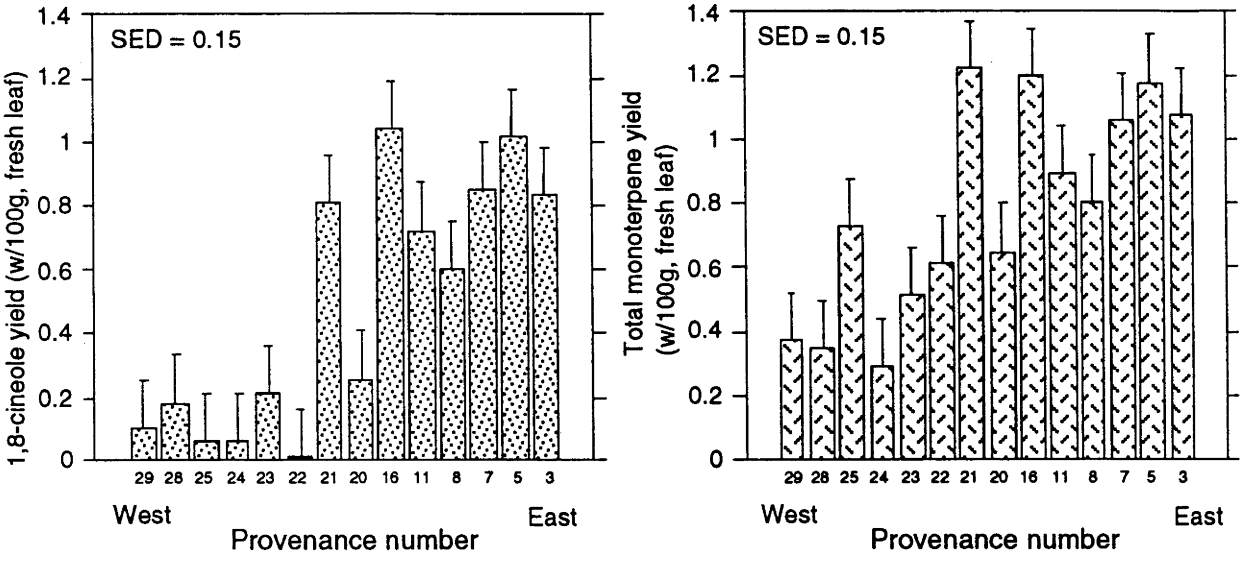


Figure 6.4 Average yield of 1,8-cineole (a) and estimate of total monoterpene content (b) of the leaf oils of 14 provenances of *E. camaldulensis* from northern Australia growing in a trial in Zimbabwe. Trees were 3.75 yr when sampled.

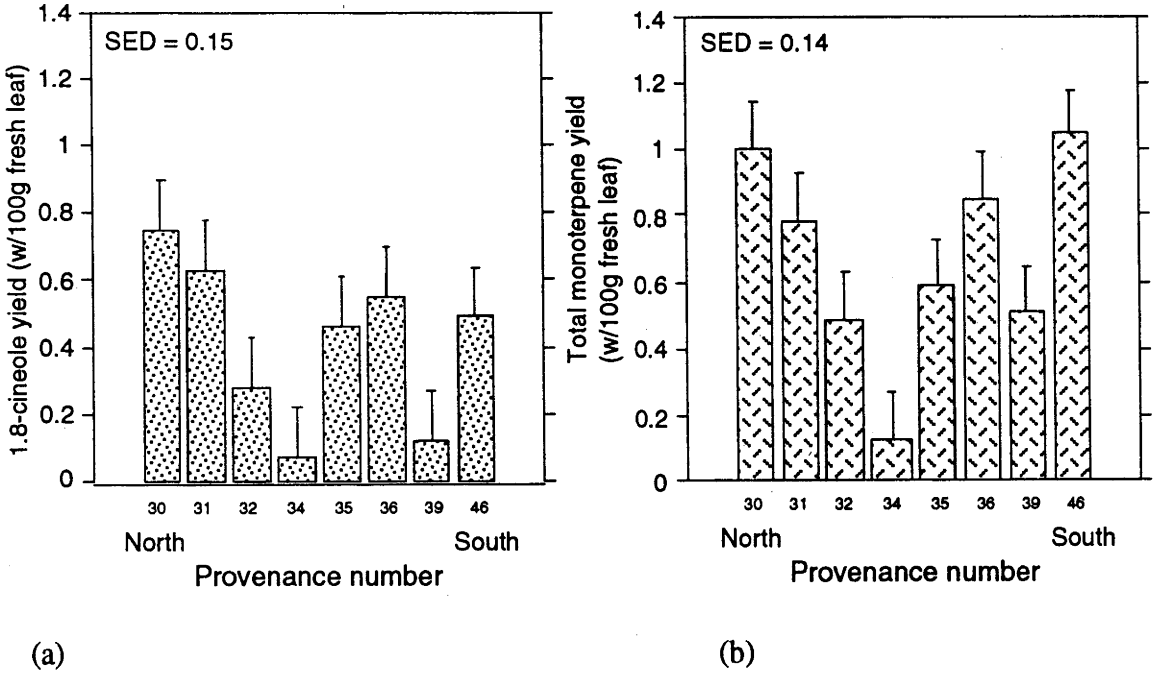


Figure 6.5 Average yield of 1,8-cineole (a) and estimate of total monoterpene content (b) of the leaf oils of 8 provenances of *E. tereticornis* from northern Queensland growing in a trial in Zimbabwe. Trees were 3.75 yr when sampled.

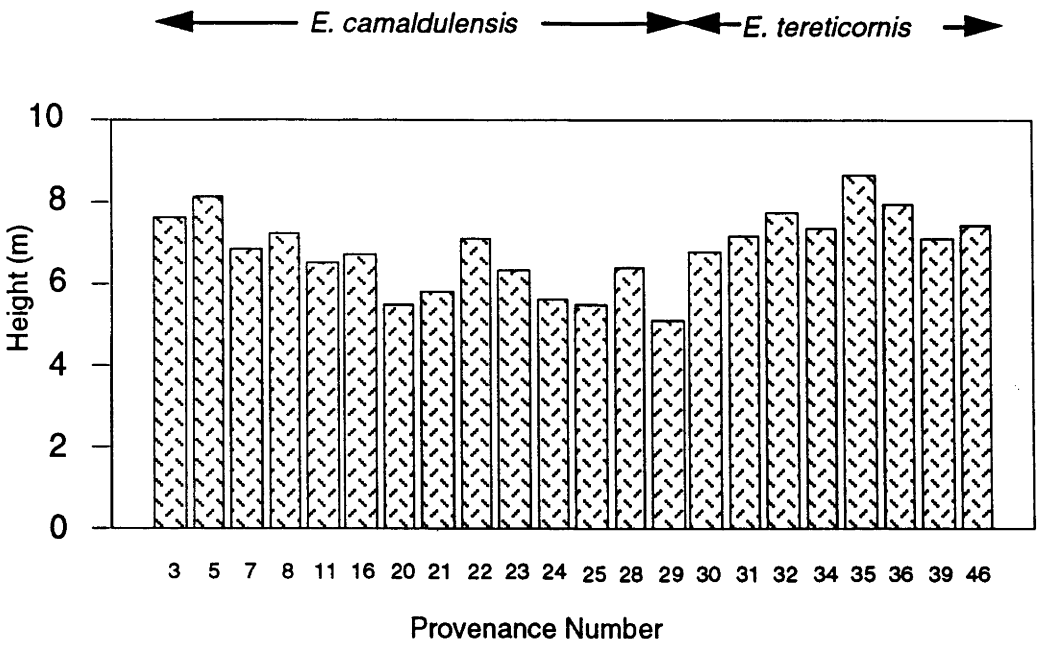


Figure 6.6 Variation in mean height (m) by provenance of trees sampled for oil analysis in progeny trials of *E. camaldulensis* and *E. tereticornis* in Zimbabwe aged 3.75 years.

Provenances giving the best combination of high growth rate and yields of 1,8-cineole that averaged greater than 1% of the weight of fresh leaf were Petford and Gilbert River Bridge. In addition, individual trees in a limited number of other provenances gave oil of a quality worthy of inclusion in a selection and breeding program should the tree or provenance in question grow well. Provenances showing potential in this category include Irvinebank and Wrotham Park *E. camaldulensis* and Morehead River and Kennedy River *E. tereticornis*. Within these higher-yielding provenances, no correlation was apparent between tree size and oil yield (w/100g, fresh leaf). Thus there may be an opportunity to increase per hectare production by selecting and propagating provenances and individuals with both fast growth and high yield of oil.

As in Experiment 2, the easterly sources of *E. camaldulensis* and the northerly sources of *E. tereticornis* reveal most potential for utilisation and improvement of their oils. Provenances from the Gulf of Carpentaria (Normanton), Northern Territory and the northwest of Western Australia are generally low in overall yields. They also usually contain undesirable amounts of α-phellandrene, although this was not quantified in this study.

The similarity of results in Experiments 2 and 3 is notable given the contrast between the trees sampled (aged, slow-growing versus young, fast-growing) and their growing locality (natural versus exotic). The reproducibility of the results indicates that natural populations might be reliably screened for their oil potential in fast-growing plantations and, as mature oil patterns appear to be exhibited at a young age (3.75 years or earlier), individual tree selection for superior oil characteristics might take place at an equally early age. This latter point is in agreement with the findings of Barton *et al.* (1991) who concluded that, for the mallee *E. kochii*, selection for oil production can be reliably carried out at three years of age.

6.3.4 Experiment 4: Survey, Petford

Oil composition varies considerably amongst the 370 trees screened in this experiment. The average yield (w/100g, fresh leaf) of cineole was 0.87% (S.D. ± 0.38%) with a range of 0 to 1.8%, and the average total yield of the major monoterpenes was 1.04% (S.D. ± 0.42%). Thirty-four trees, 9% of the sample, were of the low-cineole, high-sesquiterpene chemotype described in Experiment 1. In one instance, seven trees of the sesquiterpene chemotype were grouped in the one small area suggesting that this may be a spatial clumping of relatives and that this distinctive oil type may be strongly inherited.

The 20 best trees for oil thus far identified (selection frequency of 1 in 19) contain an average of 85% cineole and yield (w/w), on average, 1.81% (S.D. ± 0.19) total monoterpenes and 1.57% (S.D. ± 0.12) cineole on a fresh weight basis. The best tree produces more than twice the yield of cineole of the average for the population and this differential is likely to broaden as more trees are screened. It is clear that the selected genotypes provide oil not only in increased quantity relative to 'average' trees but also of enhanced quality. If these gains can be captured by selection and breeding amongst elite trees the economics of oil production from the tropical red gums would be significantly improved.

6.4 Conclusions

There is very substantial inter-and intra-specific quantitative variation in yield of oil from *E. camaldulensis* and *E. tereticornis* in northern Australia. Qualitative differences are also present, as for example the frequency of high levels of α-phellandrene in some populations and its relative absence from others. However, the significance of these differences was judged to be minor in the context of this paper and they were not pursued beyond the first experiment. There were no clear associations between the amounts of the major monoterpenes and taxonomic affinities.

Oil yields of even the best populations (1-2%) are generally below the economic threshold for viable oil production in Australia. However, these levels should be adequate to support production in countries where labour is less costly and where oil production could be a by-product of wood harvesting. The economics of oil production from the red gums will be enhanced by the large quantities of leaf generated and by the need to thin the prolific coppice that follows clearfalling in coppice rotations of these species.

Provenances of *E. camaldulensis* from the Petford region combine rapid growth and desirable oil characteristics and offer the best scope for production of 1,8-cineole-rich oil in countries in the seasonally-dry tropics where this species is grown. Other widely-planted provenances with promise for cineole production in the tropics include *E. camaldulensis* from Gilbert River Bridge and, after some genetic improvement, *E. tereticornis* from Morehead and Kennedy Rivers. There appears to be a strong relationship between average oil yields in natural populations and fast growing progeny of the same sources established in plantation in Zimbabwe. This stability augers well for the validity of combining results from Australia and elsewhere in further similar work.

Individual trees amongst promising provenances produce significantly more oil of higher quality than the average trees of the population. Therefore, significant improvement in

the economics of oil production from the tropical red gums by means of selection and breeding for these traits seems possible as oil characters in *Eucalyptus* appear to be strongly inherited (Pryor and Bryant 1958; Barton *et al.* 1991).

The identification of cineole-rich variants in *E. camaldulensis* contrasts with earlier reports of this species having no value as a source of medicinal-grade oil and highlights the importance of intra-specific genetic variation in oil content in this genus. The need for extensive sampling before categorising a species as to its oil type(s) is indicated.

CHAPTER 7. GENETIC PARAMETERS AND EXPECTED GAINS FROM SELECTION FOR MONOTERPENE YIELDS IN PETFORD *EUCALYPTUS CAMALDULENSIS*

7.1 Introduction

Some individual trees amongst the Petford provenances have produced oil of enhanced quality at about double the yield of 'average' trees. Hence, significant improvement in oil production by means of selection and breeding for these traits seems possible. However, there is little appropriate genetic information available to assist us to gauge the costs and benefits of such a program. Oil production per unit land area depends on the amount of leaf produced and the leaf oil content but there is no information published on genetic associations between oil yields and growth traits in eucalypts.

This study was based on open-pollinated (Exp. 1) and control-pollinated (Exp. 2) progeny tests of Petford *E. camaldulensis*. It provides estimates of genetic parameters for yield of 1,8-cineole, α-pinene, β-pinene, limonene, ρ-cymene and their total from leaves. These estimates are of heritability, additive and dominance genetic effects, genetic and phenotypic correlations, genotype x environment interactions and modes of inheritance. Expected gains per generation following individual selection for yield of 1,8-cineole and total monoterpenes were estimated.

