Genetic Variability and Leaf Waxes of some *Eucalyptus* Species with Horticultural Potential

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B.Ag.Sc. (Hons)

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

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Waite Agricultural Research Institute

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September 1998

Eucalyptus macrocarpa

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Abstract

Optimum pruning height for cut foliage production was initially investigated for 3-year-old trees of *Eucalyptus globulus* Labill. Trees cut at a height of 1.0 m above ground level had most stems resprouting from the trunk, but a pruning height of 0.5 m produced the longest stems. Seventeen species of *Eucalyptus* L'Hér. were subsequently subjected to trials to investigate their suitability for floriculture and the effect of pruning for cut foliage production. There was variation in leaf colour within and between species, and in the time to phase change and flowering. There were significant differences at 16 months between species for tree height, trunk diameter and lignotuber diameter with *E. globulus* having the largest dimensions. Following pruning at 19 months, there was an initial significant interaction between species and pruning height in relation to tree height, height increment, and trunk and lignotuber diameter. After one year there were significant differences between species, in the length of stems, number of stems and total weight of stems, with *E. globulus* producing the highest number and weight of stems. There was a positive correlation of trunk diameter and lignotuber diameter at 3 and 6 months since pruning, with the number of cut foliage stems produced at 12 months. For *E. gunnii* J.D.Hook. pruning to 1.0 m at 25 months produced most stems at 6 months after pruning.

Postharvest trials were conducted to assess the vase life of cut stems, and the effect of pulsing and simulated transportation on vase life. Holding solutions containing 1% or 2% sucrose and 8-hydroxyquinoline citrate (8-HQC) at 200 $mg.L^{-1}$ significantly increased vase life of *E. globulus* and *E. cinerea* F.Muell. ex Benth. over the control, but pulsing *E. cinerea* in 1%, 5%, or 10% sucrose plus 8-HQC for 2 hours at 24 °C or 24 hours at 3 °C had no effect. In simulated transport trials, pulsing overnight in 1% or 5% sucrose plus 8-HQC at 3 °C followed by 1 week dry storage at 3 °C had no effect on the vase life of cut stems of *E. sideroxylon* Cunn. ex Wools., *E. platypus* Hook., *E. spathulata* Hook., *E. cladocalyx* F.Muell. x *E. platypus,* or *E. spathulata* x *E. sargentii* Maiden, but a 5% sucrose pulse plus 8-HQC significantly increased the vase life of *E. spathulata* x *E. platypus*. A long pulse at low temperature (24 hours/3 °C) followed by

1 week dry storage was more effective than a short pulse at high temperature (2 hours/24 °C) for *E. albida* Maiden & Blakely stems and no sucrose was more effective than 1% or 5%. Thus, a 2% sucrose holding solution extended vase life of *E. globulus* and *E. cinerea.* There was no advantage of sucrose pulsing to extend vase life, or to improve vase life following dry storage, except for the hybrid *E. spathulata* x *E. platypus*.

Eighteen species of *Eucalyptus* were studied for changes in wax morphology of juvenile foliage with leaf age using Environmental Scanning Electron Microscopy. Three species were studied for wax regeneration following removal from the adaxial surface of day 16 and day 30 leaves (16 and 30 days following lamina separation, respectively) while still attached to the tree. For each leaf age, four leaves per species were sampled at 0, 1, 3, 9 and 15 days after wax removal. All species had tube wax on juvenile leaves but there were differences between the species, in the length and conformation of tubes and percentage surface area covered by wax. Tube length was greatest in day 30 leaves, but tube diameter was narrower than day 0 leaves (unfolding leaves). There were significant differences in wax structure between the proximal and distal ends of day 0 leaves of ten species, and tubes were observed crystallising from amorphous wax deposits in the proximal area only. Significant wax regeneration occurred more rapidly on day 16 leaves than day 30 leaves. Wax morphology was consistent across six species of the series *Viminales*. Thirteen species of *Eucalyptus* are recommended for the cut foliage industry.

Variation in amount and composition of leaf epicuticular wax among 17 species of *Eucalyptus* was characterised by gas chromatography (GC), thin-layer chromatography (TLC), and gas chromatography-mass spectrometry (GC-MS). Across species, wax yield ranged from 0.7 to 4.5 mg/cm^2. The major wax constituent in all species except *E. delegatensis* R. Baker, was β-diketones (24.7–83.0%), followed by wax esters (6.4–26.5%), n-alkanes (3.5–26.5%), fatty acids (3–15.9%), n-alcohols (0.6–11.1%) and aldehydes (0–9.2%). Volatile organic compounds emitted from developing *Eucalyptus* leaves have been studied. Headspace samples were collected from six species in a plantation from leaves *in situ*, using solid phase microextraction (SPME),

and were analysed by GC and GC-MS. Leaf oils were extracted and the relative amounts of volatile compounds in the extract and headspace were assessed. The major volatile constituents were α-pinene (11.4 – 57.8%, headspace; 15.3 – 32.2%, oil extract) and 1,8-cineole (3.2 – 88.6%, headspace; 20.8 – 54.7%, oil extract). Other terpenoids present in the headspace in significant quantities were alloaromadendrene, viridiflorene, thujene, α-phellandrene, γ-terpinene and limonene. In the oil extract an unidentified compound at R_t 21.2 min. (GC), alloaromadendrene, α-phellandrene, phenol derivative, viridiflorene and limonene were present in significant quantities.

Eucalyptus gunnii leaves can appear as green or glaucous phenotypes with the latter more desirable for floriculture. The epicuticular wax from these two types were compared morphologically using environmental scanning electron microscopy (ESEM), chemically using gas chromatography, and molecular markers were found to distinguish the two types using RAPDs and bulked segregant analysis. Both phenotypes had tube wax which in the glaucous type were significantly longer and thicker and the surface area was covered more densely than on the green type. When compared chemically, the glaucous wax contained a higher percentage of alkanes, alcohols and free fatty acids, but lower percentages of β-diketones and esters than the wax from the green type. The glaucous type had a greater yield of wax per unit area of leaf than the green type. Seven molecular markers were found which would distinguish the green and glaucous bulks. No individual marker could totally distinguish all green individuals from all glaucous individuals but this could be achieved using combinations of markers. These markers may facilitate the management of *E. gunnii* breeding and selection for the cut foliage industry, by providing an initial screen for glaucousness.

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Acknowledgements

This work was conducted with the support from a University of Adelaide Postgraduate Research Scholarship. I would like to thank my supervisors Prof. Margaret Sedgley, Dr. Graham Jones and Dr. Graham Collins, who have guided and encouraged me throughout my studies. Thanks also to all the growers who allowed me access to their properties and eucalypts, especially Ralph Middenway and David Manley; Rita Middleberg and Helena Oakley for statistical assistance; CEMMSA for access to the ESEM; my fellow postgrads especially Raelene Mibus, Tina Maguire and Kate Delaporte and staff in the department for their friendship. A special thanks to my parents Alf and Barbara without whom I couldn't possibly have studied full time, Kim Anderson for his love, great support and sense of humour, and to Angus, Ingrid and Grace, who can now have their mum back.

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Glossary

abaxial. The side of a leaf furthest from the axis of the branch or stem on which it grows (usually lower).

adaxial. Side nearest to axis (usually upper).

adult. The final growth phase of the leaves.

adventitious. Any organ produced in an abnormal position.

alternate. Leaves or flowers inserted individually at different heights along the branches.

axil. Angle formed between a stem and a leaf.

axillary. Arising from the axil of a leaf or a bract.

broad-lanceolate. Lance-shaped, finally pointed, length:breadth up to 5:1.

buds (accessory buds). Concealed buds produced from tissues which originate in the axil between one primary bud and the leaf and persist in the bark indefinitely, and are the source of epicormic shoots.

clinal. More or less continuous morphological variation of a species with an ecosystem (plant community).

clone. individuals derived by vegetative propagation or apomixis from a single original individual.

concolorous. The leaf is the same colour on both sides.

connate. The leaves of an opposite pair are joined around the stem.

coppice. Juvenile shoots developed from dormant buds on main trunk or lignotuber.

cordate. Heart-shaped, usually referring to the base of the leaves.

crenulate. Having small rounded teeth on the margin.

decussate. Having leaves in pairs, each pair being at right angles to those above and below.

discolorous. The upper surface of the leaf is different in colour from the lower surface.

elliptical. Roughly in the form of an ellipse, tapering fairly evenly at both ends which can be rounded or pointed.

emarginate. With a shallow notch at the end of the leaf.

entire. When the edges of the leaves are smooth.

epicormic. Growth arising from accessory buds in the trunk or stems of a tree after the branches have been cut off or destroyed.

falcate. Curved, like the blade of a sickle.

fusiform. Spindle-shaped, cigar-shaped.

gene. The unit of inheritance located in the chromosome, which controls the development of character.

genotype. Hereditary constitution of an individual, with or without phenotypic expression of the one or more characters it underlies; interacts with the environment to produce the phenotype; also individual(s) characterised by a certain genetic constitution.

genus (pl. *genera*). A group of species which resemble each other.

glands. A single cell, a group of cells, or frequently a cavity from breakdown or separation of localised cells, functioning as a collector of specific secretions, such as oil in eucalypts.

glandular. Having glands, usually implying that such are conspicuous.

glaucous. Referring to any blue-green or grey-green appearance caused by a whitish 'bloom' or wax covering.

granular. Covered with small rounded protuberances.

habit. The general appearance or characteristic growth form of the plant.

heterophylly. The existence of two or more forms of leaf on the same plant or on different plants of the same species, often as a result of juvenile vs. mature growth.

hybrid. The progeny resulting from the crossing of two unrelated parents, usually different species.

intermediate. Referring to leaves which are a gradation between juvenile leaves and the adult leaves.

juvenile. Referring to leaves of young plants or shoots from the base of older plants, particularly when these differ in shape from leaves of mature plants.

lanceolate. Lance-shaped, refers to leaves with the length:breadth up to 8:1.

lignotuber. A swollen, woody structure, at the base of many eucalypts, containing a mass of vegetative buds and substantial food reserves.

locus (pl. *loci*). The position occupied by a gene in a chromosome.

mallee. A shrubby eucalypt with several stems arising from an underground lignotuber, or the vegetation characterised by such species.

morphology. Referring to form or shape of an organism and its parts.

mucronate. Ending in a short point.

node. The swollen part of the stem from which leaves or branches arise.

oblique. The two halves of the leaf blade meet at different points on the petiole.

oblong. The sides of the leaves are parallel and narrow abruptly at both ends.

obovate. Ovate with the broadest part above the middle.

opposite. Two leaves or other organs rising from the same level on opposite sides of the stem.

orbicular. Disc-shaped, a flat body with a circular outline, length:breadth about 1:1.

ovate. Broadest at the lower end, resembling the longitudinal section of an egg, length:breadth up to 3:1.

pendulous. Hanging, pendent.

petiolate. Supported on a petiole.

petiole. Stalk of a leaf.

phenotype. Observable characteristics of an organism produced by the interaction of genes and environment.

provenance. Origin, source, place where found or produced as a cultivar or selection of a taxon.

pruinose. Covered with a powdery, waxy material.

pulverulent. Consisting of dust or fine powder.

quadrangular. Four-sided.

section. A natural group between subgenus and series used in the classification of plants.

series. A natural group between section and species used in classification of plants.

sessile. Lacking a leaf stalk.

species. A division of the genus, each species (group of individual plants) possessing characters which distinguish it from other species of the same genus.

subgenus. A natural group between genus and section used in classification of plants.

subopposite. Almost opposite.

subspecies. A form of a species having distinctive identity and occupying a particular habitat or region.

taxonomic. Referring to the classification and identification of living things, and often known as systematic botany.

taxon. General term applied to any taxonomic element, population, or group irrespective of its classification level.

variety. One of two or more forms of a species with a minor morphological distinction.

Chapter 1

General Introduction

Eucalyptus L'Héritier (Myrtaceae) is one of the world's most economically important and widely planted genera. This genus contains more than 700 species (Brooker and Kleinig, 1994) and they grow in a wide range of climatic conditions. The use of *Eucalyptus* species in Australia has, until recently, been mainly confined to the hardwood timber industry, for pulp and paper production, and for fuel as firewood. Apart from these, eucalypts are used commercially for production of essential oils for medicinal, industrial and perfumery use, and for honey production. However, for the last few years some species, such as *E. cinerea*, *E. pulverulenta* and *E. gunnii* have found favour with the Australian florist trade as cut foliage. Australian florists have used *Eucalyptus* foliage in the dried state for nearly a decade but fresh foliage is now becoming more popular with consumers. *Eucalyptus* species for cut foliage have been popular in Germany and Italy since the early 1980s (Götz, 1986; Bazzocchi *et al.*, 1987; Rumine and Bellandi, 1989). The value of the floriculture industry has increased in Australia in recent years with foliage exports in 1993/94 worth approximately $1.9 m, an increase of 186% on the previous year and indicative of an expanding world market. Australia exports fresh and preserved material to Japan, USA, the Netherlands and Germany. The increase in demand by consumers has been accompanied by an increased awareness that quality could also be improved, and research to improve these foliage crops should centre around quality attributes such as plant architecture, to increase the total production per unit tree, colour, and postharvest quality (Woodson, 1991). Currently much foliage comes from bush picking which may be endangering some rarer species, and may also suffer from insect damage, which reduces its commercial value. Large scale, managed plantations of *Eucalyptus* should provide the quality and quantity of foliage that local and overseas florists and consumers demand and help to protect rarer species.

To evaluate whether a particular *Eucalyptus* species has horticultural potential, it

must show some, if not all, of the following characteristics (Wittner, 1971).

1. Ornamental quality (e.g. flowers, fruit, foliage, growth form).
2. The foliage must be commercially acceptable for a large part of the year.
3. Usefulness for a special purpose (e.g. windbreak, ground cover, resistance to salt spray, droughts etc.).
4. Relative ease of propagation, and propagation material must be readily available.
5. Tolerance of the plant to a variety of soil types and climatic conditions.
6. The plant should have a long life span.
7. Resistance to fungal, bacterial and viral diseases and pests.

Variability within the genus *Eucalyptus* and within species is large with a large range of forms, leaf, flower and fruit morphology, and they grow in a wide range of climatic conditions. Thus, there is great opportunity to expand the current numbers of species used in the floriculture industry. A breeding and selection program is underway for improved and superior types (Delaporte *et al.*, 1998; Ellis *et al.*, 1991). With most species currently used in the industry, the juvenile foliage is favoured because the waxiness of the leaves gives them a characteristic blue or silvery colour which is very popular, and their shape is often small, rounded and attractive.

The genus *Eucalyptus*

The genus *Eucalyptus* was first named and taxonomically described by Charles L'Héritier de Brutelle in 1788. The name *Eucalyptus* is a latinised Greek compound, 'eu' meaning well, and 'kalyptos' meaning veiled or covered, an allusion to the calyx and/or petals which form a lid (operculum) over the flower bud.

It is a genus of over 700 species (Brooker and Kleinig, 1994), most endemic to Australia, but several extending to Malaysia and the Philippines. They occur in most parts of Australia with the greatest diversity located in the near-coastal regions of N.S.W. and in south-western W.A. The eucalypts are the dominant feature of the vegetation in Australia, numbering about 3/4 of the total flora. Since their original discovery in 1770 by Joseph Banks and Daniel Solander, the usefulness, variety and

adaptability of the eucalypts has caused them to be widely planted throughout the world.

Lignotubers and mallee

Eucalypts range in form from low, shrubby, multi-stemmed mallees to tall, single-stemmed forest trees. The mallee is a growth form in eucalypts in which several stems arise from a lignotuber. Lignotubers occur in some species but not in others and they are formed at an early stage of the seedling. They originate from vegetative buds in the axils of the cotyledons and the first-formed leaves of the young seedling, which form tuberous swellings and unite to form the lignotuber. They are usually buried in whole or in part in the surface soil. The lignotubers contain much bud-producing tissue capable of forming coppice shoots when the old shoot is destroyed. The seedlings can develop lignotubers at different growth stages. If the plants are stressed i.e. lack of water, the lignotubers develop within a few months of germination. On the other hand if plants are in favourable conditions, the lignotubers may develop later. When large populations of a lignotuberous species are studied a number of individual plants are found to lack a lignotuber due to variation within the species (Ladiges, 1974).

Lignotubers are persistent in mallee but present in many *Eucalyptus* species at the seedling stage but thereafter not retained. In mallee the tuberous mass may continue to grow and finally become woody although it remains living tissue with many dormant vegetative buds. The lignotuber may live on for 200-300 years (Anon., 1981). On the death of the plant stem, which may come about by fire or by cutting, the buds within the lignotuber develop and produce new stems which continue the life of the individual. When several more or less equal stems from a single lignotuber grow into a mature plant, the mallee form is produced.

Mallee eucalypts are characteristic of large areas of open-scrub formation in different parts of Australia. They usually occur on sandy soils in dry climates, but mallee scrub was present in areas of up to 500 mm rainfall before wheat farming was introduced into Australia.

Leaf phases

The leaves on a mature eucalypt plant are usually completely different in shape, size, and colour from those of the seedling. This is termed heterophylly. Four leaf phases are recognised in the development of a eucalypt plant - the 'seedling', 'juvenile', 'intermediate' and 'adult' phases.

The basic phyllotaxy of *Eucalyptus* is very simple. In all except a very few species, the leaves form in pairs on opposite sides of a square stem, consecutive pairs being at right angles to each other, that is 'decussate'. The first few to many pairs in most species remain opposite despite elongation of the stem; they may be sessile or petiolate and usually held horizontally. Later pairs which have been formed opposite as usual at the stem apex as in the mature plant, become separated at their bases by unequal elongation of the stem, the twisting of the internodes between the pairs, and twisting of the petioles. There is great divergence in the form of the leaves in the early phases but there is a significant convergence in form in the adult phase, and in most species the adult leaves are lanceolate, petiolate, apparently alternate, and green. There is no definite transition point between phases; the intermediate phase links the juvenile and adult phases.

Some species are mature in the juvenile leaf phase, e.g. *E. pulverulenta* and *E. cinerea*, although intermediate and occasionally adult leaves develop on the older plants. However they still flower on branches where the leaf form is juvenile in morphology. It seems that the juvenile leaf form is not associated with the occurrence of flowering. The transition to adult from juvenile foliage and the onset of flower bud development are not necessarily expressed at the same stage of development in all species or even in all individuals within one species.

Heterophylly is probably more striking in *Maidenaria* than in any other group in *Eucalyptus* and the changeover from the long-lasting juvenile phase in *E. globulus* to the remarkably different intermediate phase is readily seen on saplings both in the field and in cultivation. *E. globulus* is notable as well for producing the largest adult leaves in the genus. It was suggested decades ago that the juvenile leaf form may be the ancestral

form and that in passing from the juvenile to the mature stage the plant is, in effect, repeating the historical development of its species.