7.2 Experiments, objectives and methods

7.2.1 Experiment 1: Estimate of genetic parameters and gains in open-pollinated progeny tests

This experiment set out to determine the genetic parameters for the Petford population using extensive provenance/progeny trials planted in Zimbabwe as the basis for estimations. One of these trials, at Mtao, was also used to study provenance variation in Experiment 3, Chapter 6 where site details are given. Mtao is representative of regions in the seasonally-dry tropics where tropical provenances of *E. camaldulensis* grow well and the 3.75-year-old trees reflected the conditions under which they might be harvested for their oils. The other trial, at Forest Hill, is in a harsher environment for tree growth than Mtao, with less than 650 mm annual rainfall. Death of tree leaders is prevalent in the dry season and boron deficiency has been confirmed as a contributing factor (S. van der Lingen pers. comm.). Both sites have a similar soil of deep, granite-derived sands, known locally as Kalahari sands.

7.2.1.1 Genetic material and field design

The main experiment involved sampling 19 open-pollinated families from the Petford region of northern Queensland established at Mtao. The Mtao trial, planted in January 1985, used seed collected from natural populations in tropical Australia in 1983/84, as well as local and other African sources as controls, a total of 29 seed origins. It was established as 5 randomised blocks with individual family row plots of 10 trees grouped by provenance in each replication. In the trial design the Irvinebank- Emuford (9 families) provenance was treated as separate from Petford (10 families) but, in the sampling for oil analysis in this experiment, Irvinebank- Emuford was regarded as

synonymous with Petford. It was established earlier that variation in oil traits in the natural population was continuous throughout the geographic zone represented by these collections (see Chapter 6).

Sampling of the same 19 families in the second trial established in February 1985 at Forest Hill (lat. 19°20'S, long. 28°00'E) meant that comparable estimates of heritabilities for oil yields could be obtained for another site and the possibility of genotype x environment interactions in oil traits assessed. Forest Hill was established using excess stock from the main planting at Mtao. The basic design is the same as at Mtao but is unbalanced as few plants were available of some families. Of the 19 Petford families involved in this experiment, 1 family was represented in one block only, 1 in two blocks, 9 in three blocks and 8 in four blocks. Because of the poor representation of some families, estimation of phenotypic and genetic correlations was confined to the Mtao data.

7.2.1.2 Collection of plant material and tree measurements

In October 1988, 3.75 years after planting of the field trials at Mtao and Forest Hill, leaves were collected from individual trees representing each of the 19 Petford families planted at each site. For each family in each plot, three trees were selected at random. About a dozen mature leaves were collected from each tree from two branches removed at approximately two-thirds of tree height using a long-handled pruner. 281 foliage samples (4 missing trees) were obtained at Mtao and 186 (42 missing trees) at Forest Hill, placed in plastic bags and refrigerated in Zimbabwe for up to 10 days prior to being airfreighted to Australia.

To allow the estimation of genetic and phenotypic correlations amongst oil and growth traits, all trees sampled for oil analysis at Mtao were measured for : diameter at breast height over bark (dbhob); total height; height to the lowest green limb; radius of crown at its widest point; the shorter crown radius at 90° to the first. The crown measurements were used to calculate notional crown surface area using the formula for surface area of a cone. Trees were also assessed on a three-point scale for crown density (3 = dense, 2 = medium, 1 = sparse foliage). Figure 7.1 shows one of the Petford plots at Mtao and staff of the Zimbabwe Forestry Commission taking crown measurements.

7.2.1.3 Extraction and analysis of volatile oils

Ethanol extracts were prepared under quarantine in Canberra. Chemical analysis and quantification of the major monoterpenes in these extracts used standard methods (see method 3 in Table 3.1). An internal standard, n-tetradecane was added to each sample and 1,8-cineole, α-pinene, β-pinene, limonene and ρ-cymene were quantified using response coefficients determined for each of these compounds relative to the internal standard. Total yield of monoterpene compounds was approximated by assuming that three other relatively minor peaks, representing three unidentified compounds, in the early part of some chromatograms had a response coefficient of 1 relative to n-tetradecane and adding their mass to the total of the five major compounds. All oil yield figures presented in this study are expressed as the weight of oil per 100g of dry leaf (w/100g, dry leaf).

(a) (b)

Figure 7.1 Trees of Petford *E. camaldulensis* used in the oil study in Zimbabwe, (a) plots at Mtao aged 3.75 years; (b) crown measurements in progress at Mtao.

7.2.1.4 Statistical analysis

Variance components and their standard errors for replicates, treatments (families ignoring provenance) and family x block interaction at Mtao were derived by restricted maximum likelihood using the REML computer package (Robinson 1987), assuming all effects were random. This model also gave best linear unbiased predictors of family means. Residual variance in the REML model was interpreted as within-plot variance. The layout of families in provenance blocks was unfortunate, as environmental differences between provenance blocks are confounded with provenance differences. I believe such environmental differences were small as the differences between replicates for all attributes at Mtao were not appreciable.

In contrast to the regular, monoterpene-dominated chemotype, several families contained individuals representative of a distinctive chemotype dominated by sesquiterpenes. This chemotype is characterised by low amounts and proportions of the monoterpenes in relation to the dominant sesquiterpene compounds present in their oils (see Chapter 6 for a more complete description). Data for these individuals were excluded from the data sets in all statistical analyses because they violate the assumption of normality, affect the calculated variances and lead to unreasonably high estimates of heritability ($h^2_i>1$)for yield of 1,8-cineole and total monoterpenes.

Where open-pollinated families can be assumed to be truly half-sib families, the coefficient of relationship is 1/4. This cannot be assumed for eucalypts because of a likely significant level of inbreeding (Brown *et al.* 1975). For the purposes of estimating heritabilities in this study an outcrossing rate of 70% was assumed corresponding to an average coefficient of relatedness among open-pollinated progeny of r = 1/2.5 (Griffin and Cotterill 1988). Individual-tree heritabilities (denoted h^2_i) for each trait were estimated as -

$$h^2_i = \frac{2.5\,\sigma^2_f}{\sigma^2_f + \sigma^2_{fb} + \sigma^2_w} \qquad (1)$$

where σ^2_f, σ^2_{fb} and σ^2_w represent the family, family x block interaction and within plot variance components, respectively. The approximate standard errors of individual heritabilities were estimated according to Wright (1976). Genetic correlations (r_g) were calculated from estimates of additive genetic variances and covariances following Hazel *et al.* (1943) and the standard errors of these correlations were estimated according to Tallis (1959). No assumption regarding the coefficient of relationship is required for estimating genetic correlations. Phenotypic correlations (r_p) were estimated as simple correlation coefficients.

Genetic gains (denoted Δ G) per generation expected from mass selection to improve yields of 1,8-cineole and total monoterpenes were determined as -

$$\Delta G = i\, h^2_i \sigma_p \qquad (2)$$

where i is the standardised selection differential and σ_p the phenotypic standard deviation. In this study we assume that the age:age correlation is 1.0 and that selection is at an intensity of one parent in every 10 (giving i = 1.54 for a finite population size).

It should be noted that any estimate of heritability is specific to a particular genetic sample and environment. This caveat has a bearing on the planning and forecast outcome of any selection process.

7.2.2 Experiment 2: Estimation of heritabilities, additive and dominance genetic effects and modes of genetic control of foliar monoterpenes in a full-sib progeny test

7.2.2.1 Genetic material and field design

Methods of controlled pollination in eucalypts have been outlined by Hodgson (1976) and others but not specifically for *E. camaldulensis*. The procedures adopted in this project for producing control-pollinated crosses are therefore described in some detail.

Controlled pollinations

In the Emu Creek catchment in June 1988, 110 trees of known 1,8-cineole-yielding capacity were assessed for the presence of mature floral bud crops. Major limitations on tree selection were imposed by the stage of flowering (most trees had not started to flower and would have required a further month to do so) and the difficulties of vehicular and

crown access to large adult trees growing in the river beds. The objective adopted, partly because of these constraints, was to undertake crosses amongst parents spanning a range of oil types including high to low cineole parents and the distinctive sesquiterpene chemotype (assortative mating). The oil-yielding characteristics of the trees used as male or female parents or both are given in Table 7.1.

Pollen collection commenced in late June and took three weeks. Branches carrying buds and flowers were collected from pollen parents in the field. These branches were placed upright in buckets of water, all flowers stripped off and discarded and their foliage covered by plastic bags. Each day the foliage was uncovered and the buds about to lose their operculum were collected. Opercula were removed and the flowers placed on paper tissues over silica gel in glass jars with tight-fitting lids (about 20 flowers per jar). The flowers were dried at room temperature overnight, individually lifted by tweezers to the mouth of a glass vial and tapped vigorously to promote release of pollen. The labelled vials containing almost pure pollen were tightly sealed and placed in a deep freeze (-15°C) until required for pollination.

Each pollen sample was tested for viability before use by immersion of a small subsample (equal to about a pin head) in a sealed glass vial containing 4 drops of sucrose mix (30% sucrose + 1ppm boron in distilled water) and placing the vial in a germination cabinet at 30°C. After 24h the vials were removed, a small droplet placed under the microscope and an estimate made of the percentage of pollen that had produced a pollen tube.

Table 7.1 Yields of the major monoterpenes in the oils of the Petford *E. camaldulensis* parent trees used in the crossing program. Trees were classified as high (H), average (A) and low (L) cineole types based on the average of their cineole contents over three separate evaluations.

		Yield of compound (w/100g, dry leaf)				
Cineole yield class	Tree no	α-pinene	β-pinene	limonene	1,8-cineole	Total monoterpenes
H	82	0.11	0	0.27	3.30	3.68
H	58	0.07	0	0.10	3.17	3.34
H	101	0.10	0	0.13	2.98	3.21
H	I8	0.14	0.10	0.06	2.81	3.11
A	67	0.53	0.72	0.20	2.41	3.86
A	88	0.11	0	0.11	2.25	2.47
A	48	0.05	0	0.19	2.22	2.46
A	30	0.16	0.07	0.05	2.08	2.36
A	49	0.04	0	0.04	1.60	1.68
L	87	0.32	0.10	0.11	0.92	1.45
L	96	0.10	0.01	0.01	0.76	0.88
L	10*	0.18	0.80	0.20	0.54	1.72
L	51*	0.07	0.33	0.01	0.32	0.73

* low-cineole-high-sesquiterpene chemotype

Flower emasculation with curved scalpel, and bagging using woven terylene pollination bags, commenced in July 1988. The aim was to emasculate about 50 flowers per bag and to have two bags per cross on each tree located on different branches as an insurance against physical damage. Despite this strategy, losses due to wind and other sources of damage were high. About a week after bagging the first pollen was applied to the styles by very fine paint brushes (one brush per pollen source). The bagging, pollination and debagging schedule is given in Table 7.2 while key features of the process are illustrated in Figure 7.2. Mature capsules were collected during 18 November to 18 December 1988, some 4.5 - 5 months after pollination.

Table 7.2 The schedule of operations from emasculation and bagging through to de-bagging for the crossing program of *E. camaldulensis* at Petford during 1988. The crossing pattern is given in Table 7.8.

Mother tree no.	Schedule of operations Date (month and day) July 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31	Aug 1 2	Success+
10	B . . . . P . . P . . P . . P . . P	DB	14
30	B . . . . . P . . P . . P . . P	DB	14
82	B . . . . . P . . P	DB	29
87	B . . . . . P . P . . . P	DB	6*
88	B . . . . . P . . P . . P . . P	DB	86
58	B . . . . P . . . P . . P	DB	14
49	B . . . . . P . . . P . . . P	DB	36
48	B . . . . P . . P . . . P	DB	83
101	B . . P . . P . . . P	DB	67

+ % of bags giving one or more capsules at collection in Nov-Dec.
* tree was vandalised
B is emasculation and bagging, P is pollination, DB is de-bagging

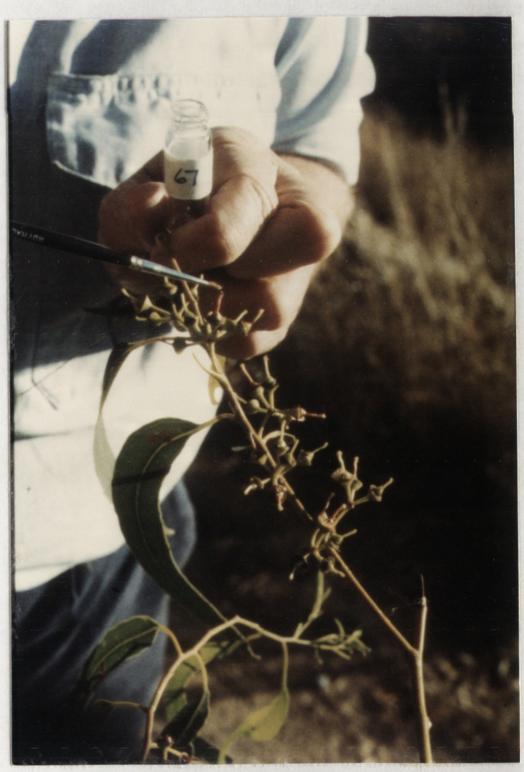
Field trial establishment

During February 1989, the control-pollinated seedlots (37) and open-pollinated seedlots (some representing different years of collection) of each of the parents in the crossing program (17) were propagated in Hawaiian dibbling tubes at the Queensland Forest Service nursery facility at Gympie in southeastern Queensland. On 5 May 1989, a progeny trial was established at Wongi near Gympie using an incomplete block design with 7 replications. Each replicate contained 9 incomplete blocks of 6 treatments in 4-tree line plots at a square spacing of 2.0m. 150g of a complete fertilizer was applied to each tree in two applications and competing weeds and grasses controlled by weedicide at regular intervals during the first year.

(a)

(b)

(c)



(d)

Figure 7.2 Steps in the control-pollinating of *E. camaldulensis*, (a) buds at the correct stage for emasculation; (b) emasculating with curved scalpel; (c) bagging emasculated flowers; (d) applying pollen with fine paint brush.

(a) (b)

Figure 7.3 The *E. camaldulensis* control-pollinated progeny test near Gympie at 12 months from planting, (a) collecting leaves for oil analysis; (b) heighting progenies.

7.2.2.2 Collection of plant material, tree measurements and analysis of volatile oils

Twelve months from planting, trees in replicates 1 to 5 were measured for height growth and sampled for their oil yields by standard methods (see Fig. 7.3). Quantification of α-pinene, β-pinene, limonene and 1,8-cineole was by GLC method 5 in Table 3.1. ρ-cymene was not prominent in the oils of any of the progenies so was not included in the analysis.

7.2.2.3 Statistical analysis

Best Linear Unbiased Estimates of offspring means at 12-months for these traits were obtained using the REML computer package to fit a model in which families were treated as fixed. Mid-parent yields were estimated by averaging the mean yields for each parent involved in a particular cross. In all there were 36 mid-parent - offspring combinations including two reciprocals and three selfs. There were no significant differences among reciprocals for the variables studied. Thus, reciprocal effects were assumed to be unimportant and ignored in further analysis.

Individual tree heritabilities (h^2_i) for each trait were first estimated by mid-parent - offspring regression as -

$$h^2_i = b_{op} \quad (3)$$

where b_{op} is the regression coefficient of offspring means on mid-parent values. Heritabilities can also be estimated from parent - offspring correlation but the regression approach is more appropriate where an assortative crossing pattern has been applied (Falconer 1981).

Recognising that estimates of additive genetic variance (V_A) for 1,8-cineole may be exaggerated because of assortative mating, sib-analysis was also applied assuming the general model -

$$Y_{ijk} = u + g_i + g_j + s_{ij} + e_{ijk} \quad (4)$$

where Y_{ijk} is the yield of the kth plot of the ijth cross; u is the population mean; g_i is the gca effect of the ith parent; g_j is the gca effect of the jth parent; s_{ij} is the sca effect of the cross between ith and jth parents such that $s_{ij} = s_{ji}$; e_{ijk} is the remainder of environmental and genetic effects between individuals in the same cross. REML analysis showed incomplete block and replicate effects were negligible, so they were omitted from this model.

The variance components associated with general combining ability (σ^2_{gca}), specific combining ability (σ^2_{sca}) and environmental effects (σ^2_e) were estimated using DIALL (Schaffer and Usanis 1969) and were equated to their genetic expectations (Hallauer and Miranda 1981).

$$\sigma^2 gca = \text{cov HS} = 1/4V_A + 1/16V_{AA}{}^{+}... \quad (5)$$
$$\sigma^2 sca = \text{cov FS} - 2\,\text{cov HS} = 1/4V_D + 1/8V_{AA}{}^{+}... \quad (6)$$

where cov HS = covariance of half-sibs; cov FS = covariance of full-sibs; V_A = additive genetic variance; V_{AA} = additive x additive epistatic genetic variance; V_D = dominance genetic variance. Assuming epistatic variance is negligible, then $4\sigma^2_{gca} = V_A$ and $4\sigma^2_{sca} = V_D$. General combining ability effects (gca) and specific combining ability effects (sca) were estimated following Griffing (1956). Individual heritabilities were calculated using the formula -

$$h^2_i = \frac{4\sigma^2_{gca}}{4\sigma^2_{gca} + 4\sigma^2_{sca} + \sigma^2_e} \quad (7)$$

where σ^2_e is the residual variance in the DIALL analysis.

The population to which these estimates apply is the Petford provenance, while the environment is that within the boundaries of the experimental site at Gympie.

7.3 Results

7.3.1 Experiment 1

The variance components and their standard errors from the REML analyses are given in Table 7.3. Highly significant differences ($p<0.001$) between families were found for yields of all 5 compounds and their total at both Mtao and Forest Hill. Figures 7.1 and 7.2 illustrate the variation in 1,8-cineole yield (unadjusted values) within and between families at Mtao and Forest Hill and give the number of sesquiterpene chemotypes occurring in each family. These figures and Table 7.4 indicate that family rankings for 1,8-cineole yield vary appreciable between sites. Average oil yields and height and diameter growth were markedly less at Forest Hill (Table 7.4).

Individual heritabilities and their standard deviations for 1,8-cineole and total monoterpene yield at 3.75 years at Mtao and Forest Hill are given in Table 7.5 (equation (1)).

Table 7.3 Variance components and their standard errors from analysis by restricted maximum likelihood for yields (w/100g, dry leaf) of essential oil traits assessed at 3.75 yrs for open-pollinated progeny of 19 families of Petford *E. camaldulensis* growing at Mtao and Forest Hill, Zimbabwe.

Source of variation	No. of observations	Variance components and standard errors ( ) total monoterpenes	1,8-cineole	limonene
Mtao:				
Replicates (R)	5	0 (0.004)	0.001 (0.004)	0 (0)
Families (F)	19	0.084 (0.036)	0.065 (0.028)	0.002 (0.001)
F x R	69	0 (0.022)	0 (0.17)	0.002 (0.001)
Within-plot	172[1]	0.304 (0.033)	0.243 (0.026)	0.007 (0.001)
Forest Hill:				
Replicates	4	0.016 (0.017)	0.007 (0.009)	0 (0)
Families	19	0.036 (0.021)	0.046 (0.021)	0.001 (0)
F x R	41	0.017 (0.018)	0.002 (0.013)	0 (0)
Within-plot	116[2]	0.163 (0.021)	0.139 (0.018)	0.003 (0)

Source of variation	No. of observations	Variance components and standard errors ( ) α-pinene	β-pinene	ρ-cymene
Mtao:				
Replicates	5	0 (0.001)	0 (0)	0 (0)
Families	19	0.019 (0.007)	0.003 (0.001)	0.001 (0.001)
F x R	69	0 (0.004)	0 (0.001)	0.001 (0)
Within-plot	172[1]	0.050 (0.005)	0.009 (0.001)	0.002 (0)
Forest Hill:				
Replicates	4	0.002 (0.002)	0 (0)	0 (0)
Families	19	0.006 (0.003)	0.003 (0.001)	0.002 (0.001)
F x R	41	0 (0.002)	0 (0)	0 (0)
Within-plot	116[2]	0.019 (0.003)	0.004 (0.001)	0.002 (0)

[1] 19 sesquiterpene chemotypes excluded and 4 missing trees

[2] 9 sesquiterpene chemotypes excluded and 42 missing trees

Table 7.4 Best Linear Unbiased Predictors for yields of 1,8-cineole and total monoterpenes in the leaves and tree height and diameter by open-pollinated family of Petford origin in 3.75-year-old *E. camaldulensis* progeny/ provenance trials at Mtao and Forest Hill, Zimbabwe.

	MTAO Yield (w/100g, dry leaf)				FOREST HILL Yield (w/100g, dry leaf)			
FAMILY	1,8-cineole	total monoterpenes	Ht (m)	Dia (cm)	1,8-cineole	total monoterpenes	Ht (m)	Dia (cm)
10716	2.17	2.56	7.7	7.0	1.55	1.74	5.8	4.9
10719	2.11	2.60	7.3	7.4	1.51	1.77	5.5	5.1
10722	1.89	2.32	8.2	7.0	1.68	1.91	6.1	5.6
10725	1.72	2.31	7.0	7.1	1.45	1.80	5.4	5.1
10728	1.74	2.02	7.5	7.2	1.48	1.74	5.5	4.9
10729	1.48	1.88	7.3	7.0	1.23	1.55	5.5	4.9
10731	1.44	1.78	7.8	7.5	1.42	1.66	5.0	5.4
10734	1.55	1.96	8.2	8.0	1.51	1.78	5.0	5.4
10737	1.56	1.92	8.0	7.5	1.37	1.67	6.1	5.5
10740	1.70	2.01	7.9	7.8	1.22	1.47	6.1	5.7
10769	1.90	2.42	7.3	7.5	1.64	1.91	5.4	4.9
10770	1.66	2.26	7.8	7.9	1.34	1.85	5.9	5.5
10771	1.83	2.25	7.7	7.4	1.58	1.88	4.7	4.3
10772	1.87	2.47	7.4	7.4	1.45	1.92	5.3	5.1
10773	1.96	2.50	7.2	7.1	1.56	1.94	4.8	4.4
10774	2.01	2.51	7.8	7.6	1.71	1.99	5.3	4.7
10775	1.60	2.47	6.1	6.8	1.18	1.76	4.8	4.2
10776	1.63	2.12	7.5	7.8	1.46	1.72	4.9	4.6
10777	1.42	2.09	7.5	7.1	0.98	1.39	5.4	4.4
Mean	**1.75**	**2.23**	**7.5**	**7.4**	**1.44**	**1.76**	**5.5**	**5.0**

Table 7.5 Heritabilities and their standard errors ( ) for total monoterpene yield and yield of 1,8-cineole (w/100g, dry leaf) in the leaf oils of Petford *E. camaldulensis* in progeny trials at Mtao and Forest Hill, Zimbabwe at 3.75 years.

Trait	h^2_i (SE)	
	MTAO	FOREST HILL
Total monoterpenes	0.54 (0.06)	0.42 (0.06)
1,8-cineole	0.53 (0.06)	0.61 (0.07)

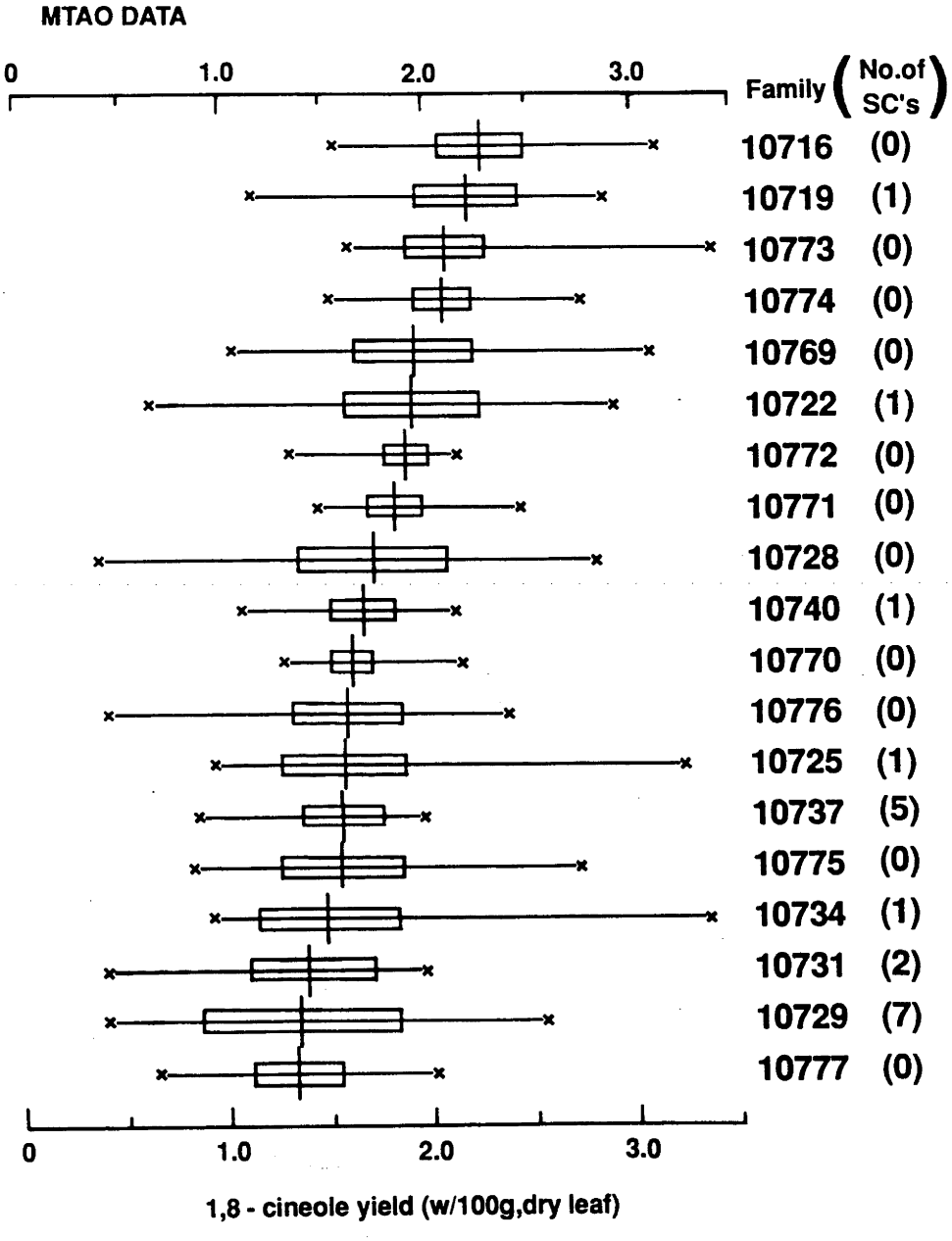


Figure 7.4 Variation in yield of 1,8-cineole within and between open-pollinated families of Petford *E. camaldulensis* at 3.75 years at Mtao, Zimbabwe. The unadjusted mean (vertical bar), 95% confidence interval (the box) and range (x) of regular monoterpene-dominated chemotypes amongst the mostly 15 trees sampled in each of 19 families is given. The number of sesquiterpene chemotypes (SC's) in each family omitted from these and other calculations is also given.