Coppice foliage produced on stems growing from the stump of a felled or burnt tree or leaves produced on the trunk or in the crown from dormant buds, usually has the form of the juvenile leaves. Further development of these shoots will result in the growth of intermediate then adult leaves. Coppice shoots arise from strands of bud-producing tissue that originate from leaf axils and persist within the phloem. Species with lignotubers generally coppice well, however, the ability to coppice declines with age but at a given age the larger stumps tend to produce more vigorous coppice (Jacobs, 1955).

Leaf waxes

Wax structure

The aerial surfaces of higher plants are covered partially or completely with amorphous wax as part of the cuticle. Various formations of crystalline wax are frequently superimposed upon these amorphous layers. The structures of the crystalline waxes range in form, and include rodlets, tubules, platelets, granules and threads. All *Eucalyptus* leaves are covered by a layer of epicuticular wax but the structure and composition of the wax is not necessarily alike in all species. There are reported to be three main types of wax present on eucalypt leaves (Hallam, 1970a,b), tube-like only, plate-like only, or tube- and plate-like waxes combined. The tubular waxes of many *Eucalyptus* species are 2-3 μm long and 0.22-0.26 μm thick and are arranged predominantly parallel to the cuticle usually fused forming a complex branching network (Baker, 1982).

Wax chemistry

Waxes are, in the strictest sense of the word, esters of long-chain acids and long-chain primary alcohols. Epicuticular waxes however, are composed of a mixture of different compounds including cyclic and long-chain aliphatic components. In most plant species, surface waxes are mainly composed of long chain aliphatic hydrocarbons. Aliphatic compounds are saturated or unsaturated open-chain or cyclic compounds not containing a benzene ring. The hydrocarbons in plant waxes have chain lengths of

between C_{20} and C_{35}. The molecules vary in polarity depending on the position and type of any substituted groups that may be present, such as carboxyl (-COOH) and hydroxyl (-OH). In eucalypts, the percentage of hydrocarbons e.g. n-alkanes, in epicuticular wax is no more than three per cent of the whole wax (Table 1).

Table 1.1. Wax composition of *Eucalyptus globulus* leaves (Horn *et al.*, 1964).

Percentage of whole wax	Constituent
56-57	β-diketones
14-15	esters of alkanoic acids & alcohols
9	free alcohols
6	free acids
2.8	flavones
1-2	sterols
2.1	hydrocarbons
6	unidentified compounds

The main constituents of many *Eucalyptus* epicuticular waxes are long chain β-diketones, and long chain primary alcohols. The β-diketone-rich waxes form tubular structures, which give the leaf a white 'bloom' or glaucous appearance and are readily rubbed off, whereas the primary alcohol-rich waxes form plate-like structures which give a more dull matt appearance to the leaf and are more resistant to abrasion (Figure 1.1) (Hallam and Juniper, 1971). These waxes occur either on different species, or on different parts of the same species, such as leaves, petioles, stems and fruit, (Juniper and Jeffree, 1983). β-diketones also occur in the waxes of *Acacia* species (Horn and Lamberton, 1962).

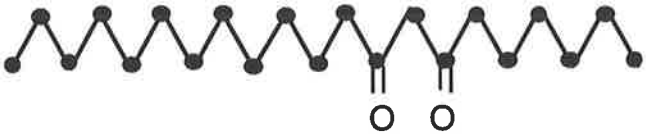
The β-diketones contain an odd number of carbons, and the chain lengths found so far are C_{29}, C_{31} and C_{33}, all of which are present in eucalypts. The hydrocarbons present in β-diketone-containing waxes do not have the same chain lengths as the β-diketones, which may suggest that the two groups are synthesised via different pathways. For example in *E. globulus* wax the major hydrocarbon is C_{29} but the major β-diketone has 33 carbons in the chain. Many chain-elongating enzyme systems with different specificities are involved in the generation of the diverse number of chain

length classes found in plant epicuticular waxes. The proposed mechanism for the biosynthesis of hydrocarbons is an elongation-decarboxylation process, where a C_{16} or C_{18} fatty acid is elongated to appropriate chain length followed by decarboxylation (Kolattukudy *et al.*, 1981). β-diketones are thought to be generated by a modified elongation-decarboxylation mechanism.

primary alcohol type :

primary alcohol type general formula : $CH_3 \bullet (CH_2)_n \bullet OH$

β - diketone type :



β-diketone type general formula : $CH_3 \bullet (CH_2)_n \bullet CO \bullet CH_2 \bullet CO \bullet (CH_2)_m \bullet CH_3$

Figure 1.1. Stylised structures of the two major wax types present in *Eucalyptus*. (Juniper and Jeffree, 1983)

The chief β-diketone in the waxes of *Eucalyptus globulus, E. cinerea* and *E. pulverulenta* is n-tritriacontan-16,18-dione [n-$C_{15}H_{31} \bullet CO \bullet CH_2 \bullet CO \bullet C_{15}H_{31}$] (Horn *et al.*, 1964). Osawa and Namiki (1981) isolated a derivative of this compound, 4-hydroxytritriacontan-16,18-dione, from *Eucalyptus* leaves and found it to possess strong antioxidative activity in water and alcohol systems. This natural antioxidant isolated from leaf waxes could potentially be produced commercially for use in the food industry. A less common isomer that has been isolated from *E. risdoni* leaf wax is n-nonacosan-12,14-dione [n-$C_{15}H_{31} \bullet CO \bullet CH_2 \bullet CO \bullet C_{11}H_{23}$] (Horn & Lamberton, 1962). Horn *et al.* (1964) measured the amount of β-diketones in many *Eucalyptus* species and found that *E. globulus* and *E. pulverulenta* epicuticular wax contained nearly one and a half times more β-diketone than *E. cinerea* but only slightly more than *E. crucis*. Wax from *E. macrocarpa* fruit contained nearly double the amount of β-diketone than that of the leaves of the same species.

Primary alcohols are a common wax class in eucalypts, but only three homologues consistently constitute major wax components. They are hexacosanol ($CH_3 \bullet (CH_2)_{24} \bullet CH_2 \bullet OH$), octacosanol ($CH_3 \bullet (CH_2)_{26} \bullet CH_2 \bullet OH$), and triacontanol ($CH_3 \bullet (CH_2)_{28} \bullet CH_2 \bullet OH$), the first two being major constituents of the leaf wax of many species of *Eucalyptus* and *Acacia.* Horn *et al.* (1964) have identified esters of alkan-2-ols, composed primarily of odd chain length homologues (C_9-C_{17}) in the leaf waxes of *Eucalyptus* species.

Eucalyptus globulus leaf wax also contains small amounts of C-methylated flavones as aglycones. Four of these compounds have been isolated, eucalyptin (4',7-dimethoxy-6,8-dimethyl-5-hydroxyflavone), 8-desmethyl-eucalyptin, sideroxylin, and 8-desmethyl-sideroxylin (5,4'-dihydroxy, 7-methoxy, 6-methyl-flavone) (Figure 1.2), the latter being present in *E. gunnii* and *E. cinerea* also (Wollenweber and Kohorst, 1981). Courtney *et al.* (1983) isolated eucalyptin from the leaf waxes of *Eucalyptus elata, E. eximia, E. gummifera, E. haemastoma, E. luehmanniana, E. maculata, E. michaeliana* and *E. youmanii,* and also 8-desmethyl-eucalyptin from the latter two species.

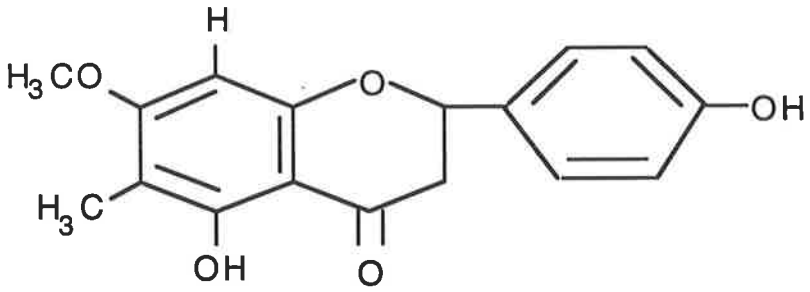


Figure 1.2. Structure of 8-desmethyl-sideroxylin (5,4'-dihydroxy 7-methoxy 6-methyl-flavone) (Wollenweber and Kohorst, 1981).

Ontogenetic variation

Developmental changes in epicuticular wax are known to occur in some species. Wax morphology on specific organs of individual species is generally constant, but there is variation in epicuticular wax deposits, particularly in the size and distribution of the particles, during the ontogeny of many plant tissues. Bukovac *et al.* (1979) found that epicuticular wax deposition on *Prunus persica* leaves increased with leaf

development until full expansion. The crystalline waxes formed during the later stages of expansion were superimposed on the amorphous layers which predominated on the surfaces of immature leaves.

Wax composition also changes during tissue development. Gulz *et al.* (1991) analysed leaves of *Tilia tomentosa* and showed that wax composition on mature leaves was different from that of leaves in the bud. Biosynthesis of some wax components did not occur until one month after unfolding. Tulloch (1973) established that the wax constituent octacosanol was formed preferentially during the early stages of growth of *Triticum durum,* whereas the production of β-diketones increased as the leaf expanded. The increased β-diketone content is associated with greater glaucousness of the flag-leaf and the leaf-sheaths compared with younger leaves.

Variation in thickness and composition of leaf wax of *Citrus* species has been shown to occur with leaf age (Freeman *et al.*, 1979). The dominant wax class in post-emergent leaves, secondary alcohols, declined during the stages of expansion and were replaced by primary alcohols and hydrocarbons. Similarly, the wax on apple fruits undergoes progressive changes during growth and subsequent storage (Skene, 1963), and the ratio between esters and ursolic acid increases during growth. During storage the fruits become greasy as a consequence of the production of short chain wax esters.

Baker and Hunt (1981), studied several different eucalypt species for developmental changes in wax composition and found that the production of individual components varied considerably throughout the course of leaf development. For *Eucalyptus globulus*, the differences found in the proportions of wax classes arose from preferential formation of individual constituents. Since the composition of epicuticular wax is reflected in wax structure, the chemical changes that occur during leaf ageing may be related to the appearance of wax fine-structure, and these changes may reflect an alteration of metabolism. The changes in production of wax components and their constituent homologues over time are a result of increased rates of synthesis. There seems to be no evidence of the decomposition of wax components during leaf expansion, which is the period when changes in leaf surface properties occur.

Wax extraction and separation

Wax can be extracted by dipping fully expanded young leaves in chloroform. Chloroform is used because it is relatively non-polar. It is one of the least selective solvents used for the removal of wax constituents, and the extraction method is relatively quick, simple and reproducible. The extracts are pooled and dried either by blowing nitrogen gas over the chloroform extract, or by rotary evaporation at room temperature. The individual constituents of epicuticular wax can be separated by thin layer chromatography (TLC) using internal standards as reference markers, or by silica acid/alumina column chromatography (Misra and Ghosh, 1992). The latter can be used as a preparative method and TLC as an analytical method.

The structure of the compounds extracted can be determined by gas chromatography-mass spectrometry (GC/MS) analysis. The chain lengths of the *Eucalyptus* alcohols belonging to both primary and secondary series can be determined by gas chromatographic analysis of the oxidation products. On oxidation the primary alcohols yield acids which are examined by GC and GC/MS as their methyl esters, and the secondary alcohols yield methyl ketones. The composition of the hydrocarbon fractions of the epicuticular waxes can be analysed by gas chromatography. Tulloch and Hogge (1978) have shown that GC/MS of β-diketones that have been converted to trimethylsilyl (TMSi) enol ethers, leads to simpler spectra and better resolution than those obtained with untreated β-diketones.

Functions of epicuticular wax

Glaucousness

Glaucousness is a feature of some species especially in the juvenile and coppice leaves of *E. bridgesiana*, *E. globulus*, *E. pulverulenta* and *E. cinerea*. Glaucousness in most cases is related to the waxy bloom which covers the juvenile leaves, so that they appear silver, blue-green or grey-green. Glaucousness is lost in many species after the juvenile phase but is generally maintained to the inflorescence structures in species which mature in a juvenile leaf phase, e.g. *E. pulverulenta* and *E. cinerea*. Glaucousness is under genetic control and the genes may act either by controlling wax chemical composition or by changing the way in which the wax is secreted. Within *Eucalyptus*

species, glaucousness is associated with frost resistance, and its frequency increases in more frosty areas (Barber, 1955). This clinal variation occurs in many frost resistant species such as *E. urnigera, E. gunnii* and *E. delegatensis*. The action of the wax may be to facilitate the rapid run-off of water from the leaf surface to prevent freezing of the leaf. Hallam (1970b) examined the contact angle of a 2 µl water droplet and showed that species having tube waxes were more water repellent than those having plate waxes.

The glaucousness of eucalypt leaves is one of the main features that make them attractive to consumers. This waxy bloom on the leaves is caused by the reflection and scattering of light on the surface, by wax crystals of different forms and angles, whose dimensions are close to or slightly above the wavelength of light (Juniper and Jeffree, 1983). The closer the wax branches are packed the more intense is the blue colour of the wax bloom. Glossy mutants have wax deposits which are either smooth films on the cuticle, or platelets which lie flat on the surface. Juvenile leaves of *Eucalyptus delegatensis* appear glaucous but it is not due to epicuticular waxes, but to irregularities in the structure of the cuticle and epidermis (Barber, 1955).

Environmental adaptations

Epicuticular wax reduces water loss during cuticular transpiration by increasing the diffusive resistance to gas exchange. The water repellency of plant epicuticular waxes plays a role in the protection of the stomata from the sudden inflow of water where the leaves are commonly wetted by rain or dew, or to prevent the ingress of fungal hyphae. Plant epicuticular waxes may also play a role in plant-insect interactions, such as acting as insect antifeedants and as obstacles to insect movement (Farrow, *et al.*, 1994; Stone and Barrow, 1994; Eigenbrode and Espelie, 1995). The waxes may also act as a physical barrier against entry of pathogens and possibly incompatible pollen tubes into the stigma.

Waxiness is often associated with the plant's adaptation to high light environments, such as alpine conditions. The presence of highly reflective wax on plants that grow in environments with high solar radiation, leads to a reduction in the energy absorbed by the leaves, and also a reduction in leaf temperature (Robinson *et al.*,

1993). Wax filaments lower the net radiation absorbed by the leaf, by increasing reflectance and thickening the boundary layer, thereby reducing the likelihood of photoinhibition.

Epicuticular wax may play a role in the trapping of volatile compounds which are produced from within the plant. The wax layer with the trapped volatiles then contributes to the external aroma of plants. It may also act as a concentrating medium for volatile compounds (Spence and Tucknott, 1983).

The cuticle and its associated waxes act as a barrier to prevent damage to the epidermal cells caused by mechanical abrasion. The wax may be weathered and removed by rainfall and abrasion but can be renewed, provided the leaf is still expanding (Hallam, 1970b).

Influencing factors on epicuticular wax

The type of wax produced is under genetic control, but the size, shape and distribution of the structure of crystalline waxes can be dramatically changed by environmental conditions. Temperature and light intensity are the principal environmental factors affecting wax deposition.

Light

Hallam (1970a) showed that the wax formed on leaves of *Eucalyptus* species which were grown in low light intensities (1-2% full sunlight) was sparse and lacked the complex branching pattern characteristic of the deposits present on unshaded plants. The form and distribution of the wax altered when light intensities fell below 20% of full sunlight. Conversely an increase in radiant energy caused an increase in the average size (from 1.5 to 6 μm) and distribution (from 125 to 225 per μm^2) of the tubular waxes of *Brassica oleracea* (Baker, 1974). Light may be an activating factor for specific enzymes which can synthesise various wax components.

Temperature and other environmental effects

The development of crystalline wax deposits can also be modified by changes in growth temperature. Baker (1974), found that as growth temperature increased, the leaf epicuticular waxes of *Brassica oleracea* tended to develop across rather than upwards from the cuticle, which resulted in the formation of dendrites, crusts and plates rather

than tubes and branching filaments. Also a decrease in temperature and humidity caused larger deposits of wax. Epicuticular wax extracted from coconut hybrids in the dry season, was found in greater quantities than when extracted in the wet season (Voleti and Rajagopal, 1991). This may be due to humidity effects, or possibly due to wax erosion in the wet season by rainfall and wind causing leaves to rub against each other.

As mentioned earlier, clinal variation of leaf wax glaucousness occurs with altitude. The higher the altitude, the more glaucous the leaves become. This has been observed in a number of *Eucalyptus* species including *E. viminalis* Labill. (Banks and Whitecross, 1971).

Effect of agricultural chemicals on leaf waxes

Agricultural chemicals such as Paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) can decrease the amount of epicuticular wax present on the leaf surface of some plants (Rao *et al.*, 1982). Marois *et al.* (1987) found that agricultural spray adjuvants, such as spreaders, stickers and penetrators, affected the epicuticular layer of grape berries (*Vitis vinifera*), which caused the rate of water movement to increase. The enzyme inhibitor and protein precipitator trichloroacetic acid (TCA) inhibits wax production and after treatment a normally glaucous plant develops leaves with less wax on the surface. These substances are used as spray adjuvants to make weeds more easily wetted and therefore susceptible to herbicides. Chemicals which are most likely to penetrate the epicuticular wax and cuticle are lipophilic compounds, due to the chemical properties and structure of the cuticle and associated waxes.

Taxonomic significance of wax structure and composition

Useful information can be obtained from wax studies for the purpose of plant taxonomy. Horn *et al.* (1964) and Hallam and Chambers (1970) chose β-diketone content, because it is the most abundant wax constituent of many eucalypts, to investigate relationships between the species. Their studies mainly supported the then current botanical classification of Blakely (1965). They found that the pattern of wax morphology confirmed the homogeneity of the tropical Corymbia or Bloodwood group, and that these species have characters in common with the closely allied genus

Angophora. Other potential variables could be used to study taxonomic relationships between *Eucalyptus* species such as percentage of a class of component in the wax, component chain length, or distribution over different parts of the plant (Tulloch, 1976). Thus with β-diketones, the chain length and the position of the diketone group could be relevant in taxonomy. Barber (1955) found a correlation between *Eucalyptus* taxonomy and melting point of leaf wax, which is related to the chemistry of the wax. More recently Stevens *et al.* (1994) used the variation in alkane and triterpene composition in epicuticular wax of *Sedum* species to study the taxonomic classification of these plants. They found that the distribution of the triterpenes was closely linked to the infrageneric classification.

Patterns of wax morphology can also have applications in taxonomy. Hallam and Chambers (1970) surveyed 315 species of *Eucalyptus* and found that the degree of ornamentation on the margins of plate waxes generally agreed with taxonomic groupings. They also suggested that the character of simple tube waxes, may be an adaptation to arid zones, and that the evolutionary trend in wax morphology is one from complex digitately edged plate waxes to relatively simple tube waxes.

Thesis aims

This project aims to investigate five aspects relating to the use of *Eucalyptus* in the cut foliage industry. These aims are:

1. To study field variation in growth rate, colour of leaves, amount of branching before and after pruning trials, and the transition time from juvenile to adult leaf phases within *Eucalyptus* species with horticultural potential.