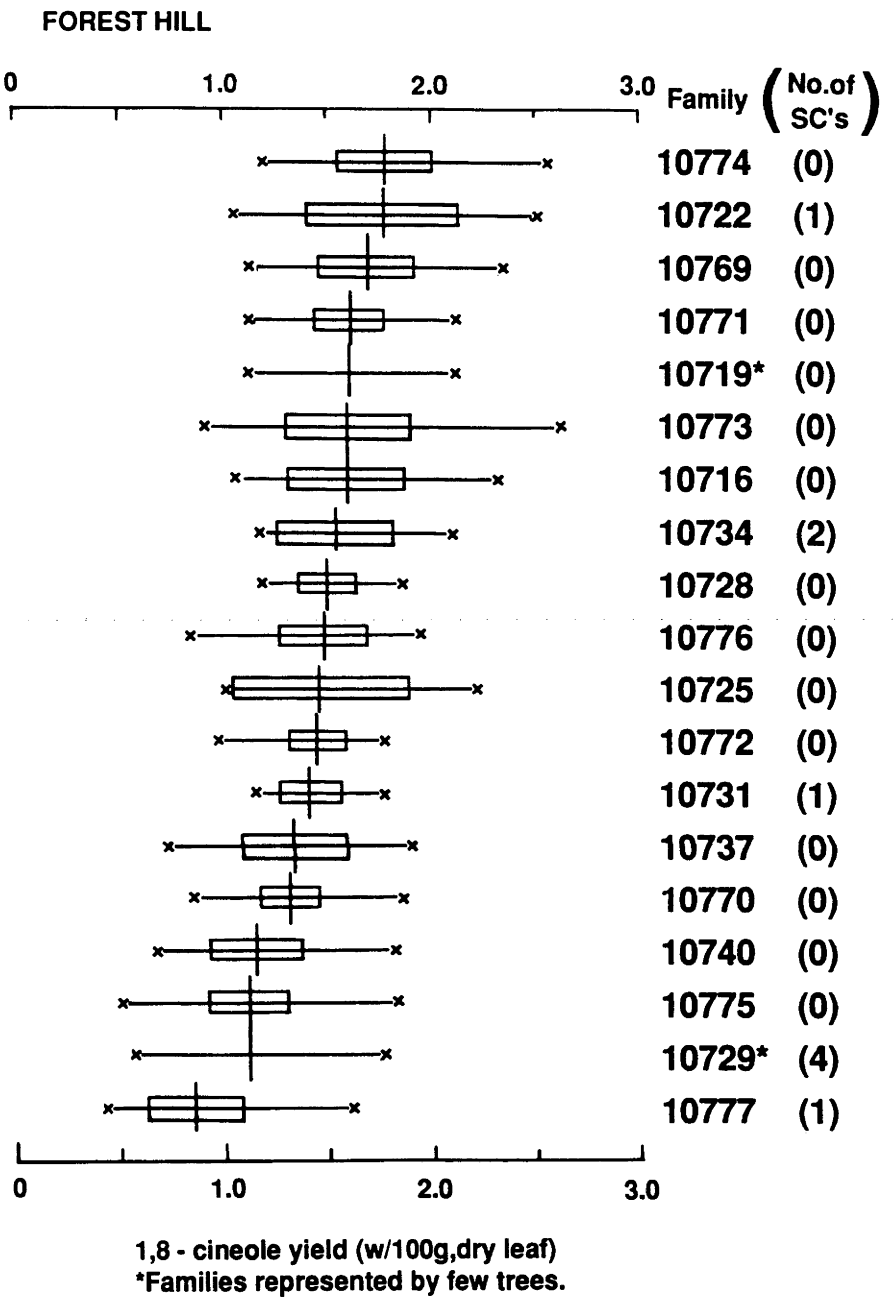


Figure 7.5 Variation in yield of 1,8-cineole within and between open-pollinated familes of Petford *E. camaldulensis* at 3.75 years at Forest Hill, Zimbabwe. The unadjusted mean (vertical bars), 95% confidence level (the box) and range (x) of regular monoterpene-dominated chemotypes amongst the 3-12 trees sampled in each of 19 families is given. The number of sesquiterpene chemotypes (SC's) in each family omitted from these and other calculations is also given.

Expected gains in oil yield in the first generation following selection of the best parent in every 10 on the basis of yield of 3.75-year-old offspring at Mtao are 25% for 1,8-cineole and 32% for total monoterpenes (equation (2)). Estimates of genetic and phenotypic correlations between 1,8 cineole and total monoterpene yield and various growth traits are given in Table 7.6a while Table 7.6b gives estimated correlations amongst the various compounds.

Table 7.6 Estimates of genetic (above the diagonal) and phenotypic (below the diagonal) correlations for 3.75-year-old Petford *E. camaldulensis* at Mtao, Zimbabwe.

(a) Correlations between growth traits and 1,8 cineole and total monoterpene yield

	Ht	dbhob	Crown surface area	Crown density	1,8-cineole yield	Total monoterpene yield
Ht		0.818 (0.139)	0.666 (0.202)	0.481 (0.303)	0.044 (0.295)	-0.481 (0.260)
dbhob	0.769		0.816 (0.171)	0.477 (0.348)	-0.100 (0.358)	-0.456 (0.333)
Crown surface area	0.618	0.751		0.467 (0.344)	-0.104 (0.341)	-0.466 (0.311)
Crown density	0.421	0.482	0.445		0.233 (0.371)	-0.139 (0.385)
1,8-cineole yield	0.041	-0.071	-0.079	0.185		0.803 (0.109)
Total monoter -pene yield	-0.427	-0.350	-0.365	-0.096	0.806	

(b) Correlations amongst yields of oil components

	α-pinene	β-pinene	limonene	1,8-cineole	ρ-cymene
α-pinene		-0.227 (0.271)	-0.064 (0.295)	-0.076 (0.294)	-0.163 (0.275)
β–pinene	-0.186		0.366 (0.267)	-0.019 (0.295)	0.056 (0.281)
limonene	-0.041	0.321		0.027 (0.309)	0.182 (0.285)
1,8-cineole	-0.088	-0.023	0.054		-0.331 (0.275)
ρ-cymene	-0.144	0.041	0.189	-0.304	

( ) standard errors for genetic correlations

Phenotypic correlations greater than about ± 0.15 are significantly different ($p<0.05$) from 0

7.3.2 Experiment 2

Controlled pollinations

The results of the crossing program are summarised in Table 7.8. Pollen germination ranged from 1% to more than 90%. Lots below 10% germination were discarded. However, this precaution may not have been essential to good seed set as pollen from tree 87, with 16% germination and one of the poorest used, gave results comparable with those from lots of very high germination (e.g. 58 and 96 with 95% germination) (Table 7.8).

With hindsight, emasculation of most trees, and particularly 10, 30 and 58, was undertaken too early, before the buds were fully mature, and resulted in a high abortion rate. If timed correctly, indicated by a colour change to the operculum from green to yellow, it should be necessary to pollinate only twice (Doran and Turner unpubl.). However, 3 or even 4 pollinations were necessary in this instance to cover all the stigmas as they became receptive (sticky). The poor result with tree 82 is almost certainly due to early emasculation and too few applications of pollen.

Table 7.8 A plan of the crosses attempted and summary of results of the controlled pollination program with Petford *E. camaldulensis*. The three numbers in each column, 1 (2) 3, are explained in the footnote.

Cineole yield class		H	H	H	A	L	L	L	L
	Tree No.	82 1(2)3	58	I8	67	87	96	10*	51*
H	82	1(38)0.54	1(3)0.02	1(2)0.04	X	2(4)0.04	X	X	X
H	58	1(13)0.28	2(6)0.08+	X	X	1(3)0.02	1(1)0.03	X	X
H	101	1(1)0.01	2(6)0.17	1(5)0.09	X	1(12)0.41	1(7)0.10	-	X
A	88	2(20)0.32	2(23)0.39	1(2)0.03	2(11)0.19	1(1)0.02	2(15)0.30	2(7)0.10	-
A	48	2(17)0.34	1(18)0.46	2(23)0.47	1(3)0.06	1(28)0.80	2(25)0.36	-	1(12)0.25
A	30	X	X	X	X	1(1)0.02	X	X	-
A	49	1(11)0.24	1(22)0.60	1(12)0.25	1(15)0.47	X	X	-	1(2)0.03
L	87	X	1(2)0.04	X	X	X	X	X	X
L	10*	X	1(6)0.19	X	X	1(2)0.06+	X	2(78)2.16	X

Additional crosses were - 88 x 88 2(19)0.21+
101 x 101 1(5)0.11+

H = high
A = average
L = low
* = low-cineole, high-sesquiterpene chemotype
1 = number of pollination bags giving mature capsules
(2)= total number of capsules collected
3 = total weight of seed extracted (g)
_ seed failed to germinate
+ seedlots with too few germinants for full representation in the field trial
X a cross which was attempted but failed
- a cross not undertaken

Oil data

1,8-cineole and total monoterpene yields at 12 months from planting for the crosses averaged by mating type amongst cineole yield classes are given in Table 7.9 and for open-pollinated progeny in Table 7.10. Yields amongst progeny of high-cineole parentage were below levels anticipated from the mid-parent values estimated from data in Table 7.1. This indicates that most have yet to exhibit their full potential. Two crosses including tree 10, a sesquiterpene chemotype, were well above expected values as the characteristics of this chemotype, as discussed in Chapter 4, had not yet appeared (Table 7.9). Despite this instability, the crosses amongst high-cineole parents (i.e. H x H) yielded 23% more 1,8-cineole than the overall average for the control-pollinated progeny. Amongst the open-pollinated families there were three represented by progeny from two different seed crops. While one family showed consistency (58), the other two (82 and 88) were variable indicating that seed-year effects might be important (Table 7.10).

Table 7.9 Yields of total monoterpenes and 1,8-cineole ( ) averaged by mating type amongst cineole yield classes in the crossing program compared to mid-parent estimates (see Table 7.1).

	Yields (w/100g. dry leaf)	
Cineole Yield class	Controlled crosses	mid-parent estimates
H x H =	2.39 (1.92)	3.34 (3.07)
H x L =	2.25 (1.52)	2.27 (1.86)
A x H =	2.19 (1.75)	2.96 (2.59)
A x A =	2.44 (1.69)	2.57 (2.11)
A x L =	1.95 (1.43)	1.89 (1.38)
L x H =	1.87 (1.31)	2.27 (1.86)
L x L =	1.62 (1.29)	1.20 (0.64)

Table 7.10 Best Linear Unbiased Estimates of 1,8-cineole and total monoterpene yield of open-pollinated families at 12 months in the field trial. Families are ranked for 1,8-cineole yield.

Yield (w/100, dry leaf)

Ranking for 1,8-cineole yield	Treatment no.	Parent no.	Total monoterpenes	1,8-cineole
1	41	101	2.81	2.21
2	42	I8	2.64	2.03
3	40	58[1]	2.31	1.99
4	39	58[1]	2.28	1.92
5	49	51	2.44	1.90
6	46	30	2.49	1.89
7	38	82[2]	2.40	1.87
8	43	67	2.96	1.76
9	47	48	2.16	1.70
10	44	88[3]	2.13	1.66
11	48	49	1.92	1.53
12	50	87	2.28	1.51
13	37	82[2]	2.14	1.51
14	51	10	1.89	1.39
15	45	88[3]	1.72	1.37
16	52	96	1.64	1.11

[1, 2, 3] seed collected in different years

Estimates of individual heritabilities for the four compounds and their total by mid-parent - offspring regression (equation (3)) and estimates of individual heritabilities by sib analysis (equation (7)) are given in Table 7.11. The diallel analysis indicated that general combining ability (gca) and specific combining ability (sca) were important sources of variation for all traits except for sca for limonene (Table 7.12). Additive variation exceeded dominance variation by the ratios of 3:1 to 8:1 for yields of α-pinene, limonene and 1,8-cineole while for β-pinene their effects were equal (Table 7.13). Gca and sca effects were calculated for each parent and cross using equation (4) and their values for 1,8-cineole yield are given in Table 7.14. Parents 101 and I8 were the top ranking for this important commercial trait.

Table 7.11 Estimates of heritabilities (h_i^2) and their standard errors ( ) from the full-sib progeny test.

Oil trait (w/100g, dry leaf)	Method of estimating h_i^2: Mid parent-offspring regression	Sib analysis
Total monoterpenes	0.28 (0.09)	-
1,8-cineole	0.25 (0.07)	0.85 (0.14)
limonene	0.26 (0.20)	0.73 (0.13)
β-pinene	0.22 (0.08)	0.49 (0.11)
α-pinene	0.86 (0.15)	0.71 (0.13)

Table 7.12 Variance components and their standard errors from DIALL analysis of oil yields of *E. camaldulensis* from the full-sib progeny test.

Source	No. of Obs.	Variance components and standard errors ( ): α-pinene	β-pinene	limonene	1,8-cineole
gca	13	0.003 (0.001)	0.003 (0.001)	0.002 (0.001)	0.045 (0.021)
sca	19	0.001 (0.001)	0.003 (0.001)	0 (0.002)	0.007 (0.004)
error	38	0.001 (0.001)	0.0003 (0)	0.003 (0.002)	0.003 (0.002)

Table 7.13 Genetic parameter estimates from the DIALL analysis of oil yields from the full-sib progeny test.