2. To perform postharvest trials on cut stems of *Eucalyptus* to determine the optimum holding solutions for increased vase life.

3. To investigate the structure of the leaf waxes and variability between species and to study *in situ* wax secretion during leaf development, and regeneration following wax removal.

4. To determine the chemical composition of the leaf waxes and leaf volatiles and variability between species.

5. To perform PCR analysis using bulk segregant analysis on a population of *E. gunnii*. These techniques in molecular biology will be used to search for markers that may be involved in the process of epicuticular wax production.

Chapter 2

Plant Material

Eighteen species of *Eucalyptus* were assessed for their horticultural potential as cut foliage. Classification follows Pryor and Johnson (1971) and Chippendale (1988).

Table 2.1. Taxonomy of eighteen species of *Eucalyptus*

GENUS	Eucalyptus		
SUBGENUS	SECTION	SERIES	SPECIES
Eudesmia	Quadria	Heteroptera	tetragona
Monocalyptus	Renantheria	Eucalyptus	delegatensis
Symphyomyrtus	Bisectaria	Kruseanae	kruseana
			brachyphylla
		Subulatae	socialis
			gillii
		Curviptera	orbifolia
			crucis
			macrocarpa
		Porantherae	albida
	Maidenaria	Viminales	bridgesiana
			globulus
			gunnii
			cordata
			pulverulenta
			cinerea
	Adnataria	Striolatae	pruinosa
		Moluccanae	albens

Ten of the species are mallees and possess a lignotuber, they include *E. tetragona, E. kruseana, E. brachyphylla, E. socialis, E. gillii, E. orbifolia, E. crucis, E. macrocarpa,*

E. albida, and *E. pulverulenta.* One species, *E. gunnii* (non-lignotuberous) was looked at in detail, because of its current importance in floriculture and distinct transition from juvenile to adult foliage, with one hundred and fifty seedlings being planted. Seed was obtained commercially from Nindethana Seed Service (WA), Blackwood Seeds (SA), Waite Arboretum and the Society for Growing Australian Plants. *Eucalyptus albida* was not planted but sourced from the Waite Arboretum. Thirty seedlings of each of the remaining 16 species were planted. All of the plants were potted on into black poly bags and were planted out in autumn of 1994.

The 6 month old seedlings were planted in March 1994 into paddock W9, at the Waite Agricultural Research Institute, University of Adelaide, South Australia, 34°58'S, 138°38'E, altitude 100 m. at spacings of 3 m x 2.5 m. The trees were planted in a computer generated, randomised order, into 10 blocks of 16 species. *E. gunnii* trees were planted as one large block. There was also a seven metre headland around the perimeter of the paddock, to allow for tractor manoeuvring. The Mediterranean climate has hot dry summers and cool wet winters, with an average rainfall of 625 mm. This was supplemented in summer with weekly watering by drip irrigation. Fertiliser application consisted of 100 g 'Complete D' (NPK 8:4:8) per tree at planting and a further 100 g NPK per tree yearly, applied by hand under the tree canopy but away from the base of the trunk.

No serious problems occurred with pests or diseases. Occasionally aphids and several species of caterpillar (*Mnesampela privata, Opodiphthera helena, Entometa fervens, Doratifera* sp.) occurred, particularly on *E. cordata, E. bridgesiana, E. crucis* and *E. globulus*, but caused little damage. These pests were removed by hand. Sawfly larvae (*Perga* sp.), gum tree scale (*Eriococcus coriaceus*) leaf miners and leaf galls also appeared on *E. gunnii*. The eucalypts were kept weed free as far as possible by applications of glyphosate herbicides as required and initially by hand weeding during the first winter/spring. Meteorological data were recorded at the Waite Campus and weather charts appear in Appendix 2.

In December 1996 the *Eucalyptus* plantation was officially name the Laidlaw Plantation after Donald Hope Laidlaw AO (Figure 2.1)

Figure 2.1. Upper: The Laidlaw Plantation, Waite Campus, University of Adelaide.

Lower: *Eucalyptus tetragona* in the foreground.

Species Descriptions And Taxonomy

Table 2.1 is adapted from Pryor and Johnson, (1971) and Chippendale (1988). It shows the taxonomic relatedness between the eighteen species which were studied during the course of this project. Three of the seven *Eucalyptus* subgenera are represented, with the subgenera Blakella, Corymbia, Gaubaea, and Idiogenes not investigated in this study.

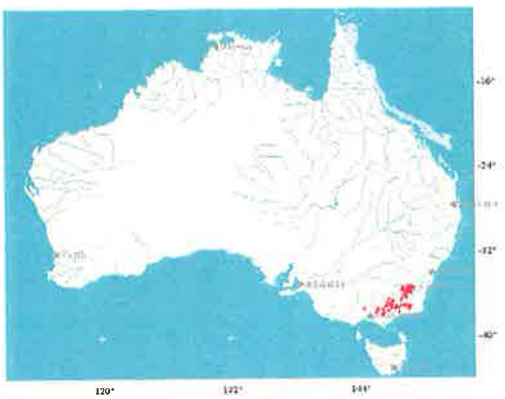
Eucalyptus tetragona (R. Br.) F. Muell.

Tallerack

Mallee to 3 m. Juvenile leaves opposite, broadly elliptic, thick, green. Adult leaves similar to juvenile leaves but glaucous. Buds quadrangular and glaucous. Favours sandy soil, but tolerates most soils. (Figures 2.1, 3.1 & 3.2).

Eucalyptus delegatensis R. Baker subsp. *delegatensis*

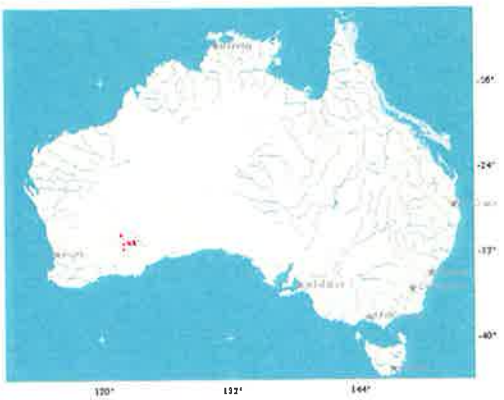
Alpine Ash



Tree to 40 m, sometimes to 90 m. Juvenile leaves sessile or shortly petiolate, opposite for a few pairs, then alternate, dull-green or glaucous, ovate. Adult leaves alternate, lanceolate, green and shiny. Prefers well-drained, deep soils, in warmer parts but prefers cool moist sites. (Figures 3.1 & 3.2).

Eucalyptus kruseana F. Muell.

Bookleaf Mallee



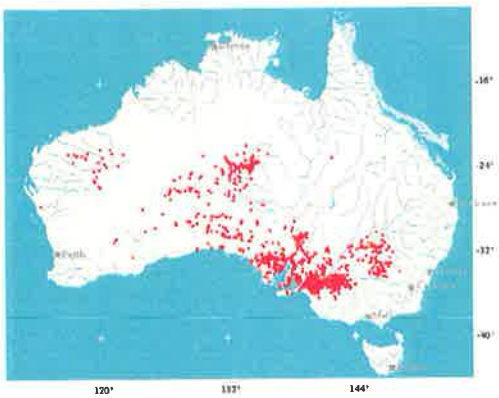
Mallee to 3 m. Juvenile leaves opposite, orbicular, glaucous, often persistent on mature plant. Adult leaves opposite, sessile, orbicular, cordate, glaucous. Grows in most soils in areas of low to moderate rainfall. (Figures 3.1 & 3.3).

Eucalyptus brachyphylla C.A.Gardner

Mallee to 3.5 m. Juvenile leaves sub-opposite to alternate, broadly ovate, glaucous. Adult leaves alternate or opposite, ovate or suborbicular, petiolate, glaucous. Suited to dry or mild temperate conditions, and limestone soils. (Figures 3.1 & 3.3).

Eucalyptus socialis F. Muell. ex Miq.

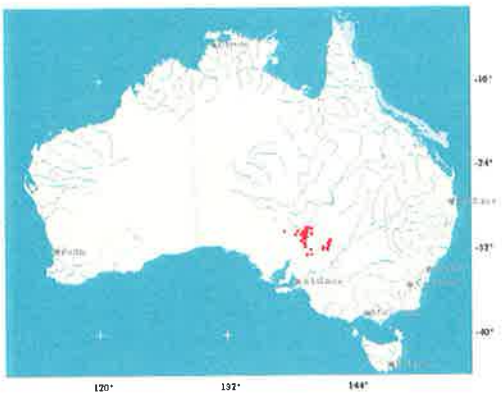
Red mallee



Mallee to 9 m, or less often, single-stemmed trees to 12 m or more high. Juvenile leaves dull green or somewhat waxy, opposite and sessile at first, then alternate, elliptic to ovate. Adult leaves alternate, lanceolate, dull grey-green. Easily grown small tree, best suited to sandy areas of low to moderate rainfall. (Figures 3.1 & 3.2).

Eucalyptus gillii Maiden

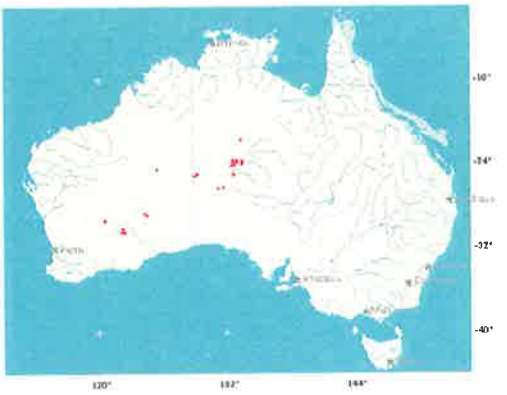
Curly Mallee



Single-stemmed, or more often mallee to 2-7 m high. Juvenile leaves opposite, sessile, glaucous, linear for a few pairs then ovate-cordate. Adult leaves only on higher branches, alternate, lanceolate, slightly glaucous. Buds glaucous, fruit waxy at first. Grows in most soils in low to moderate rainfall areas. (Figures 3.1 & 3.3).

Eucalyptus orbifolia F. Muell.

Round-leaved Mallee



Mallee, occasionally a tree, to 6 m. Juvenile leaves opposite, petiolate, suborbicular, grey-green. Adult leaves alternate, sometimes opposite, suborbicular, grey-green, pruinose. Tolerates a wide range of conditions. (Figures 3.1 & 3.2).

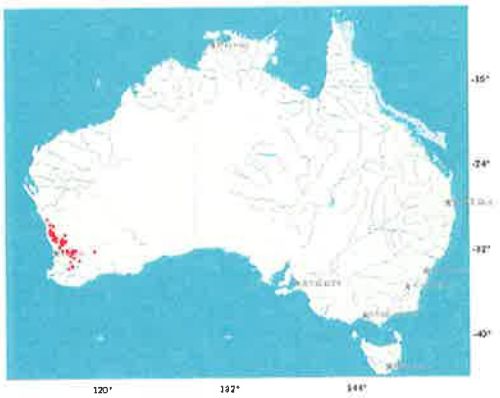
Eucalyptus crucis Maiden
subsp. *crucis*

Silver Mallee

Mallee to 7 m, spreading. Juvenile or intermediate leaves comprise the canopy. Juvenile leaves opposite, sessile, orbicular, grey-green. Intermediate leaves opposite or subopposite, petiolate to almost sessile, ovate, grey-green. Adult leaves do not develop. Buds and fruit glaucous. Suits dry to moderate, temperate areas, adapting to most soils. (Figures 3.1 & 3.2).

Eucalyptus macrocarpa Hook.

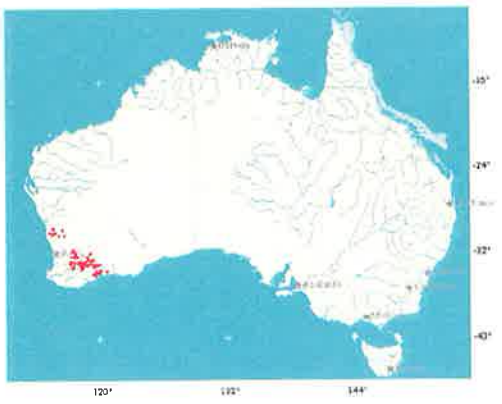
Mottlecah



Mallee to 5 m. Juvenile leaves opposite, sessile, broadly elliptic to suborbicular, grey-green or glaucous. Adult leaves opposite, sessile, broadly ovate, glaucous, thick, silvery. Best suited to light well-drained soils, but adapts to heavier soils, needs some training while the plant is still young to develop a good shape. (Figures 3.1 & 3.2).

Eucalyptus albida Maiden & Blakely

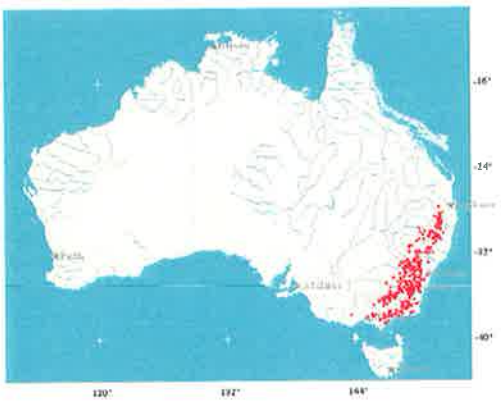
White-leaved Mallee



Mallee to 3 m. Juvenile leaves decussate, usually sessile, ovate, cordate, glaucous, often persistent as coppice on mature mallees. Adult leaves alternate, lanceolate, petiolate, green, shiny. Drought resistant, suits light or heavy soils, low to moderate rainfall. (Figures 3.1 & 3.3).

Eucalyptus bridgesiana R.T.Baker

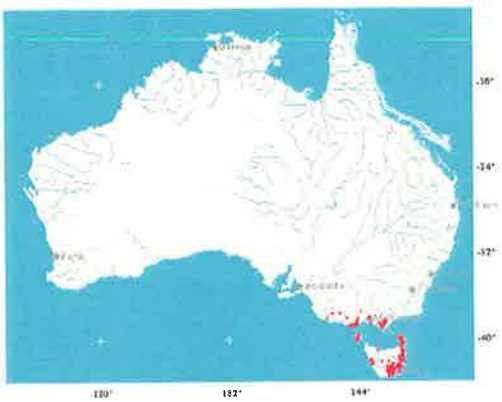
Apple Box



Tree to 22 m. Juvenile leaves opposite, sessile to shortly petiolate, orbicular to broadly ovate, crenulate, glaucous. Adult leaves alternate, lanceolate, petiolate, dark green. Grows in clayey soil. (Figures 3.1 & 3.2).

Eucalyptus globulus Labill.
subsp. *globulus*

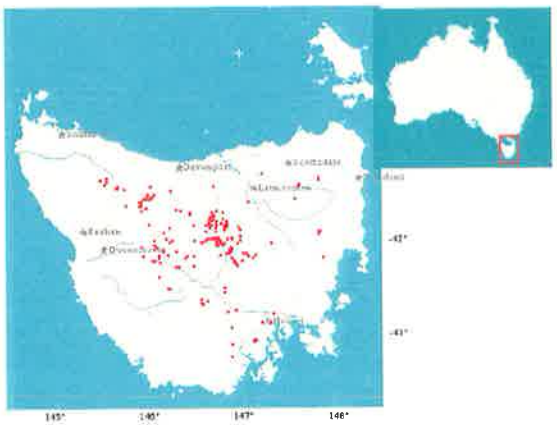
Tasmanian Blue Gum



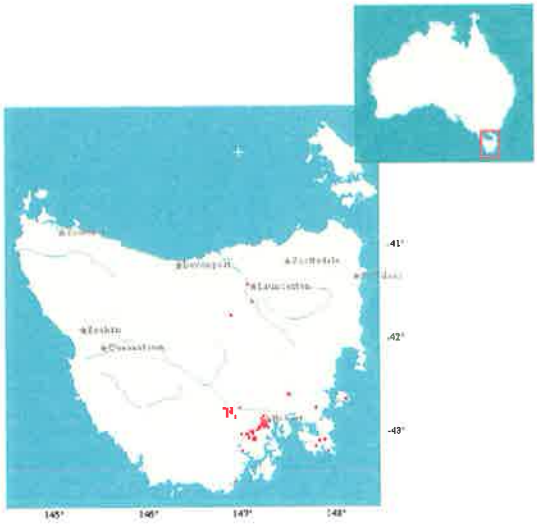
Tree to 70 m Juvenile leaves opposite, sessile, ovate, grey-green to glaucous. Adult leaves alternate, lanceolate, green. Solitary glaucous buds. Suited to most temperate conditions, including coastal. (Figures 3.1 & 3.2).

Eucalyptus gunnii Hook. f.

Cider Gum

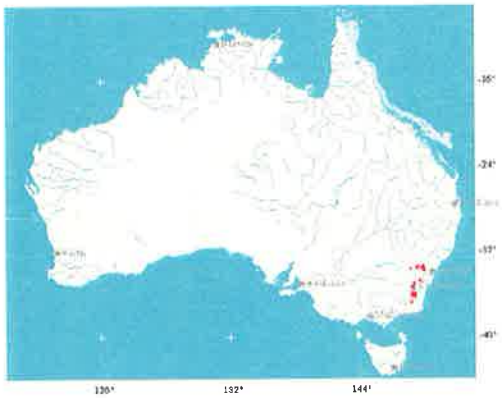


Tree to 25 m. Juvenile leaves opposite, sessile, ovate to orbicular, crenulate, grey-green, thick. Adult leaves alternate, ovate to broadly lanceolate, grey-green. Buds and fruits glaucous. Suited to cooler areas where rainfall is assured, and poorly drained areas. (Figures 3.1, 3.3 & 7.1).



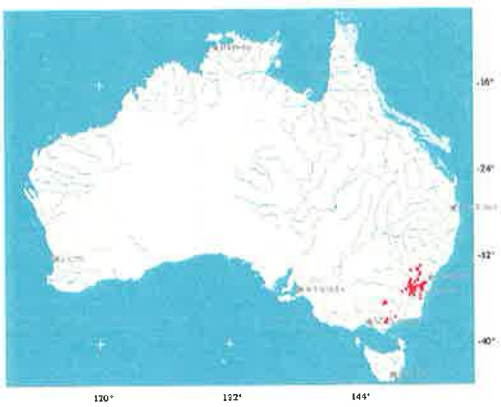
Eucalyptus cordata Labill.
Heart-leaved Silver Gum

Shrub to 3 m or tree to 21m. Juvenile leaves opposite, sessile, orbicular to cordate, crenulate, glaucous, usually persisting on mature trees. Adult leaves alternate, lanceolate, dull, grey-green or glaucous. Buds and fruits sessile and glaucous. Best suited to cool, damp conditions. (Figures 3.1 & 3.2).



Eucalyptus pulverulenta Sims
Silver-leaved Mountain gum

Tree to 9 m, or mallee to 5 m. Juvenile leaves opposite, sessile, orbicular to ovate, glaucous, usually persisting. Adult leaves alternate, lanceolate, glaucous, rarely seen. Buds sessile, glaucous. Best suited to acid soils and cool moist positions, but can be grown under warmer conditions where water is available. (Figures 3.1 & 3.3).

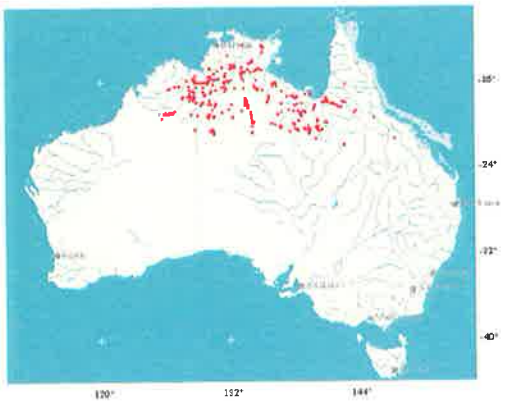


Eucalyptus cinerea F. Muell. ex Benth.
Argyle Apple

Tree to 16 m. Juvenile leaves opposite, sessile or shortly petiolate, orbicular to cordate, glaucous. Intermediate leaves opposite, sessile or shortly petiolate, cordate or broadly ovate, glaucous or almost green. Juvenile or intermediate leaves usually persist on adult trees. Adult leaves alternate, broadly lanceolate, glaucous. Buds glaucous. Requires assured moisture and dislikes lime or saline soils. (Figures 3.1 & 3.3).

Eucalyptus pruinosa Schau.

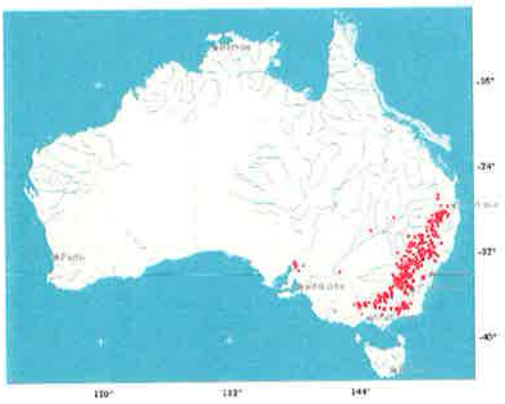
Silver Box



Tree to 10 m, often a mallee to 3 m. Juvenile leaves opposite, ovate or broadly lanceolate, glaucous. Adult leaves opposite, sessile, broadly ovate to elliptic-ovate, cordate, grey-green to pruinose. Fruits glaucous when young. Found in shallow, limestone soils and red sands. (Figures 3.1 & 3.2).

Eucalyptus albens Benth

White Box



Single-stemmed trees to 25 m high. Juvenile leaves opposite to alternate, petiolate, ovate to orbicular, grey-green to blue-green. Adult leaves alternate, broadly lanceolate, petiolate, dull, bluish-green, glaucous or grey-green. Buds and fruits glaucous, bluish-green. Suited to areas of moderate rainfall, grows in a wide range of soils. (Figures 3.1 & 3.2).

Maps of the species distribution are from the Department of the Environment, Sport and Territories and can be downloaded from:

http://www.erin.gov.au/database/TAX990R.html

Chapter 3

Species Evaluation and Pruning

Introduction

Many *Eucalyptus* species go through a change of leaf morphology from glaucous, often rounded, juvenile leaves to glossy, lanceolate-shaped adult leaves, sometimes via an intermediate phase. The time taken to reach leaf maturity varies with species and genotype. The juvenile leaves are valued for their ornamental worth, as these stems are used as a filler for flower arrangements worldwide. Plants must be pruned regularly to avoid the adult transition and to promote growth of coppice shoots with juvenile leaves.

A minority of species, such as *E. cinerea* and *E. pulverulenta,* retain the juvenile foliage indefinitely and are reproductively mature in the juvenile leaf phase. Regular harvesting of these species is required to keep the trees to a manageable size and to stimulate marketable stem length. Once the stems have reached a marketable length (> 40 cm), they are harvested as soon as the tips have hardened off, and do not wilt after picking. Stems are hardened off by withholding water from the tree two weeks prior to harvesting. The vase life of these stems can be greater than two weeks when stored in a 2% sucrose solution (Wirthensohn *et al.*, 1996).

Coppicing is a form of management in which trees are cut back regularly to encourage growth of shoots from the base of the trunk. *Eucalyptus* coppice shoots can sprout from the cut surface of the trunk, the whole length of the trunk or at the base or lignotuber. Lignotubers are partly subterranean organs containing large numbers of adventitious buds. Thus, they are a source of vegetative reproduction, if the aerial part of the tree is removed or destroyed. Not all eucalypt species produce lignotubers, in which case coppice shoots are produced from epicormic buds below the bark of the trunk. The epicormic buds begin as accessory bud-producing tissue in the axil of leaves and grow radially outward with the trunk or branch with increase in diameter (Pryor, 1976). Coppice stems initially grow faster than uncut or seedling trees because they

have the advantage of an existing root system (Blake, 1980), and Harrington and Fownes (1995) reported that the biomass obtained from coppiced *E. camaldulensis* compared to uncoppiced stands was greater up to 12 months growth.

At present only a limited range of species is used for foliage, despite the fact that many others have potential. Currently the most commonly used species for foliage are *E. pulverulenta, E. gunnii, E. cinerea* and *E. globulus*. This work examines 17 species for stem production including aesthetic and growth rate attributes. There is also very little information on pruning techniques for cut foliage of eucalypts. This work also reports the results of pruning on 17 species of *Eucalyptus,* and the effect of time of pruning on regrowth of *E. gunnii.*

Materials and methods

Species evaluation

Plant material used is described in Chapter 2 and Table 3.1. Morphological characters were measured every 3 months on a minimum of 10 plants per species: tree height, trunk diameter at 5 cm above ground level, presence or absence of lignotuber, diameter of lignotuber (above ground parts), colour of fully expanded juvenile leaves using Royal Horticultural Society colour charts. Juvenile leaf shape, leaf glaucousness, phyllotaxy, and stem shape and waxiness at the juvenile stage were assessed visually. Age at phase change to intermediate or adult leaves was noted as well as time of flowering if they occurred within the experimental period of 41 months.

Pruning trial on E. globulus

An initial pruning experiment was carried out on *E. globulus*. Trees were located on a grower's property at Inman Valley, South Australia. Fifty 3-year-old trees that were similar in size and trunk diameter and had not been pruned were selected from a population direct-seeded into a sandy loam soil, pH 5.5, with no irrigation. The trees were pruned (coppiced) in Sept. 1994 to four heights above ground level, 0.1, 0.5, 1.0, and 1.5 m, with a control group (height 2 m) that was not pruned. There were 10 trees per group and all lateral stems were cut off flush with the trunk. The total number of

new lateral stems was counted at 1, 2, 3, and 6 months. Harvested stem lengths were measured at 6 months. Data were analysed by ANOVA using Genstat 5.2.

Pruning trial on 16 species of Eucalyptus

Pruning was carried out on 16 species at 19 months of age in April 1995 at 0.5 m or 1.0 m above ground level, with a control group which were not pruned (data not presented). Lateral stems on all species were pruned flush with the trunk. There were 10 trees per treatment per species. Each treatment was assigned to 10 blocks randomly. Measurements were made at 3, 6, 9 and 12 months after pruning of the total number of new lateral stems 10 cm, and stems were harvested when the majority were of marketable length (> 40 cm) and the tips had hardened. Measurements were made of the total number of harvested stems, the total weight of stems, and the number of stems 0 - 40 cm, >40 - 50 cm, >50 - 60 cm and >60 cm. Data were analysed using ANOVA and as no significant difference between pruning heights was found for any variable, the results were pooled and re-analysed. Significant differences between the means were determined by Duncan's Multiple Range test. Relationships between variables were determined by regression analysis.

Pruning trial on E. gunnii

The *E. gunnii* were pruned at 19, 22 or 25 months of age in April, July, and October 1995 to three heights above ground level, 0.1, 0.5, or 1.0 m. There were 10 trees per treatment per age group. Ten trees were left unpruned as a control group (data not presented). Lateral stems were pruned flush with the trunk except for July and October pruned trees, where up to 12 laterals were left intact. Measurements were made at 3, 6, 9 and 12 months after pruning of the total number of new lateral stems 10 cm (not including the 12 laterals that were retained), and stems were harvested when the majority were of marketable length (> 40 cm) and the tips had hardened. Measurements were made of the total number of harvested stems, the total weight of stems, and the number of stems 0 - 40 cm, >40 - 50 cm, >50 - 60 cm and >60 cm. Data were analysed using ANOVA. Significant differences between the means were determined by Duncan's Multiple Range test.

Results

Species evaluation

There was variation among and between species for juvenile characteristics (Table 3.1). The species with the greatest variation for leaf colour was *E. delegatensis*, with trees either greyed-green 189A or yellow-green 147A. *Eucalyptus cinerea* showed the least variation for leaf colour. All species were glaucous apart from *E. brachyphylla*, and there was no difference within any species for glaucousness, juvenile leaf shape, phyllotaxy, stem shape or stem waxiness (Figures 3.1–3.3). Individual trees within species that changed leaf phase from juvenile to adult or which flowered, such as *E. tetragona*, did so within a month of each other. Not all trees within the species that flowered, did so. *Eucalyptus brachyphylla* flowered the earliest at 17 months.

Pruning trial on E. globulus

At 6 months, the trees pruned to 1 m showed significantly more regrowth of new shoots than all other treatments except those pruned to 0.5 m (Table 3.2). The drop in total shoot count between 2 months and 6 months was presumably due to competition between the large number of regrowth shoots. After 6 months, the longest average stem length was produced by the trees pruned to 0.5 m or 0.1 m. There were no significant variations in stem thickness or leaf colour or shape between the pruning regimes. All regrowth was juvenile in character. On 0.1 m pruned trees, the regrowth emerged from all areas of the remaining trunk including the cut surface. On 0.5, 1.0, and 1.5 m pruned trees, the regrowth emerged from the upper half of the trunk.

Pruning trial on 16 species of Eucalyptus

There were highly significant differences between species for tree height, trunk diameter and lignotuber diameter at 16 months ($P < 0.001$; Table 3.3). *Eucalyptus globulus* and *E. bridgesiana* were the tallest and also had the largest trunk diameter and lignotuber diameter. Following pruning at 19 months, significant interactions between species and pruning height were evident for tree height ($P < 0.001$), height increment after pruning ($P = 0.002$), for trunk diameter ($P < 0.001$) and for lignotuber diameter ($P < 0.001$), within 3 months and by 6 months, the interaction of species x pruning height

was highly significant for all parameters ($P < 0.001$)(Tables 3.4 &3.5). The tallest trees, 3 months after pruning were the control *E. globulus* and *E. bridgesiana* and species with the greatest change in height since pruning were the 0.5 m pruned *E. socialis* and the 1.0 m pruned *E. globulus*. *E. globulus* had the largest trunk diameter and *E. bridgesiana* had the largest lignotuber diameter. The control pruning group had the largest trunk and lignotuber diameters. The tallest trees with the largest trunk diameters, 6 months after pruning, were the control *E. globulus* and *E. bridgesiana*. *E. globulus* and the 0.5 m pruned group gained the most height after pruning. The control group had the largest lignotuber diameters and between the species, *E. bridgesiana* had the largest lignotuber diameter. Some species such as *E. delegatensis, E. gunnii* and *E. cordata* had no discernible lignotuber at the time of measurements although most seedlings did have small protuberances at the first node at planting time. The presence of lignotubers in *E. globulus* was obvious only at the 16 month measurements, after which the trunk outgrew the lignotuber.

The two way interaction of species x pruning height was highly significant for the number of stems ≥ 10 cm at 3 and 6 months after pruning (Table 3.6). At 3 months *E. cinerea* pruned to 0.5 m and *E. bridgesiana* pruned to 1.0 m had produced the most stems ≥ 10 cm, 29.8 and 26.3 stems respectively. By 6 months, *E. globulus* pruned to 1.0 m, *E. cinerea* pruned to 0.5 m and *E. bridgesiana* pruned to 0.5 m had produced the most stems, 38.9, 35.5 and 34.1 respectively.

One year after pruning there was a significant difference between species for number of stems produced at all lengths categories and for the total number of stems produced (Table 3.7), but there was no effect of different initial pruning height. The majority of stems produced by *E. cordata, E. kruseana, E. delegatensis* and *E. crucis* were < 40 cm in length, whereas the most stems of > 60 cm length were produced by *E. globulus,* and this species also produced the highest number of stems, while the highest proportion of > 60 cm stems were produced by *E. pulverulenta, E. pruinosa* and *E. albens*. There was a significant difference between species for total weight of stems produced after 12 months growth. *Eucalyptus globulus* produced the greatest weight of stems at 4913 g and *E. orbifolia* the least at 409 g (Table 3.8).

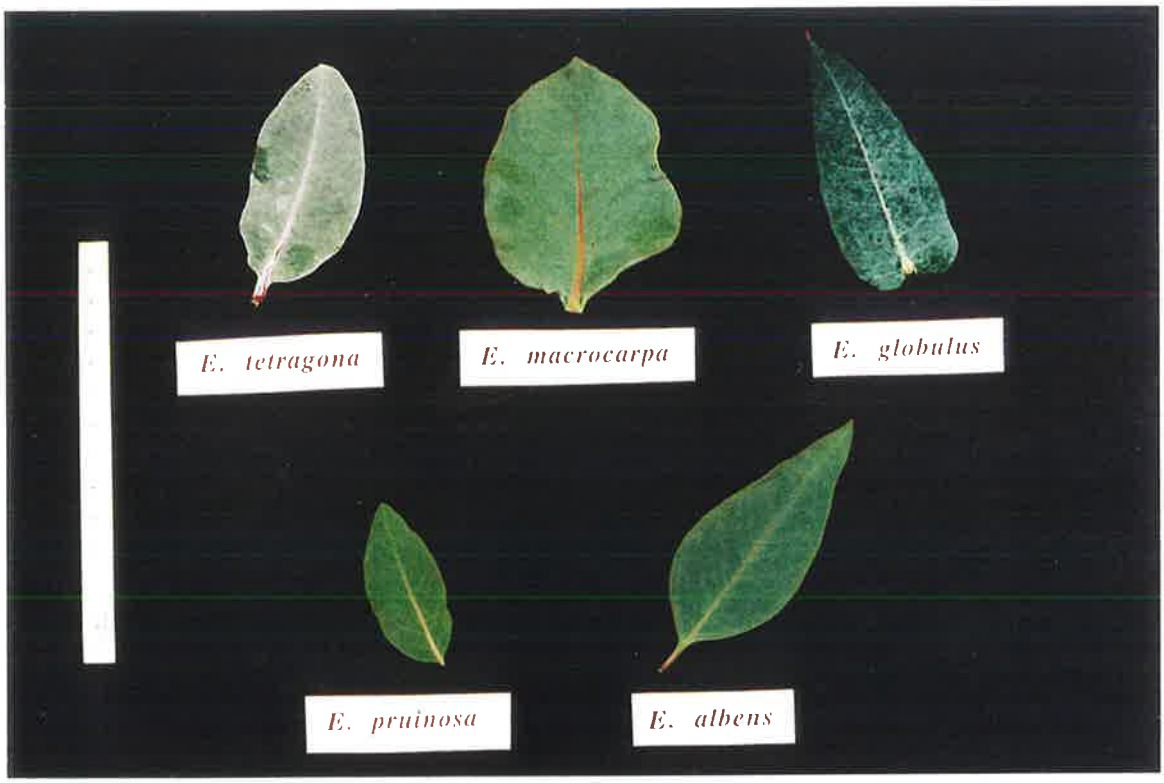
Regression analysis showed that the total number of stems produced at 1 year, was significantly related to trunk and lignotuber diameter 3 and 6 months after pruning ($r^2 > 0.6$) (Table 3.9).

Pruning trial on E. gunnii

Following pruning at 19 months at all three heights, *E. gunnii* produced relatively few new stems, so for the remaining treatments (pruning to 0.1, 0.5 or 1.0 m at 22 or 25 months) 12 laterals per tree were retained. There was a significant difference between pruning heights for number of stems 0 - 40 cm, >40 - 50 cm, >50 - 60 cm and >60 cm in length and the total number and weight of stems produced 6 months after pruning at 25 months, and for total weight of stems produced 9 months after pruning at 22 months (Table 3.10). *Eucalyptus gunnii* trees pruned to 1.0 m at 25 months produced the greatest total number of stems and the greatest weight of stems after 6 months.