Parameter	α-pinene	β-pinene	limonene	1,8-cineole
V_A	0.012	0.012	0.008	0.180
V_D	0.004	0.012	0.000	0.028
V_A/V_D	3	1	8	6.4

Table 7.14 Estimates of gca and sca effects for 1,8-cineole yield in 12 month-old *E. camaldulensis* progenies in the full-sib progeny test.

Cineole yield class	H	H	H	A	L	L	L	L	L
	Tree No.	82	58	I8	67	87	96	10*	51*
		(0.08)	(-0.06)	(0.30)	(0.05)	(-0.28)	(-0.38)	(-0.17)	(-0.16)
H	82	S	0.02 / 0.08	0.38[2] / 0.23		-0.20 / 0.05			
	(0.08)								
H	58	R	S			-0.34 / -0.11	-0.44 / -0.01		
	(-0.06)								
H	101		0.42[3] / 0.15	0.78[1] / 0.17		0.20 / -0.04	0.10 / 0.15		
	(0.48)								
A	88	0.31 / -0.04	0.07 / -0.08	0.43[4] / -0.07	0.18 / 0.10	-0.15 / 0.09	-0.25 / -0.14	-0.04 / 0.09	
	(0.13)								
A	48	0.05[5] / 0.30	-0.09 / -0.06	0.27 / -0.18	0.02 / 0.02	-0.31 / -0.06	-0.41 / 0		-0.19 / 0
	(-0.03)								
A	30					-0.10 / 0			
	(0.18)								
A	49	-0.05 / 0.21	-0.19 / 0.04	0.17 / -0.14	-0.08 / -0.1				-0.29 / 0
	(-0.13)								
L	87		R						
	(-0.28)								
L	10*		-0.19 / 0			-0.41 / 0.19			
	(-0.13)								

( ) = parental gca
gca / sca = gca and sca of cross
R = reciprocal
S = self
1 to 5 = top ranking crosses
* = sesquiterpene chemotypes

Some indication as to the number of genes that may be contributing to the inheritance of terpenes in *E. camaldulensis* can be obtained by examining the frequency distributions for each compound (Figure 7.6). The distributions for α-pinene, limonene and 1,8-cineole, although somewhat skewed towards the lower classes, were continuous indicating multiple gene control. The distribution of the β-pinene, however, was distinctly bimodal suggesting Mendelian inheritance involving few genes. A tentative hypothesis that β-pinene yield is controlled by two alleles, designated H/h, with complete dominance at the locus was made. To test this hypothesis, the progeny data were grouped into two fairly discrete classes: high, composed of plants with yields of β-pinene greater than 0.1g (w/100g, dry leaf); and low, plants with 0.1g or less. One of the three hypothetical genotypes, HH, Hh and hh was assigned to each of the parents based mainly on the performance of their offspring (Table 7.15a). Phenotypic values for each of the parents substantiated these assignments. Of the six different matings possible, four are represented by the parents in the study (Table 7.15b). Progeny data from each mating type were considered separately and combined, and ratios were obtained which show a reasonably close fit to theoretical expectations (Tables 7.15a & b).

7.4 Discussion

It must be stressed at the outset that the early nature of the data (12 months) from Experiment 2 places severe limits on the inferences that can be drawn. The results here and in Chapter 4 show that the oil yields of trees in this experiment at this time were still unstable. As a result, the main conclusions concerning genetic parameters for oil yields in Petford *E. camaldulensis* are drawn from the analysis of Experiment 1 with the preliminary data from Experiment 2 used in support. It should be noted also that, because of the unbalanced nature of the data in Experiment 1, the analysis is only approximate. The Mtao data is considered more reliable because of greater replication and fewer missing values. The estimation of genetic gains, therefore, has been confined to the Mtao data.

7.4.1 Heritabilities and gains expected from selection

Individual-tree heritabilities for 1,8-cineole and total monoterpene yields are given in Table 7.5. Estimates for 1,8-cineole and total monoterpene yield at Mtao were a high 0.53 ± 0.06 and 0.54 ± 0.06 respectively. Family heritability for 1,8-cineole yield in this study, assuming a family size of 6, was 0.62, in reasonable agreement with the corresponding figure given for *E. kochii* of 0.83 by Barton *et al.* (1991).

1,8-cineole and total monoterpene yield of leaves are expected to improve by 25% and 32% respectively (equation 2 applied to the Mtao data), following selection at an early age (about 3 years) and mating among the best 10% of trees. The mating of the best 29 of 285 trees at Mtao (by controlled pollination or by open pollination in clonal orchards) may be expected to produce an improved population of *E. camaldulensis* which would yield 2.1g of 1,8-cineole/100g of dry leaf compared with an average yield of 1.7g for unselected trees.

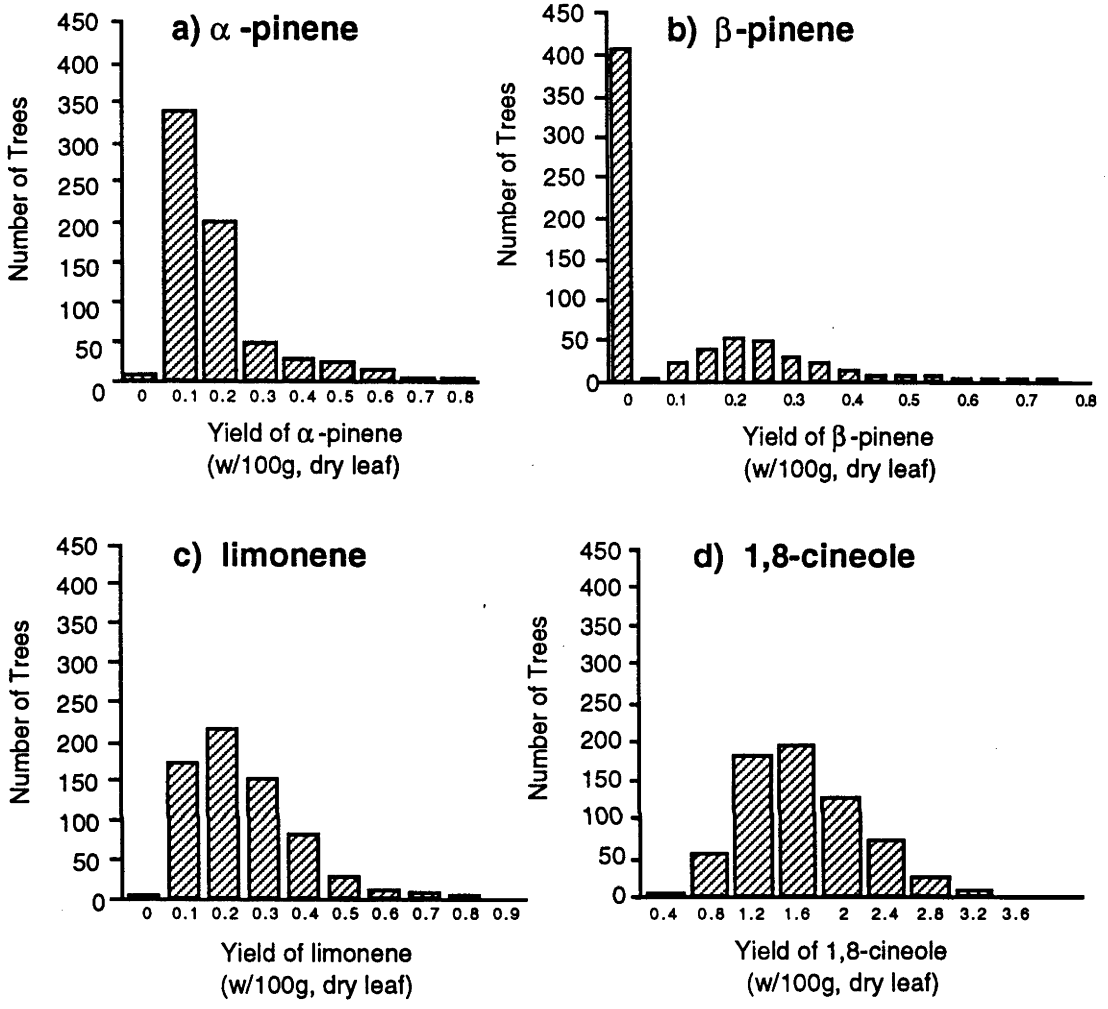


Figure 7.6 Frequency distributions of yield classes for a) α-pinene, b) β-pinene, c) limonene and d) 1,8-cineole in 1-year-old control-pollinated progenies of *E. camaldulensis*. Data from all crosses are combined.

Table 7.15(a) Observed against expected segregation ratios for ß-pinene in control-pollinated progenies.

Assumed mating type for β-pinene		hh	hh	Hh	HH	Hh	Hh	Hh	Hh
	Tree No.	82	58	I8	67	87	96	10	51
hh	82	0:10 / 0:1	0:19 / 0:1	6:14 / 1:1		5:13 / 1:1			
hh	58	0:20 / 0:1	0:7 / 0:1			13:7 / 1:1	13:5 / 1:1		
hh	101		0:20 / 0:1	7:12 / 1:1		8:12 / 1:1	8:12 / 1:1		
hh	88	0:20 / 0:1	0.19 / 0:1	9:11 / 1:1	19:1 / 1:0	5:15* / 1:1	13:7 / 1:1		
hh	48	0:20 / 0:1	0:20 / 0:1	11:8 / 1:1	8:4 / 1:0	5:11 / 1:1	9:11 / 1:1		7:13 / 1:1
hh	30					10:8 / 1:1			
hh	49	1:19 / 0:1	0:20 / 0:1	11:9 / 1:1	20:0 / 1:0				12:8 / 1:1
Hh	87		9:9 / 1:1						
Hh	10		10:9 / 1:1			4:5* / 3:1		13:6 / 3:1	

Phenotype frequencies = Observed / Expected

* = families that showed significant divergence at the 5% level between observed and expected frequencies by Chi-square tests, with df=1

Table 7.15(b) Overall result for segregation of ß-pinene in F_1 progenies

Assumed mating type	Classes of β-pinene yield (No of individuals)		Expected ratio	Chi-square test (df=1)
	High	Low		
hh x hh	1	213	0:1	ns
HH x hh	47	5	1:0	ns
Hh x hh	142	165	1:1	ns
Hh x Hh	17	11	3:1	ns

ns = not significant at 5% level

7.4.2 Genetic and phenotypic correlations

The estimated genetic and phenotypic correlations between growth traits and 1,8-cineole yield were small with large standard errors pertaining to the genetic correlation coefficients. Although these estimates lacked precision, it appears that these characters are weakly associated and selection for one is unlikely to affect the other, either adversely or favourably (Table 7.6a). Total yield of monoterpenes and growth traits, however, showed moderate and unfavourable genetic and phenotypic correlations. These genetic correlations had very large standard errors and probably mean nothing. 1,8-cineole yield and total yield of monoterpenes were, as expected, strongly associated with an r_g of 0.803 and a r_p of 0.806.

As it is probable that *E. camaldulensis* will be grown primarily for wood and with oil as a secondary product, breeders will be concerned mainly with improving growth rate and stem form. The positive genetic and phenotypic correlations between height and diameter and the crown traits of surface area and crown density are encouraging because total oil production depends not only on oil concentration in the leaves but also on total leaf biomass.

As statistical correlations between individual monoterpenes may yield information about biochemical pathways involved in terpene biosynthesis or gene linkage (Wilkinson *et al.* 1971), genetic and phenotypic correlations were calculated between the five major monoterpenes in the oil of Petford *E. camaldulensis* (Table 7.6b). Correlations were mainly small with large standard errors associated with the genetic correlations indicating that the yields of most compounds are inherited independently of one another. The largest correlations were a positive one (r_p = 0.32) between limonene and β-pinene and a negative one (r_p = -0.3) between 1,8-cineole and ρ-cymene. These appear to be as a result of chance as neither fits contemporary theory on biosynthetic pathways for these monoterpenes (J.J. Brophy pers. comm.).

7.4.3 Genotype x environment interaction

In the harsher environment at Forest Hill volume growth of progeny was only a third of that at Mtao. The prevalence of leader dieback at Forest Hill, affecting 10% of progeny at the time at collection, contributed to this differential. There appears to be an overall positive relationship between tree health, growth rate and oil yield (Figs 7.4 and 7.5, Table 7.4) between sites which is consistent with other studies on oil-bearing plants (Haagen-Smit 1949).

Family rankings for 1,8-cineole yield appeared to vary appreciably between the two sites suggesting that family x site interaction for this trait might be significant. Unfortunately, it was not possible to test for the significance of the interaction statistically and a closer look at this aspect in future research is required. The Spearman rank correlation test showed that family rankings for average 1,8-cineole yield at Mtao and Forest Hill were associated ($p<0.01$). Certainly, the families providing the top 5% of 1,8-cineole-yielding progeny were the same on each site. This suggests that in a mass selection program among progeny at Mtao and Forest Hill, selected genotypes might perform well over a range of sites.

7.4.4 Indications from Experiment 2

Heritabilities by sib-analysis were mostly greater than estimates from mid-parent regression. This was expected for 1,8-cineole as a result of an inflated estimate of additive genetic variance (V_A) from the assortative mating pattern but not for the other compounds that were not subject to any selection pressure. Estimates from mid-parent - offspring regression should improve with age as oil yields of progeny stabilise. In summary, indications from Experiment 2 are that heritabilities for 1,8-cineole and total monoterpene yield are moderate to high and consistent with estimates from Experiment 1.