Figure 3.1. Upper: Juvenile leaf specimens of *Eucalyptus tetragona, E. macrocarpa, E. globulus, E. pruinosa,* and *E. albens.*

Lower: Juvenile leaf specimens of *E. bridgesiana, E. cordata, E. crucis, E. gillii, E. brachyphylla, E. orbifolia, E. delegatensis, E. kruseana, E. albida, E. gunnii, E. pulverulenta, E. socialis,* and *E. cinerea.*


E. tetragona
E. macrocarpa
E. globulus
E. pruinosa
E. albens

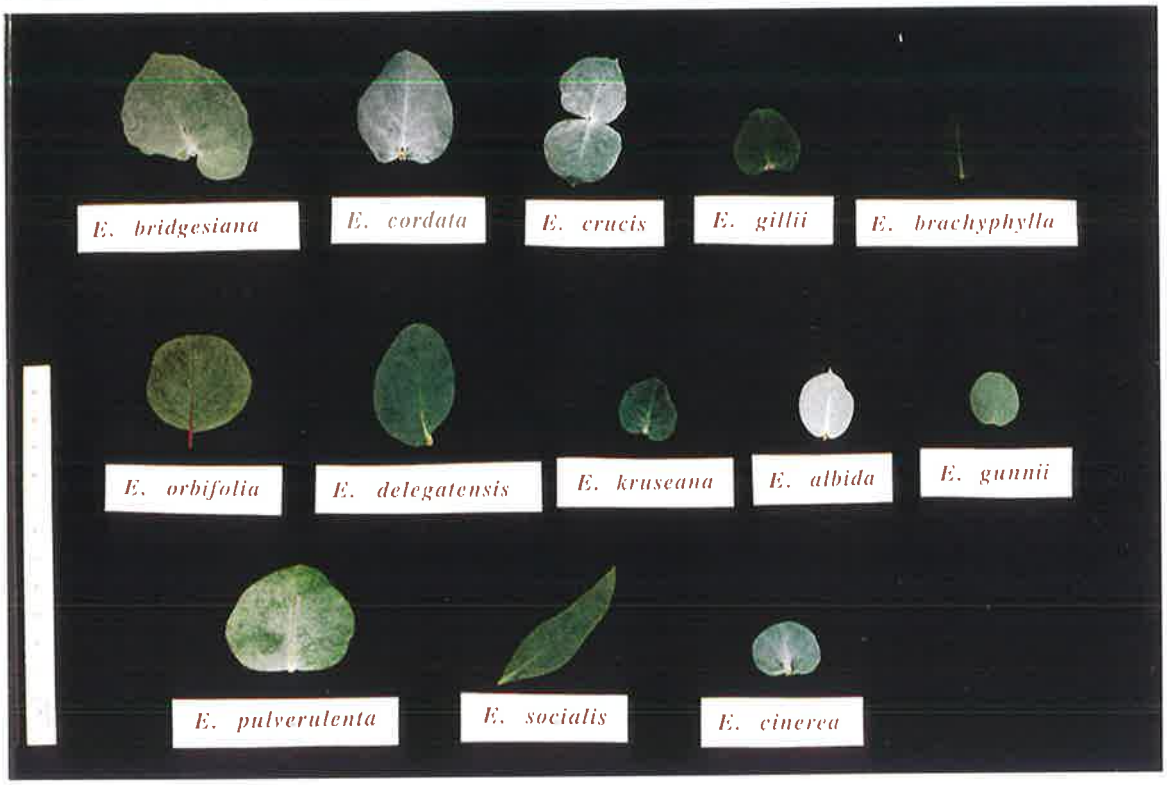
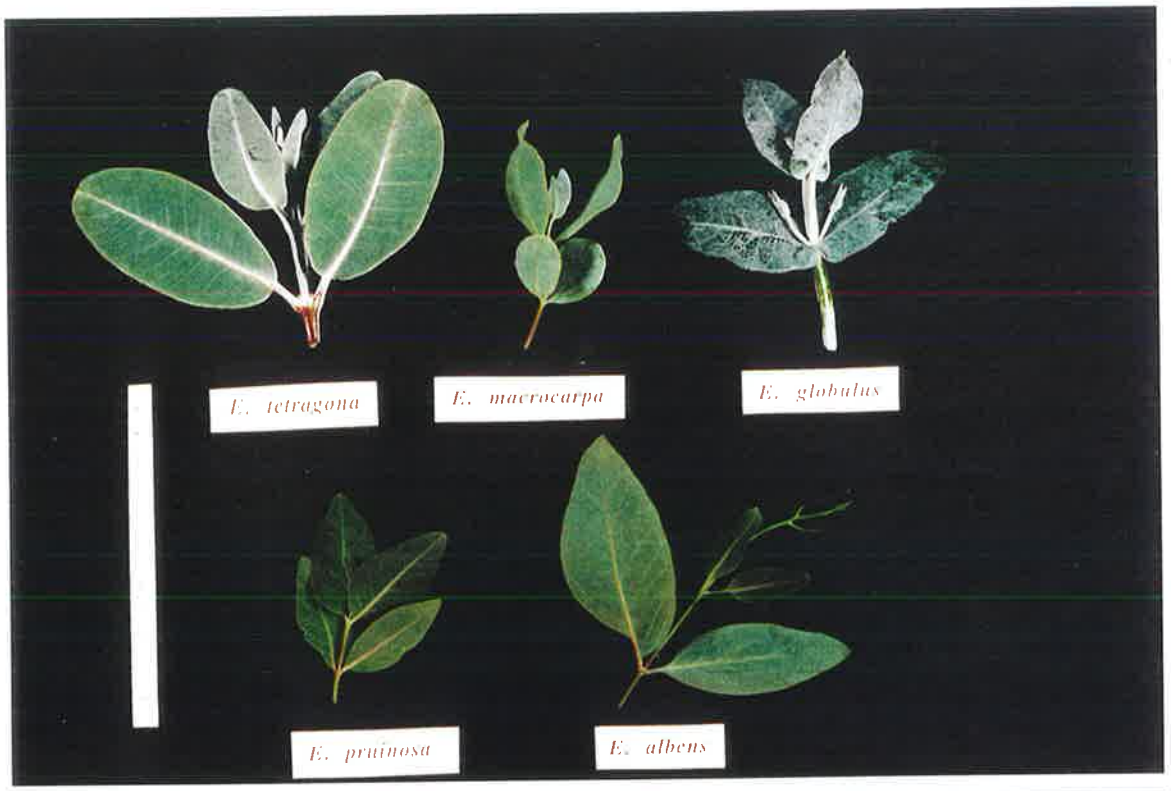

E. bridgesiana
E. cordata
E. crucis
E. gillii
E. brachyphylla
E. orbifolia
E. delegatensis
E. kruseana
E. albida
E. gunnii
E. pulverulenta
E. socialis
E. cinerea

Figure 3.2. Upper: Juvenile shoot tips of *Eucalyptus tetragona, E. macrocarpa, E. globulus, E. pruinosa,* and *E. albens.*

Lower: Juvenile shoot tips of *E. delegatensis, E. crucis, E. bridgesiana, E. socialis, E. cordata,* and *E. orbifolia.*


E. tetragona
E. macrocarpa
E. globulus
E. pruinosa
E. albens

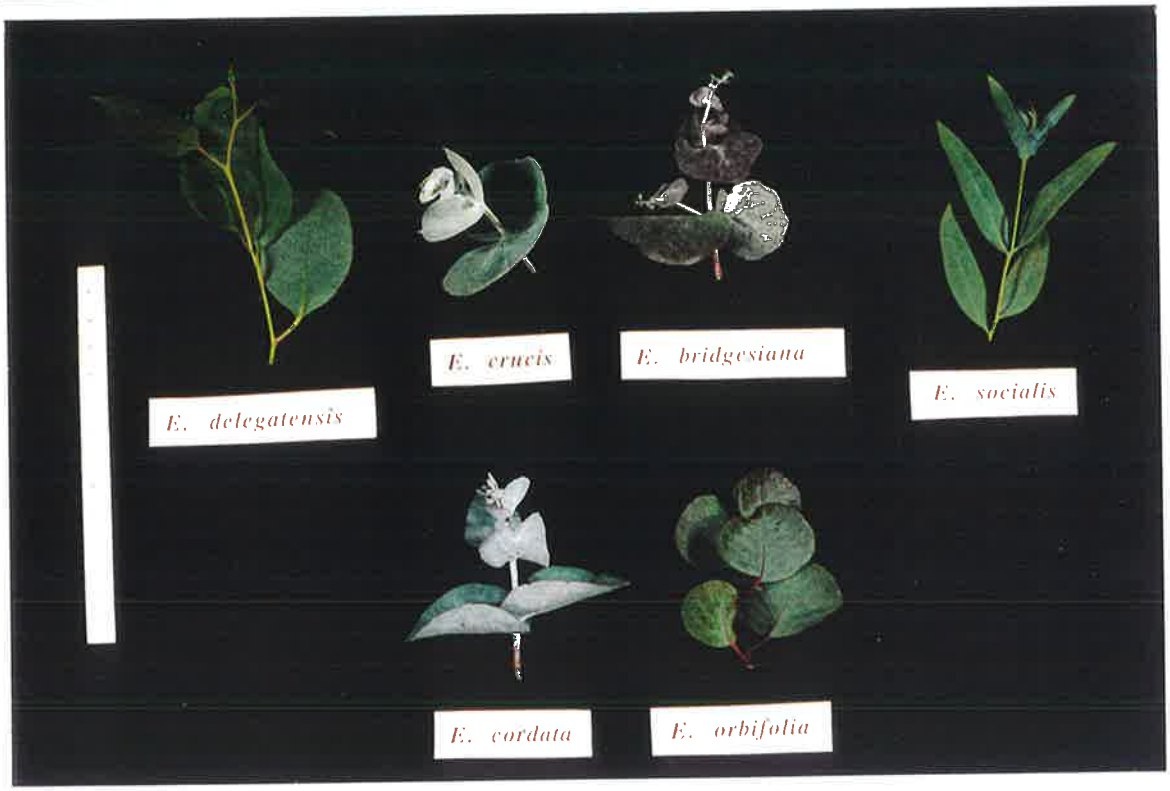

E. delegatensis
E. crucis
E. bridgesiana
E. socialis
E. cordata
E. orbifolia

Figure 3.3. Juvenile shoot tips of *E. cinerea, E. pulverulenta, E. kruseana, E. albida, E. gunnii, E. gillii,* and *E. brachyphylla.*

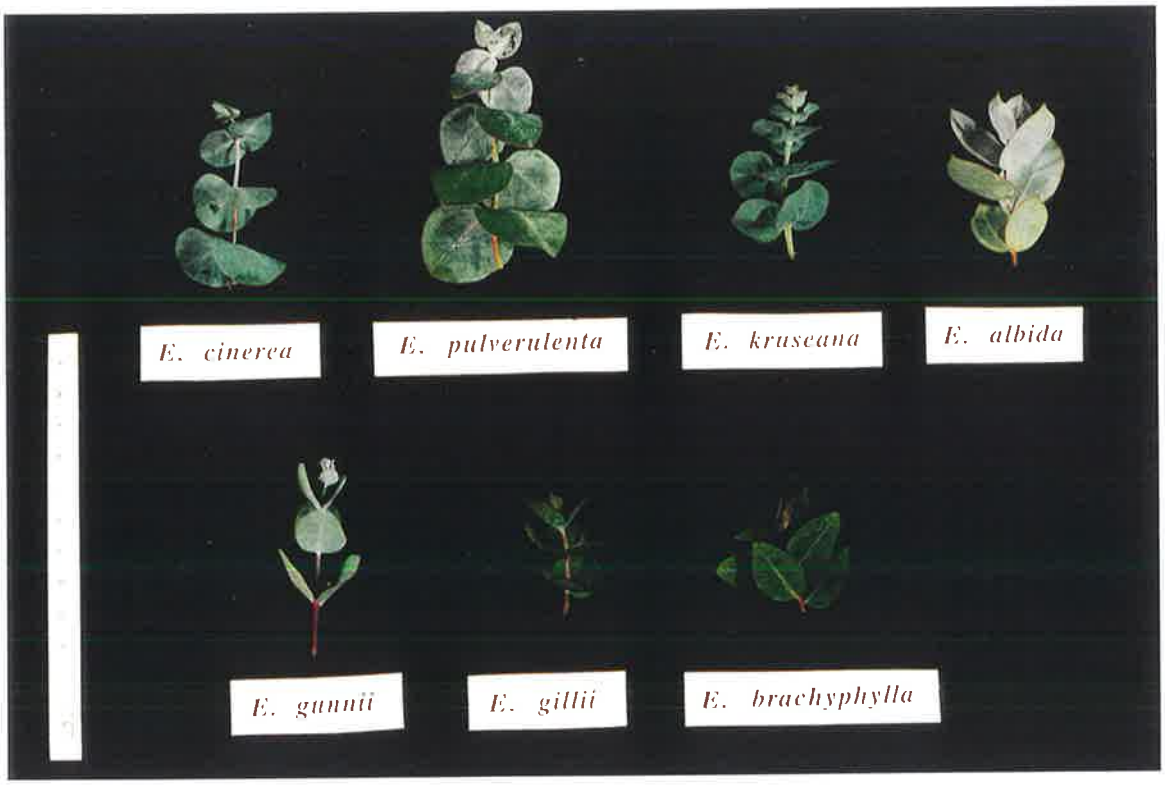

E. cinerea
E. pulverulenta
E. kruseana
E. albida
E. gunnii
E. gillii
E. brachyphylla

Table 3.1. Juvenile morphological characteristics of 17 species of *Eucalyptus*. Classification follows Pryor and Johnson (1971) and Chippendale (1988)

Species	Juvenile leaf colour[a]	Juvenile leaf shape	Phyllotaxy	Stem shape	Stem waxiness	Months to leaf phase change (%)[b]	Months to first flowering (%)[b]
Subgenus *Eudesmia*							
Section *Quadria*							
Series *Heteroptera*							
E. tetragona	189A, 188A	oblong-elliptic	opposite	square	waxy	17(100)	41 (7)
Subgenus *Monocalyptus*							
Section *Renantheria*							
Series *Eucalyptus*							
E. delegatensis	189A, 147A	ovate-orbicular	alternate	round	waxy	17(100)	-
Subgenus *Symphyomyrtus*							
Section *Bisectaria*							
Series *Kruseanae*							
E. kruseana	188A, 189A,	orbicular	opposite	round	waxy	-	30 (79)
E. brachyphylla	147A, 146A	ovate	alternate	round	non waxy	-	17 (14)
Series *Subulatae*							
E. socialis	191A, 189A	elliptic-lanceolate	alternate	square	waxy	-	32 (35)
E. gillii	189A, 191A	cordate	opposite	round	waxy	-	32 (47)
Series *Curviptera*							
E. orbifolia	189A, 191A	orbicular	alternate	round	waxy	-	33 (36)
E. crucis	188A, 189B	orbicular	opposite	square/round	waxy	-	25 (14)
E. macrocarpa	188A, 191A	broad ovate/elliptic-ovate	opposite	round	waxy	17 (100)	-

Table 3.1 continued

Species	Juvenile leaf colour[a]	Juvenile leaf shape	Phyllotaxy	Stem shape	Stem waxiness	Months to leaf phase change (%)[b]	Months to first flowering (%)[b]
Section *Maidenaria*							
Series *Viminales*							
E. bridgesiana	189A, 191A	orbicular-cordate	sub-opposite	round	waxy	23 (100)	-
E. globulus	188A, 189A	ovate-elliptic	opposite	square	waxy	23 (100)	-
E. gunnii	189A, 191A	orbicular-ovate	opposite	round	waxy	28 (100)	-
E. cordata	188A, 189B	cordate	opposite	square	waxy	-	-
E. pulverulenta	188A, 189A	orbicular	opposite	square/round	waxy	-	32 (5)
E. cinerea	188A	orbicular	opposite	square/round	waxy	-	-
Section *Adnataria*							
Series *Striolatae*							
E. pruinosa	189A, 191A	ovate	opposite	square/round	non waxy	11 (100)	32 (25)
Series *Moluccanae*							
E. albens	189A, 191A	ovate-orbicular	alternate	square/round	non waxy	14 (100)	-

[a] Most common colour shown first (> 40% of leaves and trees). Colour codes from RHS Colour Charts; - did not occur during 41 month observation period.

[b] Percentage of trees where this occurred.

Table 3.2. Effect of height after pruning on regrowth of *Eucalyptus globulus* stems.

Pruning Height (m)	Number of shoots: Time from pruning (months) 1	2	3	6	Length of stems[z] (cm)
0.1	6.3	52.3	45.4	31.7	64.9
0.5	5.4	69.3	60.7	48.6	68.9
1.0	3.3	89.4	63.3	59.3	40.3
1.5	0.8	52.4	32.8	31.1	26.8
not pruned	0.4	5.9	7.1	16.8	49.6
SEM[y]	1.43	11.22	14.69	13.53	10.83
Significance	***	***	***	*	**

[z] Measured at 6 months.

[y]SEM = standard error of mean (45 df) based on error mean square from analysis of variance.

*, **, ***Significant at $P \leq 0.05$, 0.01 or 0.001, respectively; n=50.

Table 3.3. Tree height, trunk diameter and lignotuber diameter values (± s.e.) of 17 species of *Eucalyptus* at 16 months

Species	Tree height (cm)		Trunk diameter (cm)		Lignotuber diameter (cm)	
	mean	n	mean	n	mean	n
E. tetragona	49.6 ± 8.8	16	11.4 ± 1.4	16	15.2 ± 1.7	16
E. delegatensis	65.4 ± 7.4	23	12.8 ± 1.2	23	-	
E. kruseana	74.8 ± 8.8	16	7.7 ± 1.4	16	9.4 ± 2.1	16
E. brachyphylla	35.7 ± 13.3	7	6.7 ± 2.2	7	11.3 ± 3.5	7
E. socialis	54.9 ± 8.3	18	10.2 ± 1.3	18	14.1 ± 1.5	18
E. gillii	47.9 ± 7.9	20	8.7 ± 1.3	20	14.4 ± 1.7	20
E. orbifolia	44.3 ± 9.8	13	6.0 ± 1.6	13	8.9 ± 2.7	13
E. crucis	77.9 ± 6.9	26	13.3 ± 1.1	26	-	
E. macrocarpa	48.2 ±7.9	20	9.8 ± 1.3	20	15.6 ± 1.5	20
E. bridgesiana	108.3 ± 6.4	30	22.6 ± 1.0	30	31.2 ± 1.1	30
E. globulus	130.0 ± 6.7	28	29.8 ± 1.1	28	35.3 ± 1.7	28
E. gunnii	71.1 ± 3.7	89	15.6 ± 0.6	89	-	
E. cordata	86.1 ± 6.9	26	19.0 ± 1.1	26	-	
E. pulverulenta	79.6 ± 7.4	23	11.6 ± 1.2	23	17.0 ± 1.4	23
E. cinerea	81.5 ± 7.2	24	15.8 ± 1.2	24	24.1 ± 1.2	24
E. pruinosa	47.4 ± 8.3	18	9.7 ± 1.3	18	18.1 ± 1.5	18
E. albens	71.3 ± 7.2	24	12.7 ± 1.2	24	21.9 ± 1.2	24
Mean Significance	***		***		***	

S.E., Standard error; n, number of observations; - Lignotuber not discernible; *** Significant at $P < 0.001$

Table 3.4. Tree height, trunk diameter and lignotuber diameter of 16 species of *Eucalyptus* at 22 months. The two way interaction of species x pruning height was highly significant for tree height ($P < 0.001$) and for height increment after pruning ($P = 0.002$), species and pruning height were highly significant for trunk diameter and lignotuber diameter ($P < 0.001$)

Species	Tree height ± S.E. (cm)						Trunk diameter ± S.E. (cm)			Lignotuber diameter ± S.E.		
Pruning treatment	Control (not pruned)	0.5 m	Δ height since pruned (0.5m)	1.0 m	Δ height since pruned (1.0m)	n	Species means	Pruning height means	n	Species means	Pruning height means	n
Control								33.8 ± 0.8	141		38.3 ± 1.2	75
0.5 m								25.7 ± 0.9	123		28.8 ± 1.2	79
1.0 m								27.1 ± 0.9	121		30.1 ± 1.2	79
E. tetragona	65.0 ± 14.7	59.4 ± 14.7	9.4 ± 7.9	68.3 ± 13.4	-31.7 ± 7.2	16	20.9 ± 2.4		16	27.5 ± 2.6		15
E. delegatensis	126.8 ± 16.4	55.8 ± 13.4	5.8 ± 7.2	84.0 ± 23.2	-16.0 ± 12.5	12	29.7 ± 3.0		12	-		
E. kruseana	73.8 ± 14.7	48.0 ± 14.7	-2.0 ± 7.9	87.4 ± 14.7	-12.6 ± 7.9	15	16.1 ± 2.5		15	20.3 ± 2.6		15
E. brachyphylla	43.0 ± 18.9	60.6 ± 10.4	3.5 ± 12.5	68.0 ± 23.2	-32.0 ± 12.5	7	13.1 ± 3.7		7	21.5 ± 4.7		6
E. socialis	74.7 ± 12.4	62.8 ± 13.4	12.8 ± 7.2	80.9 ± 12.4	-19.1 ± 6.7	20	18.4 ± 2.2		20	28.0 ± 2.3		20
E. gillii	77.3 ± 13.4	56.7 ± 12.4	6.7 ± 6.7	70.0 ± 12.4	-30.0 ± 6.7	20	16.0 ± 2.2		20	22.0 ± 2.3		20
E. orbifolia	109.3 ± 16.4	60.3 ± 16.4	10.3 ± 8.9	94.3 ± 16.4	6.8 ± 8.9	12	12.2 ± 2.8		12	16.9 ± 3.0		12
E. crucis	125.0 ± 10.9	49.6 ± 11.6	-0.4 ± 6.3	87.8 ± 11.6	-12.3 ± 6.3	25	23.2 ± 1.9		25	30.2 ± 2.4		19
E. macrocarpa	91.5 ± 13.4	53.0 ± 12.4	3.0 ± 6.7	80.8 ± 13.4	-19.2 ± 7.2	19	19.1 ± 2.2		19	27.3 ± 2.4		19
E. bridgesiana	210.6 ± 10.4	60.6 ± 10.4	10.6 ± 5.6	105.3 ± 10.4	5.3 ± 5.6	30	42.2 ± 1.8		30	51.9 ± 2.1		25
E. globulus	259.2 ± 10.9	58.1 ± 10.4	8.1 ± 5.6	119.6 ± 10.9	19.6 ± 5.9	28	53.0 ± 1.8		28	-		
E. cordata	161.8 ± 11.6	57.7 ± 10.9	7.7 ± 5.9	101.9 ± 11.6	1.9 ± 6.3	25	36.0 ± 1.9		25	-		
Mean Significance							***	***		***	***	