Additive genetic effects are the only source of genetic variation which can be utilised in the cumulative improvement of trees by recurrent selection from one generation to the next. Non-additive genetic effects (estimated as sca) can, however, be exploited when improved genetic material is propagated vegetatively for use in establishing plantations. These preliminary results indicate that additive variance is substantially greater than non-additive variance for the four major oil compounds, although again it is stressed that V_A for 1,8-cineole was not reliably estimated. The three control-pollinated families with the greatest 1,8-cineole yields (101 x I8, 82 x I8 and 101 x 58) ranked highest apparently because at least one of the parents was a good general combiner and moderately high levels of sca were also present. The implications of this result for breeding strategy are discussed in Chapter 8.

Major gene control of several terpenes has been often reported for the pines where different terpenes appear to be under major gene control in different species (e.g. β-pinene in *Pinus contorta* (White 1984); 3-carene in *P. monticola* (Hanover 1966); β-pinene and myrcene in *P. elliottii* (Squillace 1971); β-pinene, myrcene, limonene and β-phellandrene in *P. taeda* (Squillace *et al.* 1980)). However, the mode of genetic control of major terpenes in eucalypts does not appear to have been discussed prior to this study. Much of the early work proposing monogenic control of constituents of coniferous resins has been refuted by Birks and Kanowski (1988) because proportional data have been treated statistically as absolute quantities leading to unsubstantiated conclusions. In the current study, absolute values are used and should be free of these deficiencies.

In Petford *E. camaldulensis* multiple gene control is indicated for α-pinene, limonene and 1,8-cineole yields. On the other hand, it appears that inheritance of β-pinene yield may be controlled largely by two alleles at a single locus, with the alleles for high yield being dominant over the low. The progenies of crosses assumed to be homozygous and heterozygous were found to fit the Mendelian segregation ratios quite well, although the few exceptions are a problem and perhaps indicate that modifying genes may also be involved (Table 7.15a).

7.5 Conclusions

Genetic parameters were estimated for yield of 1,8-cineole and total monoterpenes as a fraction of leaf weight in nineteen open-pollinated families of Petford origin at 3.75 years in two progeny/provenance trials of *E. camaldulensis* in Zimbabwe. Both traits appear to be highly heritable and, as expected, strongly genetically correlated, with narrow-sense individual heritabilities near 0.50. Expected gain in the first generation following individual selection in the trials of one tree in 10 for either trait is about 25-32%.

Genetic correlations between growth traits and 1,8-cineole yield were small. This indicates that both traits might be improved concurrently in the same population thus enhancing the economics of growing Petford *E. camaldulensis* for wood and medicinal oil. However, the presence of moderate and unfavourable genetic correlations between growth traits and total yield of monoterpenes warrants further study. It was not possible to gauge the significance of apparent family x site interaction for 1,8-cineole yield in this study. However, a test of rank correlation showed an association ($p<0.01$) between family rankings on each site and the highest-yielding trees on both sites came from the same families. This raises the possibility of being able to select individuals for cloning that yield well over a range of site conditions.

The results of the controlled-pollination experiment, although tentative because of the young age of the progeny, support the main findings from Zimbabwe that yields of foliar monoterpenes in Petford *E. camaldulensis* are under strong genetic control. Important additional information provided by this experiment was that levels of additive genetic variation appear to be far more important in determining yields of the various compounds than non-additive variation. However, useful amounts of non-additive variation, in the form of sca, are present amongst the best crosses with potential for utilisation in mass vegetative propagation. While multiple gene inheritance was implied by frequency distributions of yields of α-pinene, limonene and 1,8-cineole, the segregation of β-pinene into two classes suggests that the yield of this compound is controlled largely by two alleles at a single locus, with high yield dominant over low yield.

CHAPTER 8. BREEDING STRATEGY

8.1 Introduction

E. camaldulensis is perhaps the world's most widely-used tree species for plantings in arid and semi-arid lands. In addition to extensive but largely unrecorded plantings for shade and shelter in many parts of the world, over 500,000 ha of plantations had been established by the mid-1970s (Jacobs 1979). It is probable that this figure has now doubled, due to expanded planting in the tropics. The success of this species as an exotic is attributed to its superiority to other trees in production of wood on infertile dry sites, its tolerance of extreme drought and high temperature combined with rapid growth when water is available, deep penetration of roots, tolerance of frost, good coppicing ability, and usefulness of the wood (Midgley *et al.* 1989). The main use of the wood of planted *E. camaldulensis* has been for poles, posts, firewood, charcoal and paper pulp.

Planting in tropical areas with a long dry season, especially in southeast Asia and Brazil, is increasing. At present, most plantings of *E. camaldulensis* in this climatic zone rely on seed collected from local unselected trees of unknown origin. Because of the scant attention given to the genetic quality of seed, the productivity of the resulting plantations is well below the full potential of the species and is conceivably declining further through inbreeding. There is a growing awareness, however, of the value of using the climatically-adapted northern Australian provenances and of initiating tree breeding programs to enhance productivity.

Petford provenance of *E. camaldulensis* is one of the best known and most widely-used provenances in the world's seasonally-dry tropical zone (Midgley *et al.* 1989). Seed from this provenance is the basis for several tree breeding programs commenced in recent years. The countries involved include China (Nikles 1987), Kenya (Milimo pers.comm.), Nepal (White 1986a), Thailand (Raymond 1991) and Zimbabwe (Barnes 1984).

We now know that Petford provenance is a potential source of *Eucalyptus* oil. *Eucalyptus* oil can be extracted in simple low-cost stills by villagers close to plantations. Its production where Petford provenance thrives could result in the establishment of important cottage industries bringing much social benefit. This has already happened in several countries using other eucalypt species (e.g. in Bolivia (Eberlee 1991), China (Song 1990) and India (Boland 1980)). We also know from the preceding Chapters that cineole production in Petford *E. camaldulensis* is under strong genetic control which, combined with significant variability, gives scope for rapid and substantial improvement by tree breeding.

It is unlikely, however, that, in the developing countries where Petford provenance is widely used, a major tree breeding program aimed solely at improving oil yields could be economically justified. For example, Thailand spends somewhere in the order of A$1,000,000 per day on imported pulp and paper products (K. White pers. comm.) while the annual import bill for *Eucalyptus* oil is about A$500,000 (Ministry of Commerce, Thailand pers. comm.). Because of the imbalance between supply and demand in wood products, the top priority in these countries must be increased wood production through selecting and breeding trees superior in traits such as health, vigour, form and wood properties. *Eucalyptus* oil therefore, will be a valuable additional product

from *E. camaldulensis* plantations being grown primarily for wood. Improvement of oil traits in this species might be a secondary aim in some breeding programs but it is unlikely to qualify as a top priority.

In this Chapter, one practical way of incorporating breeding for improved oil production into a strategy aimed at improving growth traits is discussed. Use is made of the information on variation in and genetic parameters for foliar oil yields in *E. camaldulensis* provided by the research described in earlier Chapters.

8.2 Basic concepts of tree breeding strategies

Tree improvement programs aim to develop new plantations superior to their ancestors in one or several key economic traits. The *modus operandi* of most contemporary programs is to start with a carefully chosen breeding strategy implemented through a dependant breeding plan. The breeding strategy provides the philosophy of the management of genetic improvement while the breeding plan prescribes the 'nuts and bolts' for implementing the selected strategy. Typically the plan includes a set of objectives and a flow chart illustrating what is to be done each month for several years ahead, and is subject to regular revision, every 2-5 years (Eldridge *et al.* in press).

Most worthwhile breeding strategies recommend starting with a well-adapted broad genetic base population. The base population is then subjected to a particular method of selection. Selected trees are then mated to maximise long term genetic gain and minimise the effects of inbreeding within the limits imposed by human and economic resources.

Selection and mating are key activities in breeding. They accumulate genes which influence yield and adaptation, steadily increasing over successive generations the frequency of superior phenotypes. Every successful breeding strategy, therefore, requires efficient methods of selecting superior material including the progeny tests in which selection is carried out, appropriate measurement techniques and selection technology (e.g. selection indices). Mating can be done by open pollination or controlled pollination, carefully minimising the potential of inbreeding and allowing for genetic material from other sources to be incorporated. In pursuing its principal functions of efficient selection and mating, a strategy should aim to assess the variation within a species, generate genetic information about it and ensure that genetic resources for future selection are conserved (Barnes 1987; Matheson 1990).

The cyclic or recurrent nature of the selecting, testing and mating processes as part of an overall breeding strategy is illustrated in Figure 8.1. Every effective breeding strategy involves maintaining a hierarchy of three major types of population which can continue to meet the demand for genetically improved planting stock for the fourth population, the wood producing plantations (Eldridge 1984; Griffin 1989; Matheson 1990). They are the base, breeding and propagation populations. Awareness of the concept of maintaining distinct types of population within the cycle, even if circumstances dictate that some populations are combined in the one planting, is essential in planning the operations of genetic improvement (Libby 1973).

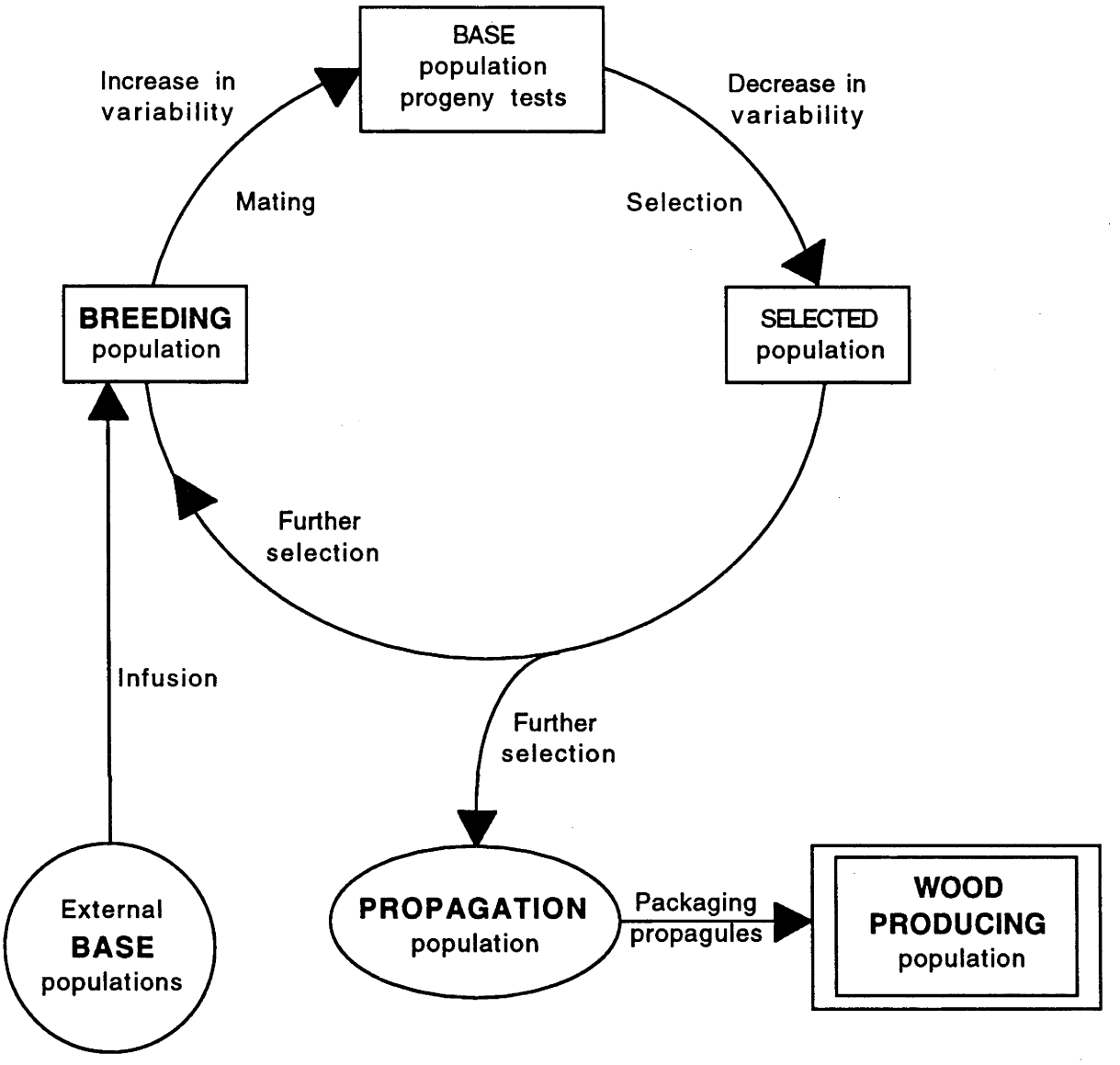


Figure 8.1 The major components and activities of the breeding cycle as adapted from White (1987).

8.3 A breeding strategy for Petford *E. camaldulensis*

8.3.1 Common constraints

Many countries now embarking on improvement programs with Petford *E. camaldulensis* share common constraints that influence the choice of the most efficient and cost-effective breeding strategy. They are -

Availability of genetic resources - Most have established provenance trials and determined that the Petford seed source is amongst the top-performing provenances. Despite this, there is still a tendency to economise by relying on seed from local land races of unknown origin for plantation establishment. The more progressive planting projects have imported seed of Petford provenance from Australia. Unfortunately, the records of plantation establishment using this seed are often incomplete, making it hard to determine on the ground which plantations are of Petford origin. Uncertainty about the existing genetic resources makes the conventional breeding approach of mass selection and clonal seed orchards unattractive.