Table 3.4. continued

Species	Tree height ± S.E. (cm)						Trunk diameter ± S.E. (cm)		Lignotuber diameter ± S.E.	
Pruning treatment	Control (not pruned)	0.5 m	Δ height since pruned (0.5m)	1.0 m	Δ height since pruned (1.0m)	n	Species means	n	Species means	n
E. pulverulenta	125.1 ± 11.6	50.0 ± 11.6	0 ± 6.3	97.3 ± 12.4	-2.7 ± 6.7	23	23.3 ± 2.0	23	30.7 ± 2.4	19
E. cinerea	155.5 ± 11.6	57.4 ± 11.6	7.4 ± 6.3	100.8 ± 11.6	0.8 ± 6.3	24	31.9 ± 2.0	24	43.1 ± 2.1	23
E. pruinosa	94.2 ± 13.4	60.2 ± 14.6	10.2 ± 7.9	76.8 ± 14.7	-23.2 ± 7.9	16	20.1 ± 2.4	16	33.7 ± 2.6	16
E. albens	114.4 ± 12.4	60.6 ± 12.4	10.6 ± 6.7	111.1 ± 10.4	11.1 ± 5.6	24	23.8 ± 2.0	24	40.5 ± 2.1	24
Mean Significance							***		***	

S.E., Standard error; n, number of observations; - Lignotuber not discernible; *** Significant at $P < 0.001$

Table 3.5. Tree height, trunk diameter and lignotuber diameter of 16 species of *Eucalyptus* at 25 months. The two way interaction of species x pruning height was highly significant for tree height and trunk diameter ($P < 0.001$), species and pruning height were highly significant for change in height after pruning and for lignotuber diameter ($P < 0.001$)

Species	Tree height ± S.E. (cm)					Trunk diameter ± S.E. (cm)				Lignotuber diameter ± S.E. (cm)		
Pruning treatment	Control (not pruned)	0.5 m	1.0 m	Δ height since pruned	n	Control (not pruned)	0.5 m	1.0 m	n	Species means	Pruning height means	n
Control				na							45.2 ± 1.3	74
0.5 m				26.7 ± 2.5	100						31.7 ± 1.3	72
1.0 m				7.2 ± 2.4	105						33.3 ± 1.3	72
E. tetragona	78.4 ± 16.5	72.6 ± 16.5	82.8 ± 15.1	2.2 ± 7.5	16	26.8 ± 4.8	21.4 ± 4.8	21.5 ± 4.4	16	29.5 ± 2.8		16
E. delegatensis	189.3 ± 18.5	68.0 ± 21.4	84.0 ± 37.0	0.6 ± 14.4	8	56.7 ± 5.4	20.6 ± 6.3	14.8 ± 10.8	8	-		
E. kruseana	77.4 ± 16.5	61.0 ± 18.5	94.0 ± 16.5	2.3 ± 8.3	14	24.2 ± 4.8	15.1 ± 5.4	16.8 ± 4.8	14	25.3 ± 3.1		13
E. brachyphylla	49.7 ± 21.4	55.0 ± 26.1	65.0 ± 26.1	-14.7 ± 12.3	7	11.6 ± 6.3	17.6 ± 7.7	15.0 ± 7.7	7	23.2 ± 4.9		22
E. socialis	88.4 ± 14.0	64.5 ± 15.1	88.7 ± 14.0	1.3 ± 6.9	20	23.1 ± 4.1	21.6 ± 4.4	18.2 ± 4.1	20	29.2 ± 2.6		19
E. gillii	88.3 ± 15.1	59.6 ± 14.0	75.3 ± 14.0	-8.0 ± 6.6	20	21.4 ± 4.4	13.0 ± 4.1	15.2 ± 4.1	20	26.9 ± 2.5		20
E. orbifolia	119.5 ± 18.5	65.8 ± 18.5	94.7 ± 21.4	5.0 ± 9.5	11	16.5 ± 5.4	14.2 ± 5.4	10.0 ± 6.3	11	19.4 ± 3.8		9
E. crucis	135.3 ± 12.3	54.0 ± 15.1	107.7 ± 15.1	5.9 ± 7.1	21	28.9 ± 3.6	21.0 ± 4.4	23.8 ± 4.4	21	33.2 ± 3.1		14
E. macrocarpa	107.2 ± 15.1	63.1 ± 14.0	81.5 ± 15.1	-3.1 ± 6.9	19	26.0 ± 4.4	19.4 ± 4.1	18.3 ± 4.4	19	32.8 ± 2.5		19
E. bridgesiana	233.2 ± 11.7	101.9 ± 12.3	122.4 ± 11.7	36.8 ± 5.7	29	59.0 ± 3.4	39.1 ± 3.6	38.5 ± 3.4	29	55.7 ± 2.4		22
E. globulus	331.7 ± 12.3	128.7 ± 21.4	171.1 ± 14.0	74.8 ± 8.4	19	69.4 ± 3.6	55.7 ± 6.3	54.1 ± 4.1	19	-		
E. cordata	200.4 ± 13.1	73.4 ± 16.5	114.3 ± 15.1	18.8 ± 7.5	19	53.2 ± 3.8	24.0 ± 4.8	33.2 ± 4.4	19	-		
Mean Significance				***						***	***	

Table 3.5. continued

Species	Tree height ± S.E. (cm)					Trunk diameter ± S.E. (cm)				Lignotuber diameter ± S.E. (cm)		
Pruning treatment	Control (not pruned)	0.5 m	1.0 m	Δ height since pruned	n	Control (not pruned)	0.5 m	1.0 m	n	Species means	Pruning height means	n
E. pulverulenta	134.1 ± 13.1	64.0 ± 13.1	106.7 ± 15.1	10.2 ± 6.7	22	29.2 ± 3.8	18.2 ± 3.8	27.2 ± 4.4	22	39.4 ± 2.6		18
E. cinerea	197.8 ± 13.1	79.8 ± 13.1	115.4 ± 13.1	22.4 ± 6.2	24	53.2 ± 3.8	28.9 ± 3.8	24.6 ± 3.8	24	49.4 ± 2.3		23
E. pruinosa	94.5 ± 15.1	61.8 ± 16.5	87.6 ± 16.5	-0.6 ± 7.8	16	22.9 ± 4.4	16.7 ± 4.8	23.1 ± 4.8	16	40.6 ± 2.8		16
E. albens	136.9 ± 14.0	74.3 ± 14.0	120.5 ± 11.7	22.4 ± 6.1	24	39.6 ± 4.1	18.4 ± 4.1	25.3 ± 3.4	24	47.4 ± 2.4		23
Mean Significance				***						***		

S.E., Standard error; n, number of observations; na, not applicable; - Lignotuber not discernible; *** Significant at $P < 0.001$

Table 3.6. Regrowth of stems of 16 species of *Eucalyptus* after 3 and 6 months following pruning at 19 months. The two way interaction of species x pruning height was highly significant for stem growth after 3 and 6 months ($P < 0.001$)

Species	Number of stems ≥ 10 cm ± S.E.							
	3 months				6 months			
	Control (not pruned)	0.5 m	1.0 m	n	Control (not pruned)	0.5 m	1.0 m	n
E. tetragona	0.6 ± 5.2	7.2 ± 5.2	5.7 ± 4.9	16	1.4 ± 11.9	10.0 ± 11.9	10.5 ± 10.8	16
E. delegatensis	4.5 ± 5.9	1.3 ± 4.8	0 ± 8.3	12	17.8 ± 13.2	10.3 ± 15.3	9.0 ± 26.5	8
E. kruseana	6.4 ± 5.2	7.8 ± 5.2	10.0 ± 5.2	15	2.8 ± 11.9	21.3 ± 13.2	19.6 ± 11.9	14
E. brachyphylla	2.3 ± 6.8	1.0 ± 8.3	6.0 ± 8.3	7	3.3 ± 15.3	17.5 ± 18.7	8.0 ± 18.7	7
E. socialis	3.1 ± 4.4	6.0 ± 4.8	4.3 ± 4.4	20	5.9 ± 10.0	26.8 ± 10.8	20.7 ± 10.0	20
E. gillii	3.8 ± 4.8	4.7 ± 4.4	4.6 ± 4.4	20	5.7 ± 10.8	19.1 ± 10.0	18.9 ± 10.0	20
E. orbifolia	1.8 ± 5.9	3.0 ± 5.9	1.8 ± 5.9	12	6.0 ± 13.2	11.5 ± 13.2	7.0 ± 15.3	11
E. crucis	6.7 ± 3.9	0.4 ± 4.1	3.5 ± 4.1	25	7.3 ± 8.8	2.1 ± 10.8	9.8 ± 10.8	21
E. macrocarpa	0.7 ± 4.8	1.0 ± 4.4	1.0 ± 4.9	19	1.2 ± 10.8	11.4 ± 10.0	2.8 ± 10.8	19
E. bridgesiana	9.2 ± 3.7	24.7 ± 3.7	26.3 ± 3.7	30	14.5 ± 8.4	34.1 ± 8.8	32.7 ± 8.4	29
E. globulus	13.2 ± 3.9	6.7 ± 3.7	21.7 ± 3.9	28	24.4 ± 8.8	28.0 ± 15.3	38.9 ± 10.0	19
E. cordata	6.5 ± 4.1	8.6 ± 3.9	8.0 ± 4.1	25	12.3 ± 9.4	22.4 ± 11.9	19.2 ± 10.8	19
E. pulverulenta	2.3 ± 4.1	7.5 ± 4.1	3.0 ± 4.4	23	2.3 ± 9.4	17.0 ± 9.4	14.0 ± 10.8	22
E. cinerea	14.9 ± 4.1	29.8 ± 4.1	21.1 ± 4.1	24	20.8 ± 9.4	35.5 ± 9.4	30.9 ± 9.4	24
E. pruinosa	0.3 ± 4.8	3.2 ± 5.2	13.2 ± 5.2	16	1.3 ± 10.8	7.4 ± 11.9	18.0 ± 11.9	16
E. albens	8.7 ± 4.4	3.9 ± 4.4	7.4 ± 3.7	24	2.3 ± 10.0	15.1 ± 10.0	11.7 ± 8.4	24

S.E., standard error

Table 3.7. Stem lengths of 16 species of *Eucalyptus* harvested at 12 months after pruning. There was no significant difference in pruning height for any variable and results are pooled

	Length of stems								Total no. of stems	Total weight of stems (g)
	0 - 40 cm		>40 - 50 cm		>50 - 60 cm		> 60 cm			
	No.	(%)	No.	(%)	No.	(%)	No.	(%)		
Species										
E. tetragona	8.8^{de}	38.3	6.9^{b}	30.0	3.3^{cd}	14.3	4.0^{ef}	17.4	23.0^{cd}	2093^{bc}
E. delegatensis	7.5^{e}	56.4	2.0^{b}	15.0	2.5^{cd}	18.8	1.3^{f}	9.8	13.3^{d}	428^{e}
E. kruseana	28.9^{ab}	65.5	9.4^{b}	21.3	3.8^{cd}	8.6	2.0^{f}	4.6	44.1^{cd}	959^{de}
E. brachyphylla	17.8^{cd}	59.0	9.3^{b}	31.0	1.3^{d}	4.0	1.8^{f}	6.0	30.0^{cd}	706^{de}
E. socialis	8.6^{de}	17.7	11.1^{b}	22.9	11.6^{b}	23.9	17.2^{cd}	35.5	48.5^{cd}	1666^{cd}
E. gillii	12.3^{de}	34.6	9.0^{b}	25.4	5.7^{bcd}	16.1	8.5^{def}	23.9	35.5^{cd}	1208^{cde}
E. orbifolia	5.3^{e}	29.9	3.4^{b}	19.3	3.4^{cd}	19.2	5.6^{ef}	31.6	17.7^{d}	409^{e}
E. crucis	22.8^{bc}	54.7	9.0^{b}	21.7	4.5^{bcd}	10.8	5.3^{ef}	12.8	41.5^{cd}	1000^{de}
E. macrocarpa	3.3^{e}	21.9	2.7^{b}	17.9	3.4^{cd}	22.6	5.7^{ef}	37.6	15.2^{d}	1447^{cd}
E. bridgesiana	27.5^{abc}	26.6	25.5^{a}	24.7	21.1^{a}	20.4	29.2^{b}	28.3	103.3^{b}	2938^{b}
E. globulus	27.5^{abc}	19.6	34.0^{a}	24.3	26.7^{a}	19.1	51.8^{a}	37.0	140.0^{a}	4913^{a}
E. cordata	34.3^{a}	59.0	11.0^{b}	18.9	7.0^{bcd}	12.1	5.8^{ef}	10.0	58.1^{c}	1549^{cd}
E. pulverulenta	9.1^{de}	25.6	5.6^{b}	15.7	6.4^{bcd}	18.0	14.5^{cde}	40.7	35.6^{cd}	1418^{cd}
E. cinerea	25.7^{abc}	27.4	25.3^{a}	27.0	19.9^{a}	21.2	22.9^{bc}	24.4	93.7^{b}	2955^{b}
E. pruinosa	6.0^{e}	23.7	4.2^{b}	16.6	5.3^{bcd}	20.9	9.8^{def}	38.8	25.3^{cd}	1076^{de}
E. albens	2.9^{e}	8.3	6.4^{b}	18.2	9.4^{bc}	26.6	16.6^{cd}	46.9	35.4^{cd}	1483^{cd}
Mean	***		***		***		***		***	***

*** Significant at $P < 0.001$; Mean values within a column followed by the same letter are not significantly different at $P \leq 0.01$.

Table 3.8. Effect of species and pruning height on total number and weight of stems of 16 species of *Eucalyptus* 12 months after pruning. The difference between species was significant at $P < 0.001$

	Total no. of stems			Total weight of stems (g)		
Pruning height	0.5 m	1.0 m	mean[A]	0.5 m	1.0 m	mean[A]
Species						
E. tetragona	28.4	17.6	23.0^{cd}	2680	1506	2093^{bc}
E. delegatensis	11.0	20.0	13.3^{d}	303	800	428^{e}
E. kruseana	46.2	42.4	44.1^{cd}	1000	927	959de
E. brachyphylla	39.5	20.5	30.0^{cd}	930	483	706de
E. socialis	48.7	48.3	48.5^{cd}	1795	1555	1666^{cd}
E. gillii	32.3	39.2	35.5^{cd}	996	1456	1208^{cde}
E. orbifolia	23.3	10.3	17.7^{d}	540	235	409^{e}
E. crucis	52.0	32.8	41.5^{cd}	1261	782	1000de
E. macrocarpa	16.5	13.8	15.2^{d}	1648	1245	1447^{cd}
E. bridgesiana	97.9	108.1	103.3^{b}	2602	3240	2938^{b}
E. globulus	93.7	159.9	140.0^{a}	3523	5509	4913^{a}
E. cordata	59.5	57.0	58.1^{c}	1408	1663	1549^{cd}
E. pulverulenta	28.4	44.0	35.6^{cd}	1435	1398	1418^{cd}
E. cinerea	92.0	96.0	93.7^{b}	2683	3318	2955^{b}
E. pruinosa	25.0	25.6	25.3^{cd}	1010	1141	1076de
E. albens	34.1	36.2	35.4^{cd}	1341	1582	1483^{cd}
Significance			***			***

***, Significant at $P < 0.001$; [A] Mean values within a parameter followed by the same letter are not significantly different at $P \leq 0.01$ according to Duncan's Multiple Range test.

Table 3.9. Regression equations and correlation coefficients (r^2) between total number of stems one year after pruning (y), with tree height (x_1), trunk diameter (x_2) and lignotuber diameter (x_3) measured at different times during development for 16 species of *Eucalyptus*

Measurements taken	Tree height (cm)	Trunk diameter (cm)	Lignotuber diameter (cm)
		Before pruning	
January 1995	$y = 48.9 + 0.38x_1$	$y = 8.3 + 0.1x_2$	$y = 13.4 + 0.12x_3$
(16 months)	$r^2 = 0.55$ (224 d.f.)	$r^2 = 0.59$ (224 d.f.)	$r^2 = 0.60$ (130 d.f.)
		Three months post pruning	
July 1995	$y = 60.5 + 0.52x_1$	$y = 14.5 + 0.22x_2$	$y = 21.7 + 0.2x_3$
(22 months)	$r^2 = 0.45$ (224 d.f.)	$r^2 = 0.69$ (224 d.f.)	$r^2 = 0.63$ (130 d.f.)
		Six months post pruning	
October 1995	$y = 73.8 + 0.24x_1$	$y = 15.7 + 0.25x_2$	$y = 23.9 + 0.22x_3$
(25 months)	$r^2 = 0.27$ (224 d.f.)	$r^2 = 0.69$ (224 d.f.)	$r^2 = 0.63$ (130 d.f.)
Significance	***	***	***

*** Significant at $P < 0.001$.

Table 3.10. Effect of pruning height and age at pruning on stem regrowth of *E. gunnii*

Height of pruning (m)	Number of stems (%)				Total number of stems	Total weight of stems (g)	Time after pruning to marketable stems (mnths)
	0 - 40 cm	>40 - 50 cm	>50 - 60 cm	> 60 cm			
				Pruning at 22 months			
0.1	29.3 (54)	10.0 (19)	7.7 (14)	7.0 (13)	54.0	1122	9
0.5	22.9 (33)	14.8 (21)	12.5 (18)	19.5 (28)	69.7	2753	9
1.0	15.0 (23)	12.5 (19)	16.0 (25)	21.0 (33)	64.5	3122	9
Significance	ns	ns	ns	ns	ns	*	
				Pruning at 25 months			
0.1	21.3 (33)	18.6 (29)	9.9 (15)	14.4 (22)	64.2	1466	6
0.5	27.1 (35)	20.7 (27)	12.7 (16)	16.9 (22)	77.3	1831	6
1.0	42.6 (31)	40.0 (29)	26.8 (19)	28.3 (21)	137.7	3251	6
Significance	**	**	***	*	***	***	

***, **, *, Significant at $P < 0.001, < 0.01, < 0.05$

Discussion

Of the 17 species studied, 11 have potential as cut foliage on aesthetic grounds. They are *E. tetragona, E. kruseana, E. socialis, E. gillii, E. crucis, E. bridgesiana, E. globulus, E. gunnii, E. cordata, E. pulverulenta* and *E. cinerea*. The foliage market requires leaves to be more rather than less glaucous. Generally, the more glaucous the leaf, the more wax that is present and the longer the wax tubes on the epicuticular surface (Wirthensohn and Sedgley, 1996). The extra wax load on leaves may be a character that could be manipulated with breeding.