For most breeding programs, the major genetic resource available is the native stands near Petford in northern Queensland. In recognition of this fact, CSIRO's Australian Tree Seed Centre has made extensive individual-tree seed collections in the Petford region and stocks some 400 such seedlots for breeding purposes.

Incomplete information on the biology of the species - To develop a breeding strategy it is vital to have information about the biology of the species involved. Knowledge of the reproductive mechanisms, potential for asexual reproduction, relationships between characters of economic importance, and extent of variation and the heritability of such characteristics are all required (Raymond 1991). The information on these biological aspects of *E. camaldulensis* is incomplete. However, it is important to summarise what is known as it affects the options available for developing a suitable strategy.

E. camaldulensis is regarded as an outcrossing species but, in common with most eucalypts, will have a degree of self pollination due to its floral biology. Controlled pollination can be highly successful but requires suitable equipment, accurate timing and technical expertise. Extensive use of controlled pollination is not considered a practical option in most countries. *E. camaldulensis* has the ability to root readily from cuttings if the correct techniques are employed (White 1986b; Reuveni *et al.* 1990; Kijkar 1991); cuttings are used routinely for plantations in Morocco (Marien 1991). Deployment of superior genetic material using vegetative propagation may be a feasible option in some breeding programs.

Information about variation in key economic traits within the species has been reviewed by Midgley *et al.* (1989) and Eldridge *et al.* (in press). For tropical provenances, significant genetic variation in most traits occurs at both the within-provenance and between-provenance level.

Untrained personnel and inadequate facilities - Successful tree breeding needs scientific and technical expertise and a commitment from management to provide facilities and funds in the long-term. Fortunately, in many of the countries wishing to breed Petford *E. camaldulensis*, there is this commitment and a core of well-trained staff to draw upon. In

the following discussion, resources of this nature are not considered to be a major constraint to the type of breeding strategy proposed.

8.3.2 Outline of an appropriate strategy for improving growth traits

The breeding strategy proposed can be defined as recurrent selection for general combining ability with open pollination in a single population keeping family identity (Eldridge *et al.* in press). Details of the strategy are given below and in diagrammatic form in Figure 8.2.

* Obtain open-pollinated seed from about 300 - 400 unrelated trees of Petford origin from CSIRO. Additional open-pollinated seed from 50 - 100 superior phenotypes selected locally might also be included.

* Establish several progeny tests each of 10 hectares (or more) on sites representative of those where Petford *E. camaldulensis* is or might be planted. Use the best possible up-to-date experimental designs such as alpha-designs (John 1987) and a minimum of 25 or 30 seedlings per family.

* Measure and, using appropriate analysis and selection procedures, (i) cull heavily within all families and (ii) cull only the very worst families in the first-generation progeny tests. Culling may take place at various stages over four years.

* At six years, collect open-pollinated seed from the best 2-3 trees in all remaining families to establish the second generation.

* Establish second generation progeny tests including the best trees of the first generation plus an infusion of new material from Australia, local plantations and any other promising source.

* Cull the first-generation tests heavily at about 7 years for future seed production by removing families identified, using the best possible analysis and selection procedures, as having low general combining ability at the first measurement of the second generation (say after one growing season).

* The culled first-generation test is then managed as a commercial seed orchard, with <100 trees per ha.

* Vegetative propagation, via rooted cuttings from girdled plus-trees in the progeny tests may also be used to allow rapid deployment of superior phenotypes to the production population.

* Repeat the cycle for subsequent generations.

This strategy combines the base, breeding and propagation populations in a single plantation on each site type for each generation. These plantations serve sequentially as progeny tests of trees selected in the previous generation, as a basis for selection and breeding for the next generation, and finally as commercial seed orchards. While the strategy requires a high level of skill and efficiency in establishment and assessment of tests, and in data analysis for combined index selection on both individual and family merit (Eldridge *et al.* in press), it is based on open pollination. It is, therefore, relatively

simple to apply, cheaper than many other options and provides a high rate of cumulative genetic gain over time especially with fast-growing short rotation coppicing species like *E. camaldulensis*.

This strategy is eminently suitable for many of the countries wishing to improve growth traits in Petford *E. camaldulensis*. As a consequence, it or similar strategies are widely recommended by geneticists (e.g. Barnes 1984, Nikles 1987, Raymond 1991).

8.3.2.1 Expected gains

A disadvantage of open-pollinated mating is that the majority of the selection pressure is applied to the female parent whereas in the case of controlled pollination the breeder is effectively applying selection pressure to both male and female parents. This leads to a reduction in expected gain on a per generation basis (Cotterill *et al.* 1989). There is also the problem of non-random mating with its potential to lower gains through its effects on estimation of family breeding values and effective selection pressure (Griffin and Cotterill 1988; Griffin 1989).

Nevertheless, very useful gains can be made. Shelbourne (1989, 1991), using realistic selection intensities for orchards of this type, estimated the genetic gain per generation for a trait of medium heritability (i.e. 0.2) to be 8.5%. Even greater gains were actually achieved in practice by Franklin and Meskimen (1984). These workers, pioneers in applying this strategy to *E. grandis* in Florida, achieved large genetic gains in growth rate (84%) and frost tolerance (15%) by the third generation of selection.

8.4 Selection criteria and limitations of the OP strategy for improvement of oil yields

In order to maximise overall genetic gain it is necessary to select on as few characters as possible, so that gain in each character is maximised. Most breeding programs for *E. camaldulensis* will focus on the key economic traits of growth rate and stem form. While there is little direct information about the association of these characters in *E. camaldulensis*, there is some indication in other eucalypt species that stem form may be positively correlated with growth traits (Volker *et al.* 1990) and that this correlation is reasonably strong. The selection of big healthy trees with large crowns can be expected to boost oil production on a unit area basis. However, in this study we are primarily concerned with improvement of yield of oil from the leaves. If growth rate and oil yield had a strong positive genetic correlation, selection for both would be simple. However, this study (see Chapter 7) shows that they are not strongly correlated and can be considered as independent traits.

Therefore, the inclusion of oil traits as selection criteria in a combined selection index with growth traits will diminish the rate of improvement in the key economic traits of growth rate and form while allowing only small gains to be made on the secondary traits of oil yield and oil quality. There is also another significant constraint to adopting this approach. Extraction and analysis of oils greatly increases the work load of evaluating the many trees in progeny tests over several sites. With the limited staff and analytical facilities available for detailed chemical analysis in many of the target countries, it is impractical to consider this as a realistic option.

Clearly, the selection criteria for most orchards must remain focussed on the key economic traits of vigour and stem form rather than complicating the strategy with traits of uncertain economic return. How then, under this hypothesis, can improvements in oil traits be made?

8.5 Nucleus breeding for the production of outstanding families for oil traits

8.5.1 Background

In 1989, Cotterill *et al.* introduced a new concept to tree improvement which they called 'nucleus' breeding. A similar concept has been successful with sheep breeding since the early 1970s (Jackson and Turner 1972). The authors proposed a reorganisation of the traditional population structures used in tree improvement to create a two-tier breeding population involving :-

(a) A large 'main' breeding population, relying on open pollination for regeneration.

(b) An elite 'nucleus' formed by controlled pollination in an assortative mating pattern of 40 or so of the most outstanding individuals from the main population to provide maximum genetic gain in this separate small breeding population.

Progeny tests of control-pollinated families are used to provide material for clonal programs. A two-way flow of material (about 10 selections per generation) is proposed between 'main' and 'nucleus' to enhance the 'main' while reducing the tendency towards inbreeding in the 'nucleus'.

Cotterill *et al.* (1989) showed that potential gains in the 'nucleus' population, in a reasonably heritable trait, such as sectional area in *P. radiata*, eclipsed by 10% those expected from traditional strategies of cumulatively improving a single large breeding population in the first generation of selection. Nucleus breeding involves concentrating the best breeding individuals, financial investment and breeding effort together in an elite nucleus population. It fits very well with clonal forestry and seems to have excellent prospects for application to eucalypt breeding generally.

8.5.2 Modification of the main strategy to incorporate an elite 'nucleus' for oil traits

'Nucleus' breeding for oil traits dovetails extremely well into the conventional open-pollinated breeding strategy where the main objective is to produce more wood. It is also manageable in terms of expertise, staff availability and cost. The proposed scheme is outlined below and is based on the following assumptions :-

* That production of *Eucalyptus* oil is secondary to wood production because of the primacy of demand for wood products. Therefore, traits like health, vigour and form will have priority in the 'main' breeding population.

* That the *Eucalyptus* oil requirements of most countries can be met from a relatively small area of high-oil-yielding *E. camaldulensis* plantation.

For example, using extrapolations from various papers giving leaf biomass estimates for *E. camaldulensis* (e.g. Bunyavejchewin *et al.* 1987; Sahunalu *et al.* 1987) and a conservative estimate of yield of oil of 0.7g per 100g of fresh leaf (see White 1988), the area of routine Petford plantation required to be felled annually in Thailand to meet that country's internal oil needs is estimated to be about 1,000 ha (Doran unpubl.). This will decrease significantly once oil yields are improved by breeding and a system is developed whereby additional leaf production is obtained from the thinning of coppice shoots as part of normal plantation silviculture.

Because only a relatively small plantation estate is required in any one country for commercial *Eucalyptus* oil production, it is practical to propose that development of this industry be concentrated on a specific region(s). This region should be one where Petford provenance thrives and where it is possible to set up village cooperatives to undertake the labour-intensive work of leaf collection and extraction. Such cooperatives have succeeded in the economic production of oil in places such as Bolivia (Eberlee 1991), China (Song 1990) and India (Boland 1980) with much social benefit.

Ideally the region should be a site for the establishment of one of the several 'main' breeding populations proposed country-wide. This breeding population will then become the focus for selection and controlled pollination to establish the elite 'nucleus' for oil production. The scheme is outlined below and shown diagrammatically in Figure 8.2 :-

* By four years of age the 'main' breeding population will have been thinned down gradually on the basis of the key selection criteria of health, vigour and stem form. Mild selection between families, retaining say 350 of the original 400 families, and moderately intense within-OP-family selection retaining say 5 of the original minimum of 25 progeny per family would be a realistic approach.

* All 1750 trees remaining in the pool should be screened for 1,8-cineole yield using rapid methods similar to those described in Chapter 3.

* The highest-yielding individuals in the best families will become candidates for an assortative controlled pollination program that should be undertaken once all 40 or so parents are flowering. This program would use the family estimates of gca for oil traits gained from the initial screening to predict which crosses will produce superior oil-yielding progeny. The benefits of this approach in improving genetic gains are demonstrated in Chapter 7.

* The control-pollinated families generated would be raised in progeny tests and the best individuals in the best families selected by combined index selection with oil traits given equal weight to growth traits. These selections would then be available for mass vegetative propagation.

* The tendency of the nucleus towards inbreeding would be reduced by including selections from new families in later generations of the 'main' breeding population. There is also scope for introduction of clones and pollen from Australia developed in a joint CSIRO/ANU program (Anon 1990).

* The 40 or so parents selected as the basis for the 'nucleus' could be propagated vegetatively through girdling and collection of cuttings from basal shoots (Kijkar 1991). As rooted cuttings they would be established in clonal tests. Selections

that root readily and grow well might be used immediately for clonal oil-producing plantations.

Adoption of this strategy has a number of advantages over the alternative of attempting to improve oil traits in the 'main' breeding population. In the proposed 'nucleus' strategy, selection for oils is delayed until the fourth year when the initial thinning for growth traits in the 'main' population has been completed. At this stage the worst families have been removed and the poorest growing individuals in the selected families culled from the 'main' breeding population. Analytical work and selection for oil traits is therefore limited to those families and individuals that have grown well to half rotation age and produce large quantities of leaf biomass (see Chapter 7). Four years is also sufficient time for trees to have expressed their mature oil characteristics and oil-yielding potential (see Chapter 4). By using controlled pollination, best advantage can be taken of the favourable genetic parameters for oil yields reported in Chapter 7. The expertise is available in most countries to undertake a controlled pollination program of this magnitude (e.g. in Thailand - Ngamkhajornwiwat and Moncur in press).

8.5.3 Expected gains

One of the main advantages of the 'nucleus' strategy for improvement of oils over trying to breed for these traits in the 'main' breeding population is that the favourable genetic parameters for these characters can be harnessed to give a high rate of gain. As an example, we might assume a selection of the 40 best trees for 1,8-cineole yields in the leaves is undertaken amongst the 1750 trees remaining in the 'main' population after first thinning. If these selections were to be simply interbred in a conventional clonal seed orchard using open pollination, this should lead to a production plantation with 39% more 1,8-cineole in its leaves. This is based on the heritability estimate and phenotypic standard deviation for this trait in the Mtao provenance trial (using formula (2) in Chapter 7). Average production of 1,8-cineole on a per hectare basis should be further enhanced through improved leaf biomass production as some selection pressure has been applied in the 'main' breeding population for health and vigour.

The 'nucleus' strategy of assortative crossing of best (gca) mates and testing in progeny tests, however, can be confidently predicted to lead to clones of an oil-yielding capacity far better than their parents. For example, a progeny of the high oil-yielding cross, 101 X I8, growing in the Gympie field trial (see Chapter 7), has rapid height growth in combination with 1,8-cineole yields consistently above 4% (w/100g, dry leaf). This is 55% greater than the mid-parent value and about double the yield of 'average' trees. The use of selections like this in clonal plantations would improve yields dramatically.

The major limitation of this scenario is the problem of inbreeding. With so few parents in the elite 'nucleus', inbreeding and/or the risk of reliance on a very narrow genetic base could become a constraint by the third generation. To counter this tendency it is important to have infusions of new families from the 'main' population and from other programs such as the Australian work mentioned previously.

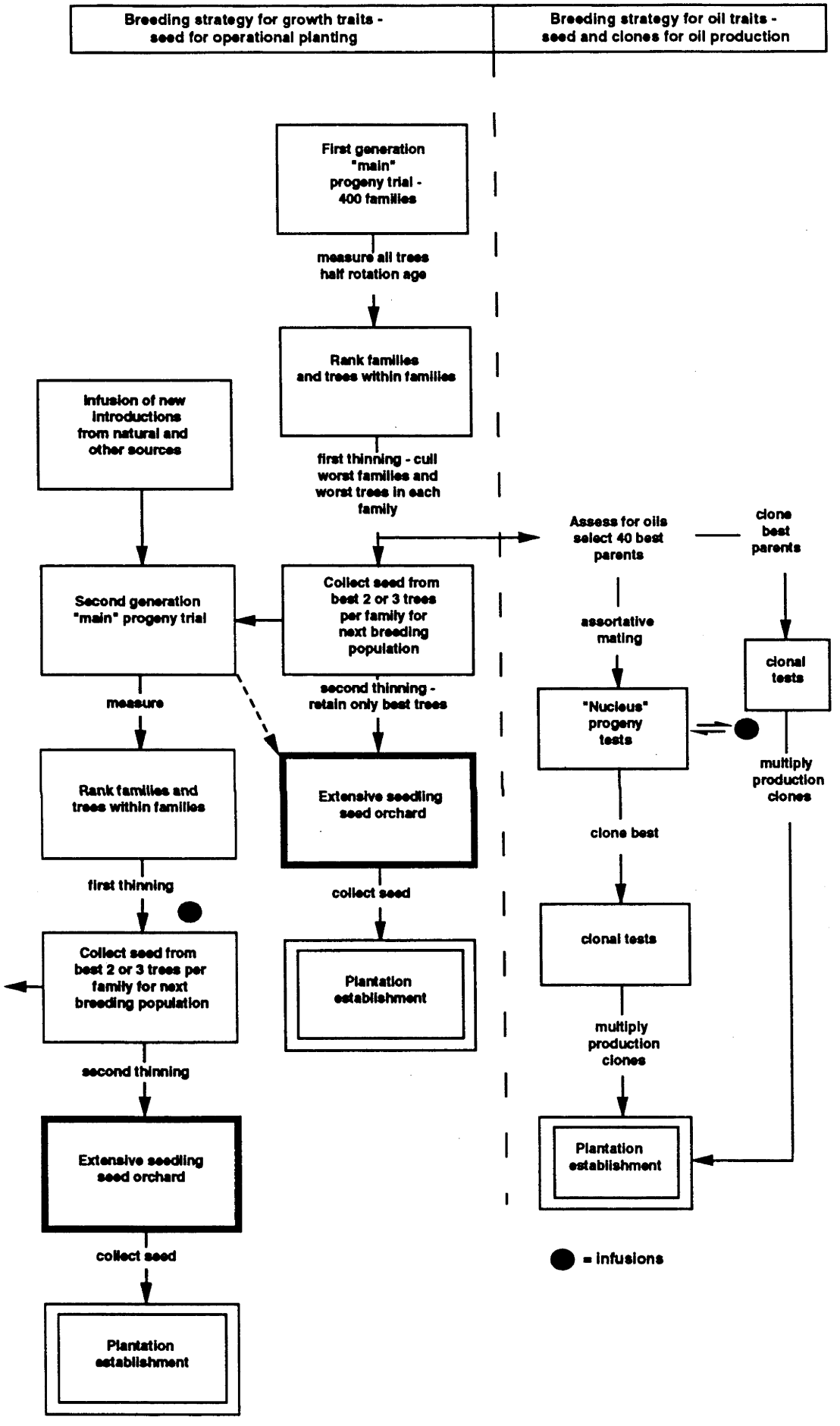


Figure 8.2. Schematic diagram representing the open-pollinated breeding strategy for growth traits and the establishment of a "nucleus" for improvement of oil traits.

8.6 Conclusions

The establishment of a small oil-elite 'nucleus' breeding population by controlled pollination of selections in the 'main' breeding population is recommended as an effective and efficient means of integrating breeding for essential oils into a main-stream breeding strategy where selection for growth traits has precedence. Controlled pollination to a pattern where best (gca) mates are crossed to form the 'nucleus' ensures maximum advantage can be made of favourable genetic parameters like high heritability of 1,8-cineole yield. The probability of developing elite oil-producers for use in clonal forestry is increased by this strategy.

The requirements for chemical analysis are high for 'nucleus' breeding for oil traits but are considered manageable. Chemical evaluation of all trees in the 'main' breeding population prior to each thinning, as would be required in breeding for this trait in a conventional way, would require large inputs and in many situations would prove impractical. A disadvantage of 'nucleus' breeding is the rapid reduction of the genetic base and the resulting increased tendency towards inbreeding. However, as long as this constraint is recognised, family identity and pedigree are maintained and opportunities taken to infuse new genotypes into the 'nucleus', this tendency could be controlled for several generations.

CHAPTER 9 SUMMARY OF KEY FINDINGS AND RECOMMENDATIONS

9.1 Introduction

Preliminary work in this study and in Nepal showed that some tropical red gum provenances and particularly those of *E. camaldulensis* gave 1,8-cineole-rich oil and appeared to offer potential as a commercial source of medicinal-grade *Eucalyptus* oil. Few tropical eucalypts lend themselves to this purpose so this discovery was of considerable interest especially as one of the best provenances for oil yield was Petford, a remarkably successful seed source in the world's seasonally-dry tropical zone. The quality of these cineole-rich oils met international standards but the average yields of about 1-2% (w/100g, fresh leaf) were modest as a basis for commercial production.

The main aim of the experiments developed in the current study was to gather the information necessary to decide if tree breeding offered scope for improving 1,8-cineole and total monoterpene yields in the leaves of the tropical red gums and, if affirmative, how best to undertake a selection and breeding program for these traits. Aspects investigated were :-

* Laboratory and field sampling methodologies.

* The age when the oil-yielding capacity of trees can be evaluated reliably.

* The influence of non-genetic factors (namely season, site, year, water stress and physiological age of leaves) on oil yields and implications of this for selection and breeding.

* Genetic variation in 1,8-cineole and total monoterpene yields between and within selected populations.

* Genetic parameters (heritability, genetic correlations, family x site interactions, levels of additive and non-additive variance) for yields and patterns of inheritance of prominent monoterpenoid compounds in the oils of Petford *E. camaldulensis*.

* How best to incorporate breeding for improved oil yields into a commonly-applied main-stream breeding program aimed principally at achieving gains in economically important growth traits such as wood production and stem straightness.

9.2 Summary of key findings and recommendations

9.2.1 Laboratory and field methodologies

The ethanolic extraction method is a reliable means of estimating individual red gum oil yields. In addition, it is a robust and practical technique when a large number of trees

need to be screened for oil, such as in a selection and breeding program. The low level of variation found in oil yields of mature leaves about the crowns of individual trees and the small seasonal variation in yield in natural stands of *E. camaldulensis* contribute to the robustness of the method during the collection and screening process.

MIR spectroscopy is a promising method of directly assessing 1,8-cineole yield of *E. camaldulensis* with the key advantages of speed of analysis and rapid application in the field. Further testing and development of this methodology is highly recommended.

9.2.2 The age at which trees can be reliably evaluated for their oil-yielding capacity

A reasonably reliable assessment of ranking of individual plants for 1,8-cineole yield can probably be obtained from seedlings of *E. camaldulensis* as young as two years of age, although absolute values might be subject to some further increases with time. Two years was also the time needed for progeny carrying genes for an undesirable low-cineole-high-sesquiterpene oil type to give unambiguous expression of this characteristic.

9.2.3 Influence of non-genetic factors on oil yields and implications for selection and breeding

In nursery trials, leaf maturity and genotype were shown to have a profound influence on estimates of 1,8-cineole and total yield of monoterpenes of young ramets (about 2 years old) of *E. camaldulensis*. Yields of oil peaked in young leaves that were fully expanded but soft and of high moisture content (i.e. at 3-4 months). During their adolescence they were sensitive to environmental influences such as drought stress which lowered yields. However, after maturation and lignification of leaves at about 5 months of age, the oil yield tended to stabilise at a level determined by their individual genotype.

Estimates of oil yield in field trials of 1 to 4-year-old *E. camaldulensis* were shown to be influenced by the environmental factors of season, site and year of harvest. The extent and direction of variation in yield depended very much on the individual tree such that no general pattern could be defined. There did not appear to be a relationship between seasonal variation in yield and climatic factors. It was deduced from indicators like leaf moisture content that leaves of variable age had been sampled, despite attempts to sample leaves of similar age at each sequential harvest. Leaf age effects, a significant source of variation, were confounded inadvertently with environmental effects in these studies.

Nevertheless, the variation found in the field trials was representative of what a tree breeder might encounter if selecting 'plus' trees for oil yields within plantations of differing age, on different sites and at different times of the year. The key finding was that, while absolute values did vary due to several non-genetic factors, the top (and lowest) ranking trees on each site maintained their ranking throughout the entire two years of the experiment. This consistency augers well for reliable mass selection within sites. Progeny testing should then be used to compare the performance of the initial selections under uniform conditions representative of where the trees will be grown for commercial oil production.

9.2.4 Genetic variation in 1,8-cineole and total monoterpene yields between and within selected populations

Surveys of the oil yields of various northern Australian provenances of *E. camaldulensis* and *E. tereticornis*, growing in natural stands and in fast-growing trials in Zimbabwe, were undertaken. These showed that only certain provenances gave leaf-oils in sufficient quantity (1-2% of fresh leaf) and of suitable quality (e.g. >70% cineole) for use as commercial sources of *Eucalyptus* oil. Petford provenance of *E. camaldulensis* ranked amongst the best for oil traits and, because of its established reputation as a fast-growing provenance and wide use internationally, this provenance became the focus for the genetic studies that followed the surveys.

Some individual trees of *E. camaldulensis* in the Petford region were found to produce oil of enhanced quality at about double the yield (as a fraction of leaf weight) of 'average' trees. Clearly sufficient variation was present in the Petford population to allow significant improvement in oil yields by selection and breeding if the genetic parameters for key traits were favourable.

9.2.5 Genetic parameters and patterns of inheritance of prominent monoterpenoid compounds and especially 1,8-cineole in the oils of Petford *E. camaldulensis*

Narrow-sense heritabilities for yield of the principal monoterpenoids in the leaves of Petford *E. camaldulensis* in progeny trials in Zimbabwe at 3.75 years were high. The key commercial traits of 1,8-cineole and total monoterpene yield had individual heritabilities near 0.50 and, as expected, were in favourable genetic intercorrelation. Strong genetic control of monoterpene yields was also indicated by the preliminary estimates of heritability from a controlled-cross progeny trial near Gympie.

Genetic correlations between growth traits and 1,8-cineole yield were small, although the presence of moderate and unfavourable genetic correlations between growth traits and total yield of monoterpenes warrants further study. A comparison of family data from Mtao and a harsher site for growth, Forest Hill, showed that, while family x site interaction for 1,8-cineole yield appeared to be present, the highest-yielding trees on both sites came from the same families. This raises the possibility of being able to select for cloning individuals that yield well over a range of site conditions.

The results of the controlled-cross experiment at 1 year from planting, although tentative, indicate that levels of additive variance appear to be far more important in determining yields of the various compounds than non-additive variation. However, useful amounts of non-additive variation, in the form of specific combining ability (sca), are present amongst the best crosses with potential for utilisation in mass vegetative propagation. While multiple-gene inheritance was implied by frequency distributions of yields of α-pinene, limonene and 1,8-cineole, the segregation of β-pinene into two classes suggests that the yield of this compound is controlled largely by two alleles at a single locus with high dominant over low.

9.2.6 A practical approach to incorporating breeding for improved oil production into a 'main-stream' breeding strategy for improving growth traits in *E. camaldulensis*

It was assumed that improvement in oil traits as a breeding objective will always be secondary to the objective of improving the key economic traits of wood production and stem straightness in breeding programs for Petford *E. camaldulensis*.

The breeding strategy deemed to be the most practical and cost-effective for improvement of growth traits in Petford *E. camaldulensis* was defined as recurrent selection for general combining ability with open-pollination in a single population, keeping the identity of a large number of families. This strategy was first used with *E. grandis* in Florida (Meskimen 1983). It was highly successful and has since been adopted by tree breeders in several countries for the improvement of various tree species.

In this plan, combined base and breeding populations are established on several sites. They are progressively thinned to become seedling seed orchards. Each site has up to 400 families under test. The aim of the plan is to increase stem wood yields; key selection criteria are health, growth rate and form. While oil yield might be included as a fourth selection criterion in such a program, the work load of screening for this trait would be substantial and would soon overload most in-country analytical services. Such effort is also unwarranted as only a relatively small area of plantation estate is needed to meet the requirements of most countries for *Eucalyptus* oil.

'Nucleus' breeding for oil traits, accompanying a 'main' breeding population, was suggested as a practical alternative means of incorporating breeding for oil production into the 'main stream' breeding strategy. The establishment and management of the oil-nucleus and the strengths and weaknesses of this approach were discussed in detail in Chapter 8. The high rate of gain at reasonable cost, drawing on available expertise in tree breeding, were highlighted as positive features of the plan. Ways were suggested of circumventing a problem with early narrowing of the genetic base and the resultant consequence of inbreeding by infusing new selections from the second-generation 'main' population and introducing new genetic material from Australia.

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