Some of the species studied require early and regular pruning to maintain juvenile leaves, for example *E. pruinosa* changes leaf phase at 11 months. Early pruning of trees regulates and controls growth, flowering, fruiting and influences tree form. Eucalypts grown for foliage production are pruned more vigorously than other horticulturally important trees as no emphasis is placed on flowering or fruiting. All significance is on the regrowth of many juvenile stems which are the marketable product of these eucalypts. Some species are precocious and will flower on juvenile foliage, for example *E. brachyphylla* was observed to flower after 17 months, and this is a positive feature which adds value to the foliage. Stems can be harvested within the first eighteen months of planting, so growers can obtain an early return on investment compared to other horticultural enterprises. Foliage exports from Australia in 1993/94 were worth approximately $1.9 m which was an increase of 186% on the previous year, indicating an expanding world market.

This study has shown there is great variation between species for height, trunk diameter and lignotuber diameter. However with each species there was no difference in growth parameters when pruned to 0.5 m or 1 m 12 months after pruning at 19 months. All species studied have the ability to coppice, and the vigour of regeneration depends on the species. Greater than 50% of regrowth stems of 11 species were >40 cm in length, and there was considerable variation in regrowth stem number. *Eucalyptus kruseana, E. crucis* and *E. cordata* had short stems following pruning and these would be suitable for posies. Other variables affecting coppicing ability include shoot origin, season of cutting, diameter, height of stump, age and spacing (Blake 1983). This

experiment has found positive regressions between the number of regrowth stems and trunk diameter and lignotuber diameter. This could be used for prediction of yield. The pruning trial on *E. gunnii* showed that pruning at 19 months was damaging, and this also resulted in low stem numbers for *E. delegatensis, E. orbifolia* and *E. macrocarpa.* However by delaying the pruning of *E. gunnii* until 22 months produced marketable stems after 9 months or after 6 months when pruning was performed 25 months after planting. By pruning to 1 m, more stems were produced. This finding is in partial agreement with Bowersox *et al.* (1990) who found that tree size had no effect on the number of coppice shoots or shoot length but was affected by coppice stump height. A stump height of 60 cm for *E. saligna* doubled the number of shoots and increased the length of the longest shoot compared to lower cut stumps. Similarly, in a pruning trial on *Leucaena leucocephala*, Dutt and Urmilla (1987) found that significantly more sprouts were produced per stump at coppicing heights of 25-100 cm, than at ground level and that the height and diameter of coppice stems was greater for coppicing heights of 50-100 cm compared to ground level or 25 cm. In *Morus alba* (mulberry) Fotadar *et al.* (1995) found an increase in leaf yield with increasing pruning heights from the base of the tree.

This work has shown that a greater number of shoots regenerate from *E. gunnii* when pruned in spring than when pruned in winter or autumn. This is in agreement with other workers who have found that there was a decline in the number of shoots and length of shoots when some *Eucalyptus* species were cut in summer and a maximum number when cut in winter or early spring, the dormant season, when root reserves are at their highest (Blake, 1972; Cremer, 1972, 1973). Kruger and Reich (1993) studied coppicing effects on *Quercus rubra* and found that when seedlings are cut at the dormant stage rather than the active growing stage, the growth rate of sprouts is higher than uncut seedlings.

Eucalypts have strong apical dominance and one or more shoots may become quickly dominant, therefore, these shoots should be cut back or tip pruned when necessary to encourage desirable shoots. This will encourage the tree to branch as low as possible and then stems can be picked constantly when hardened off. Growth rates of

different species depends on soil type, rainfall, and other climatic conditions. When deciding which species and provenance to use, the soil type and irrigation conditions are factors which need to be considered, for example eastern Australian provenances are more suitable for regularly irrigated sites due to their great capacity to harvest available water (Johansson and Tuomela, 1996).

Little work has been done to show how long a single tree will keep producing epicormic shoots. Cremer (1972), while studying accessory buds of 21 species of *Eucalyptus,* found that the potential to produce epicormic shoots after several generations of removal, did not decrease. Similarly Penfold and Willis (1961), believes that coppicing can be carried out virtually indefinitely, due to the reservoir of epicormic buds. It has been documented that mallee lignotubers may last as long as 200 years (Anon., 1981) or 900 years (Tyson *et al.*, 1998). This author feels that the trees should be able to produce stems for many years as long as they are regularly fertilised to replace lost nutrients and kept free of disease and pests. Judd *et al.*, (1996) recommend that fertiliser ratios of N:P should be 1:1 in the first year increasing to 2:1 in later applications for *E. globulus*.

Up to 10% of some *Eucalyptus* species fail to produce epicormic shoots each time the trees are cut (Bulman, 1995). In this study 25% of the trees failed to reshoot. This could have been due to insect attack, extreme weather conditions, mechanical damage or physiological reasons. Important factors when pruning are to minimise the risk of decay entry by cutting on a slight angle to facilitate water runoff, and by using sharp equipment which produces wounds that heal quickly. After pruning, trees should be watered and fertilised to replace lost nutrients and encourage rapid new growth. New shoots should appear after a month depending on the species.

When all characteristics are taken into account, the best species from this study for cut foliage are *E. globulus, E. bridgesiana,* and *E. cinerea.* The first two species need to be pruned regularly to maintain juvenility and promote regrowth, while *E. cinerea* needs be pruned only to retain a manageable size. Species such as *E. orbifolia* and *E. delegatensis* were too slow regrowing and while *E. tetragona* and *E. macrocarpa*

produced amongst the lowest number of stems, it is suggested that the stems of these two species be left longer to encourage flowering and thus be of added value.

Chapter 4

Postharvest Treatment of Cut Stems

Introduction

Juvenile *Eucalyptus* foliage provides an important filler crop in floral decorations, for world floriculture markets, but little research has been conducted on its production or postharvest handling. *Eucalyptus* foliage has been cultivated in the United States for some years (Nowak and Rudnicki, 1990), and Rumine and Bellandi (1989) investigated 11 species for adaptability to the Italian climate. They concluded that *E. cinerea, E. stuartiana* F.Muell. ex Miq., *E. parvifolia* Cambage, *E. gunnii* J.D.Hook., and *E. maidenii* F.Muell. gave the best quality foliage. At present the worldwide industry is based on relatively few species, with *E. gunnii* the most widely cultivated; to fulfil the requirement for a wider range of foliage types, a selection and crossing program has been commenced (Ellis *et al.*, 1991).

The efficiency with which postharvest storage and handling are carried out is very important (Halevy and Mayak, 1979, 1981). Sucrose concentrations that are too high can damage *Eucalyptus* foliage, causing browning of leaf margins (Jones and Sedgley, 1993). An important part of postharvest handling is correct storage and transportation conditions, because foliage must often travel to distant markets with the risk of quality loss. Work carried out by Forrest (1992) on *E. gunnii* showed that foliage could be stored wet or dry for up to 4 weeks at 5 °C without damage. Similar work with *E. crenulata* Blakely & Beuzev. and *E. gunnii* by Jones *et al.* (1993, 1994) found dry storage at 1 °C for up to 35 days did not affect longevity. This study has been conducted to establish the most appropriate postharvest solutions for a range of species and hybrids.

Materials and methods

Plant material

All material was sourced from close to Adelaide, South Australia (lat. 34°58'S, long. 138°38'E). *Eucalyptus globulus* plants were located on a grower's property at

Inman Valley. *Eucalyptus cinerea* and *E. albida* stems were harvested from the Waite Agricultural Research Institute Arboretum. Stems of *E. sideroxylon, E. platypus, E. spathulata, E. cladocalyx* x *E. platypus, E. spathulata* x *E. platypus,* and *E. spathulata* x *E. sargentii* were collected from plantations at Murray Bridge. The hybrids were the result of a crossing program (Ellis *et al.*, 1991).

Vase life

Stems of *E. globulus* and *E. cinerea,* 40–50 cm long, were placed into 750 ml bottles containing either 0, 1%, or 2% sucrose with 8-hydroxyquinoline citrate (8-HQC) at 200 $mg.L^{-1}$, pH 5. Bottles were placed in a growth cabinet maintained at 20 °C with a 12-h photoperiod, 70% relative humidity, and illuminated at 315 $\mu mol.m^{-2}.s^{-1}$. The number of leaves on each stem was recorded initially and at regular intervals, and vase life was considered to be over when 50% of the leaves commenced wilting or browning.

Pulsing

Stems of *E. cinerea* were pulsed in 0, 1%, 5%, or 10% sucrose with 8-HQC for either 2 h at 24 °C, 24 h at 3 °C, or for 24 h at 20 °C. After pulsing, stems were placed in water with 8-HQC and tested for vase life as described above.

Pulsing and simulated transportation

Stems of *E. sideroxylon, E. spathulata, E. platypus, E. cladocalyx* x *E. platypus, E. spathulata* x *E. platypus* and *E. spathulata* x *E. sargentii* were placed into pulsing solutions of 0, 1%, or 5% sucrose with 8-HQC. Pulsing was conducted for 18 h at 3 °C. *Eucalyptus albida* stems were placed into pulsing solutions of 0, 1%, 5%, or 10% sucrose with 8-HQC for 2 h at 24 °C or for 24 h at 3 °C. After pulsing stems were stored dry, wrapped in newsprint, and placed 40 per transport carton in a room at 3 °C for 1 week. Stems were then placed into water with 8-HQC. Growth cabinet conditions and measurements were the same as for the vase life experiments.

Statistical analysis

Data were tested for analysis of variance using Genstat 5.2. Dunnett's test (P = 0.95) comparing treatment means to controls, is presented where appropriate. The

statistical designs were: 1) Vase life of *E. globulus* and *E. cinerea*: 24 stems of *E. globulus* x 3 treatments = 72 stems with two stems per bottle = 36 bottles divided into three blocks within a growth cabinet. For *E. cinerea* there were 36 stems divided into 3 treatments x 3 blocks x 4 replications with one stem per bottle. 2) Pulsing experiments: 3 pulsing solutions x 4 replications = 12 stems per genotype with two stems per bottle; bottles divided into two blocks within a growth cabinet. 3) *Eucalyptus albida* pulsing experiment: stems were pulsed with 4 pulsing solutions x 2 time/temperature regimes x 4 replications = 32 stems with two stems per bottle. Bottles were divided into two blocks within growth cabinet.

Results

Vase life

Sucrose solutions of 1% and 2% extended the vase life of *E. globulus* and *E. cinerea* by ≥ 2 days (Table 4.1). Stems of *E. globulus* had a vase life of 13 days and *E. cinerea* up to 19 days in 2% sucrose.

Pulsing.

There was no significant effect of pulsing with sucrose for up to 24 h on the vase life of *E. cinerea* stems that were subsequently held in water. Vase life ranged from 12 to 15 days following pulsing.

Pulsing and simulated transport.

Pulsing *Eucalyptus* stems of three species and hybrids for 18 h at 3 °C with sucrose solutions of 1% or 5% before dry storage for 1 week at 3 °C, gave no advantage over the control pulse solution except for the *E. spathulata* x *E. platypus* hybrid (Table 4.2), where 5% sucrose for 18 h at 3 °C doubled vase life. All other species and hybrids had a vase life of 5-6 days.

Vase life of *E. albida* stems was not improved over the control after pulsing with 1% to 10% sucrose at 3 °C or 24 °C before 1 week of dry storage at 3 °C (Table 4.3). Control stems lasted up to 18 days and treated stems up to 16 days. The longer cooler pulse was significantly better than the short warm pulse. The warm (24 °C) pulse with

sucrose was deleterious relative to the control, with vase life reaching only 3 days. This trend was evident after 4 days.

Table 4.1. Effect of sucrose on the vase life of cut stems of *Eucalyptus globulus* and *E. cinerea*. All solutions contained 8-HQC at 200 mg.L^{-1}.

	Vase life[zy] (days)	
Sucrose (%)	*E. globulus*	*E. cinerea*
0	6.5 b	14.9 b
1	9.7 b	16.8 a
2	12.7 a	18.8 a
SEM[x]	1.808	0.497
Significance	*	***

[z]Vase life was considered over when 50% of the leaves showed wilting or browning
[y]Mean comparisons with control using Dunnett's test at $P = 0.95$ (22 df) = 3.65 days. Within columns, values followed by a common letter are not significantly different.
*, ***Significant at $P \leq 0.05$ or 0.001, respectively.
[x]SEM = standard error of mean based on error mean square from analysis of variance.

Table 4.2. Effect of sucrose concentration in overnight (18 h, 3 °C) pulsing solutions before dry storage for 1 week at 3 °C on the vase life of cut stems of three *Eucalyptus* species and three hybrids. Stems were placed in water after dry storage. All solutions contained 8-HQC at 200 mg.L^{-1}

Species	Sucrose (%)	Vase life[zy] (days)
E. sideroxylon	0	6.9 b
	1	6.2 b
	5	6.4 b
E. platypus	0	6.4 b
	1	5.7 b
	5	6.1 b
E. spathulata	0	4.7 b
	1	6.3 b
	5	6.4 b
E. cladocalyx	0	5.6 b
x *E. platypus*	1	5.7 b
	5	4.6 b
E. spathulata	0	6.1 b
x *E. platypus*	1	5.2 b
	5	12.3 a
E. spathulata	0	6.5 b
x *E. sargentii*	1	6.4 b
	5	5.9 b
SEM[x]		1.16
Significance		**

[z]Vase life was considered over when 50% of the leaves showed wilting or browning.
[y]Mean comparisons with controls using Dunnett's test at $P = 0.95$ (20 df) = 2.36 days. Within columns, values followed by a common letter are not significantly different.
**Significant at $P = 0.005$.
[x]SEM = standard error of mean based on error mean square from analysis of variance.

Table 4.3. Effect of pulsing regime before dry storage for 1 week at 3 °C on the vase life of cut stems of *Eucalyptus albida*. Stems were placed in water after dry storage. All solutions contained 8-HQC at 200 mg.L^{-1}.

Treatment			Vase life[zy] (days)
Sucrose (%)	Time (h)	Temperature (°C)	
0	2	24	13.7 b
	24	3	18.1 a
1	2	24	2.6 c
	24	3	14.9 a
5	2	24	2.7 c
	24	3	16.2 a
10	2	24	2.3 c
	24	3	16.4 a
	SEM[x]		1.48
	Significance		***

[z]Vase life was considered over when 50% of the leaves showed wilting or browning.
[y]Mean comparisons with control using Dunnett's test at $P = .95$ (7 df) = 4.28 days. Within columns, values followed by a common letter are not significantly different.
***Significant at $P<0.001$.
[x]SEM = standard error of mean based on error mean square from analysis of variance.

Discussion

These results are in agreement with previous research showing that holding solutions containing up to 2% sucrose are beneficial, whereas a pulse of up to 10% sucrose is not (Forrest, 1992; Jones *et al.*, 1993, 1994). In our study, *E. cinerea* had a vase life of 19 days when held in 2% sucrose, but only 13-15 days after pulsing with 5% sucrose and holding in water. Sucrose solutions greater than 5% were not beneficial to the stems due to browning and wilting of the leaves. This work also confirms and extends previous results that highlight variability in species response. Jones *et al.* (1993, 1994) report that vase life of *E. crenulata* is improved by 0.25% sucrose, whereas *E. gunnii* responded to concentrations up to 2%. This study found that stems of *E. cinerea* responded equally to 1% and 2% sucrose, while 2% was more effective for *E. globulus*. *Eucalyptus cinerea* also had a longer vase life than *E. globulus*, and *E. albida* was the most tolerant of simulated dry transport. This difference existed despite the fact that all of the trees were grown in similar environments and stems were tested under controlled conditions.

This is the first study to investigate sucrose pulsing before dry storage of eucalypt stems, and is also the first to assess new hybrids. There was no advantage of a sucrose pulse before simulated dry transport on vase life, except for the hybrid *E. spathulata* x *E. platypus*. There was no obvious morphological reason why the hybrid stems showed such a dramatic effect of prestorage sucrose pulsing on vase life. Stems of *E. albida,* which were pulsed for 2 hours at 24 °C before storage, had a shorter vase life than those pulsed at 3 °C for 24 hours, an effect observed at all sucrose concentrations and in the control. Apparently, *Eucalyptus* foliage will not tolerate even moderately high temperatures for even a short period, unlike many cut flower stems that are routinely pulsed at temperatures between 20 °C and 27 °C to open flower buds (Halevy and Mayak, 1981).

Of particular significance is the variability in species response shown by this and previous studies, and the vase life superiority of the *E. spathulata* x *E. platypus* hybrid over both parental species following dry storage. The results suggest that introduction of

vase life and storage longevity into a *Eucalyptus* selection program may lead to rapid improvements in cut stem quality.

Chapter 5

Epicuticular Leaf Wax Structure and Regeneration

Introduction

Epicuticular wax is present on adult and juvenile leaves of *Eucalyptus* species. Structurally wax can occur as plates, tubes or a mixture of plates and tubes (Hallam, 1964). Tube wax on a leaf causes light to scatter which gives the glaucous appearance of a white or grey-green 'bloom'. This wax bloom and the attractive juvenile leaf shape of some species, makes eucalypt foliage a popular product in the expanding native floriculture industry which is currently worth $27.75 million in exports. Eucalypt foliage is used as a filler in flower arrangements and can be used as fresh foliage or dried and preserved in glycerine. Eucalypts may have glaucous juvenile and adult leaves, such as *E. cinerea,* glaucous juvenile leaves and glossy mature leaves, such as *E. globulus,* or glossy juvenile and mature leaves, such as *E. regnans*. Generally the wax structure is the same, except in a few cases, such as *E. brevifolia,* which has plate wax on mature leaves and tube wax on juvenile leaves (Hallam and Chambers, 1970). The physiological significance of epicuticular wax has been reported to include such functions as water repellency (Jeffree *et al.,* 1971), reflectance of high irradiation (Robinson *et al.,* 1993), plant pathogen resistance (Royle, 1976), reducing cuticular transpiration (Kolattukudy, 1980), frost hardiness (Barber, 1955), and insect antifeedants (Eigenbrode *et al.,* 1991) or deterrents (Phelan and Miller, 1982).

Important aspects of epicuticular wax include its chemical constitution, morphology or structure, and origin within the leaf. The chemical composition of the wax has a large influence on its structure. The main constituents of many *Eucalyptus* epicuticular waxes are long chain β-diketones and long chain primary alcohols (Horn *et al.,* 1964). The β-diketone-rich waxes form tubular structures, whereas the primary alcohol-rich waxes form plates (Horn *et al.,* 1964; Hallam and Chambers, 1970; Jeffree *et al.,* 1976). More recent studies on the chemical characteristics of *Eucalyptus* leaf wax include Courtney *et al.* (1983) and Garrec *et al.* (1995). The physical environment in

which the plant is grown plays a role in the wax structure of some other genera. Baker (1974) found that an increase in radiant energy, and decreased temperature and humidity led to an increase in wax deposits in *Brassica oleracea,* but an increase in temperature caused the waxes to develop horizontally, rather than vertically from the cuticle. This resulted in the formation of dendrites and plates rather than tubes and branching filaments. Other environmental effects on epicuticular wax have been reported, including high night temperature (Welker and Furuya, 1994), wind (van Gardingen *et al.,* 1991) and snow cover (Reiley *et al.,* 1995).

Wax morphology on specific organs of individual species is generally constant, but there is variation in deposits, particularly in the size and distribution of the particles during the ontogeny of many plant tissues. Bukovac *et al.* (1979) found that epicuticular wax deposition on *Prunus persica* leaves increased with leaf development until full expansion, and Freeman *et al.* (1979) showed that leaf wax concentration of *Citrus* species, initially increased and subsequently declined with leaf age, and the proportion of soft wax decreased with leaf age. Wax is present on the leaf before the lamina separates from the apex (Hallam and Chambers, 1970; Weiller *et al.,* 1994) and there seems to be no evidence of decomposition of wax components during leaf expansion, (Baker and Hunt, 1981).

There is still speculation on how wax components move through the epidermal cell wall and cuticle and where they are modified. Jenks *et al.* (1994), found that tubular epicuticular wax on *Sorghum bicolor* was secreted from papillae on epidermal cork cells. The wax is produced in these specialised epidermal cells where the endoplasmic reticulum is involved in the synthesis of wax precursors. These wax precursors were vesiculated and transported out of the cells by exocytosis. No secretory organs have been identified in eucalypts, but Hallam and Juniper (1992) separated three different molecular weight proteins from eucalypt leaf wax, which they proposed acted as carrier proteins for wax precursors through the hydrophilic cell wall and the cuticle.

Wax removal is a problem in the floriculture industry as it detracts from the appearance of the foliage. Tube wax is very easily removed from the leaf thereby downgrading the quality of the product (Jones and Sedgley, 1993). This study

investigates the structure of epicuticular wax as the leaf develops in 18 species of *Eucalyptus* and the regeneration of leaf wax after removal. The species were chosen because of their potential or current use in the commercial cut foliage market. Previous studies on *Eucalyptus* have looked at preserved material or replicas of fresh leaves (Hallam, 1964, 1970a, 1970b; Jones and Sedgley, 1993) or they have used frozen leaves coated or uncoated (Jeffree and Sandford, 1982). This study uses an Environmental Scanning Electron Microscope (ESEM). The advantages are that it provides the opportunity to study leaves fresh and does not require any pretreatment, so the waxes are more likely to be viewed in an unaltered, natural state.

Materials and methods

Unfolding leaves (designated day 0) were tagged on 18 species of *Eucalyptus*. Juvenile leaves of three trees per species were sampled on day 0, day 16, and on full leaf expansion (day 30), for epicuticular wax examination. Epicuticular wax was studied on proximal and distal areas of unfolding leaves (day 0) of all species except *E. albida.*

Regeneration of wax was investigated following removal from day 16 and day 30 leaves of *E. brachyphylla, E. cordata* and *E. kruseana*. Wax was removed by rubbing all visible bloom from the adaxial surface of the leaves while still attached to the tree. For each leaf age, four leaves per species were sampled at 0, 1, 3, 9 and 15 days after wax removal.

Juvenile leaves were examined fresh on the day of sampling. Sections, 5 x 5 mm, were carefully cut from the middle of the leaves, or from the proximal and distal areas of 0 day leaves, and placed on a cold stage, adaxial surface uppermost, of an Electroscan environmental scanning electron microscope (ESEM) at 4-7 Torr, 6-7 mm working distance, 10 kV, 4-10 ^{o}C and 100 μm aperture. Digital images were taken of the leaves and image analysis was performed on a Macintosh LC 475 computer using the public domain NIH Image program (written by Wayne Rasband at the US National Institute of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov). Measurements were taken of tube length and tube diameter at ten randomly selected areas on the image, measurement of percentage of the adaxial leaf

surface covered by wax utilised the whole image, and observations were made on the gross morphology of the wax. Data were analysed using ANOVA.

Results

All species observed had tube waxes and there were significant differences between species in tube length and percentage surface area covered by wax (Table 5.1). *Eucalyptus albida* and *E. tetragona* (Figure 5.1) had the longest tubes at 12.1 and 8.9 µm respectively and *E. delegatensis, E. pruinosa* (Figure 5.2) and *E. albens* had the shortest at 2.1, 2.4 and 2.6 µm respectively. *Eucalyptus albida, E. macrocarpa* and *E. tetragona* (Figure 5.1) had the most surface area covered by wax, and *E. albens, E. brachyphylla* and *E. delegatensis* had the least, with species such as *E. cinerea* intermediate (Figure 5.3). Wax tube diameter was not significantly different between species, the range was 0.23 - 0.42 µm. All species belonging to the section *Maidenaria*, series *Viminales* had tubes arranged in fan-shaped clusters (Figure 5.4).

From the images, the following descriptions of wax morphology were made on each species;

1. *E. tetragona*: long, rarely branched tubes. Dense, close cover of wax. (Figure 5.1).
2. *E. delegatensis* subsp. *delegatensis* short, branched tubes, open cover. (Figure 5.6).
3. *E. kruseana*: medium length, occasionally branched tubes, arranged in 'tufts', close cover. Amorphous wax blobs were visible at the proximal end of the day 0 leaf.
4. *E. brachyphylla:* short, branched tubes, sparse cover of wax. Amorphous wax layer was visible on day 0 leaves and a distinct zone of change could be seen where the amorphous wax gives way to branched tubes. (Figure 5.5).
5. *E. socialis*: medium length branched tubes, open cover. Day 0 leaves showed tubes emerging from amorphous wax 'blobs' at the proximal end of the leaf. (Figures 5.8–5.10).
6. *E. gillii*: medium length, branched tubes, close cover. Tubes appear in tufts on day 0 and day 16 leaves.
7. *E. orbifolia*: long tubes increasing in length two-fold from day 0 to day 30. Close cover at day 0 (tufts) to open cover at day 30. Day 0 leaves showed amorphous wax blobs at the proximal end of the leaf and structured wax at the distal end.

8. *E. crucis* subsp. *crucis*: medium length, branched tubes in tufts, close cover. Day 0 leaves showed tubes growing from amorphous wax blobs at proximal end of leaf.
9. *E. macrocarpa*: tufts of long tubes, occasionally branched, close cover. Day 0 leaves showed tubes emerging from amorphous wax blobs at the proximal end of the leaf.
10. *E. albida:* long tubes, occasionally branched, dense, close cover.
11. *E. bridgesiana*: medium length, branched tubes in tufts, close cover at day 0, more open cover by day 30.
12. *E. globulus*: medium length, branched tubes in tufts, close cover.
13. *E. gunnii*: medium length tubes arranged in tufts, close cover at day 0, open cover by day 30. Amorphous wax blobs were visible on the proximal end of day 0 leaf Tubes were seen emerging from this wax. (Figure 5.7). Wax at distal end arranged in tufts. Spiral wax was seen on a day 16 leaf.
14. *E. cordata*: long, branched tubes in tufts, close cover. (Figures 5.11–5.16).
15. *E. pulverulenta*: long tubes in fan-shaped tufts at days 0 and 16. Close cover at day 0, more open cover by day 30. (Figure 5.4).
16. *E. cinerea*: medium length, branched tubes in tufts, close cover. (Figure 5.3).
17. *E. pruinosa*: short, branched tubes, open cover (Figure 5.2). Occasionally longer, thicker tubes appear.
18. *E. albens*: short, branched tubes, open cover.

Amorphous wax was visible at the proximal end of day 0 leaves of some species. In *E. brachyphylla* it was a featureless layer which appeared fluid (Figure 5.5). Amorphous deposits were present in *E. kruseana, E. socialis, E. orbifolia, E. crucis, E. macrocarpa, E. delegatensis* (Figure 5.6) and *E. gunnii* (Figure 5.7). In both types, wax tubes appeared to be crystallising out of the film or deposit over a zone of transition.

Eucalyptus bridgesiana, E. cinerea, E. delegatensis, E. globulus, E. orbifolia, E. pulverulenta and *E. tetragona* showed no significant differences in tube wax measurements between the proximal and distal areas of day 0 leaves, although *E. cinerea* and *E. pulverulenta* showed a difference in percentage surface area covered

with amorphous and tube wax, with the distal areas having the greater percentage in both cases (Table 5.2). There were significant differences in *E. albens* and *E. pruinosa* for wax tube length and diameter between the distal and proximal areas, in *E. gillii, E. kruseana, E. macrocarpa* and *E. socialis* for wax tube length only and in *E. brachyphylla, E. cordata, E. crucis* and *E. gunnii* in tube diameter only.

There was a highly significant effect of leaf age on wax tube length and diameter (Table 5.3, Figures 5.8–5.10). Day 30 leaves had longer wax tubes and narrower tube diameters than day 0 leaves. Percentage surface area covered by wax was not significantly different for the different leaf ages.

The three species, *E. brachyphylla, E. cordata* and *E. kruseana* showed significant wax regeneration following removal. This can be seen for all measurement parameters (Table 5.4, Figures 5.11–5.16). However, the investigated species differ in the period of time when the longest wax tubes can be observed (*E. brachyphylla*: 15 days on day 16 leaves, *E. cordata*: 9 days on day 16 leaves, 15 days on day 30 leaves, *E. kruseana*: 9 days on day 16 leaves). For all species the day 16 leaves regenerated tubes as long as or longer than the leaves from which wax was not removed, only *E. brachyphylla* regenerated tube length to the control level for the day 30 leaves. The period of time for the regeneration of the widest tubes also differ within these species (*E. brachyphylla*: 15 days on day 16 and day 30 leaves, *E. cordata*: 3 days on day 16 leaves, 9 days on day 30 leaves, *E. kruseana*: 1 day on day 16 leaves, 15 days on day 30 leaves). All the species, for both leaf ages, regenerated wax tubes of similar diameter to the controls. There was regeneration of wax in percentage surface area covered in all three species, but *E. cordata* did not regenerate to the level of the untreated species mean for either leaf age. *Eucalyptus brachyphylla* regenerated similar to the species mean for both leaf ages and *E. kruseana* regenerated more than the species mean on the 16 day old leaves only. *Eucalyptus brachyphylla* had regenerated the most by day 9 in 16 day old leaves, although this was not significantly different from day 1 in either leaf age group. *Eucalyptus cordata* had regenerated the most by day 9 in both leaf age groups. *Eucalyptus kruseana* regenerated significantly more in day 16 leaves than day 30 leaves by day 9 after removal.

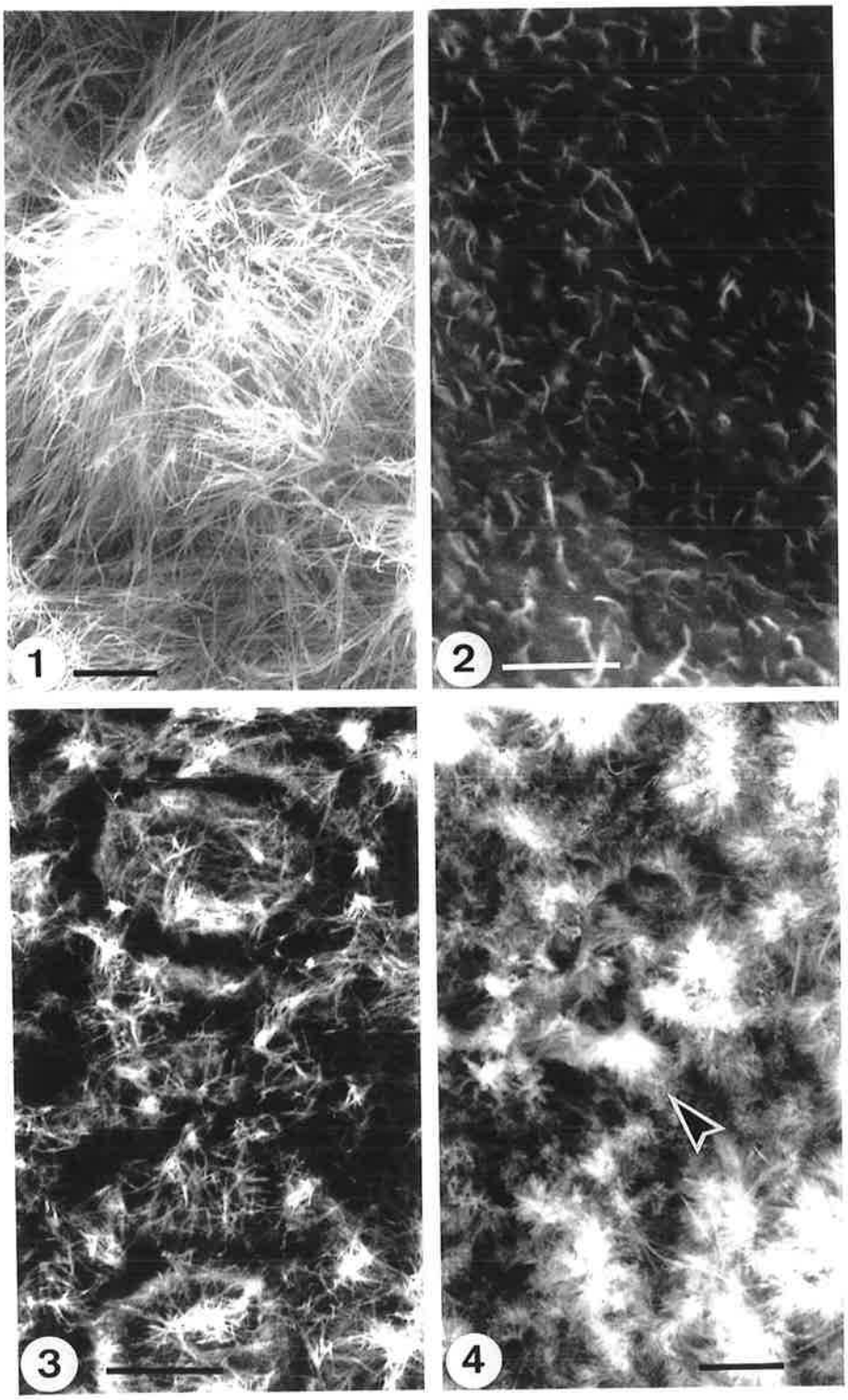
Environmental scanning electron micrographs of adaxial leaf surfaces.

Figure 5.1. *E. tetragona* at day 9 showing dense cover of long wax tubes. Scale: 10 μm.

Figure 5.2. *E. pruinosa* at day 0 showing sparse cover of short wax tubes. Scale: 5 μm.

Figure 5.3. *E. cinerea* at day 30 showing intermediate cover of intermediate length wax tubes. Scale: 10 μm.

Figure 5.4. *E. pulverulenta* at day 3 showing fan-shaped wax clusters (arrow). Scale: 5 μm.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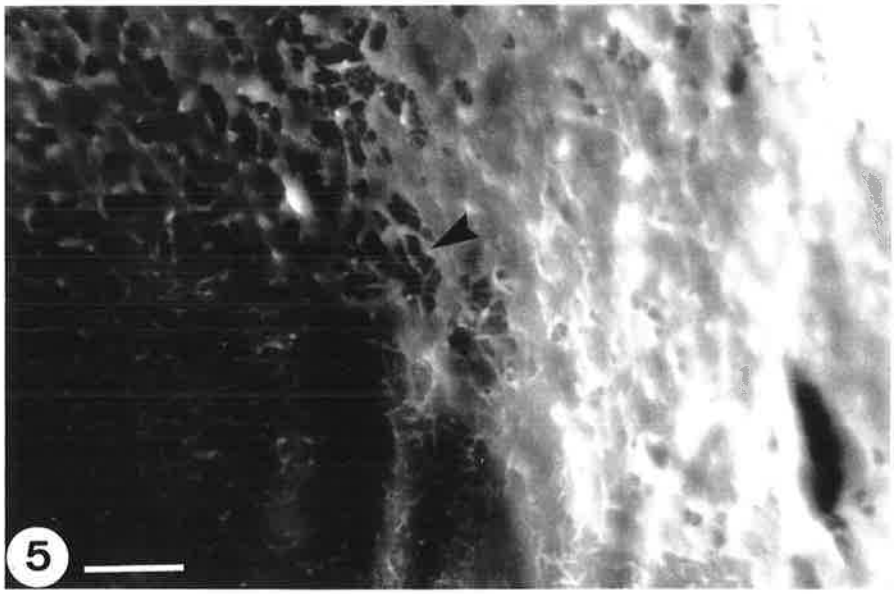
4

Environmental scanning electron micrographs of proximal adaxial surfaces of day 0 leaves.

Figure 5.5. *E. brachyphylla* showing zone of transition between amorphous and structured wax (arrow). Scale: 10 µm.

Figure 5.6. *E. delegatensis* showing wax tubes crystallising from amorphous wax (arrow). Scale: 10 µm.

Figure 5.7. *E. gunnii* showing wax tubes crystallising from amorphous deposits (arrow). Scale: 10 µm.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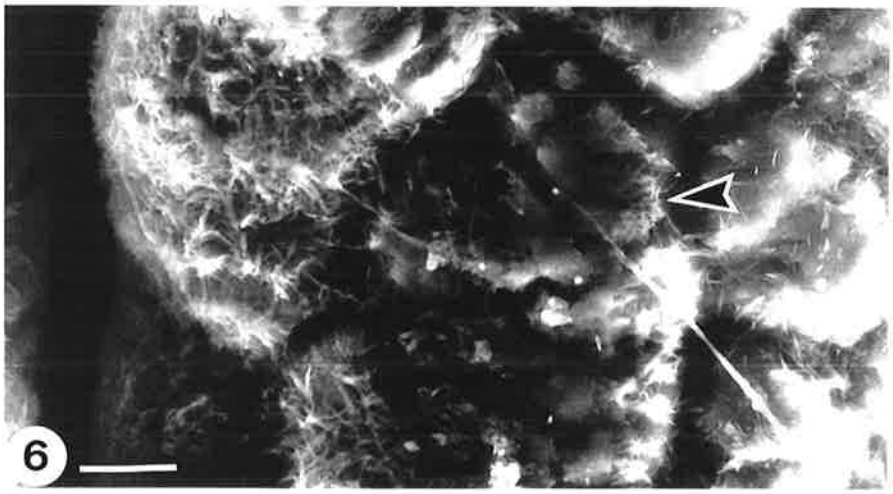
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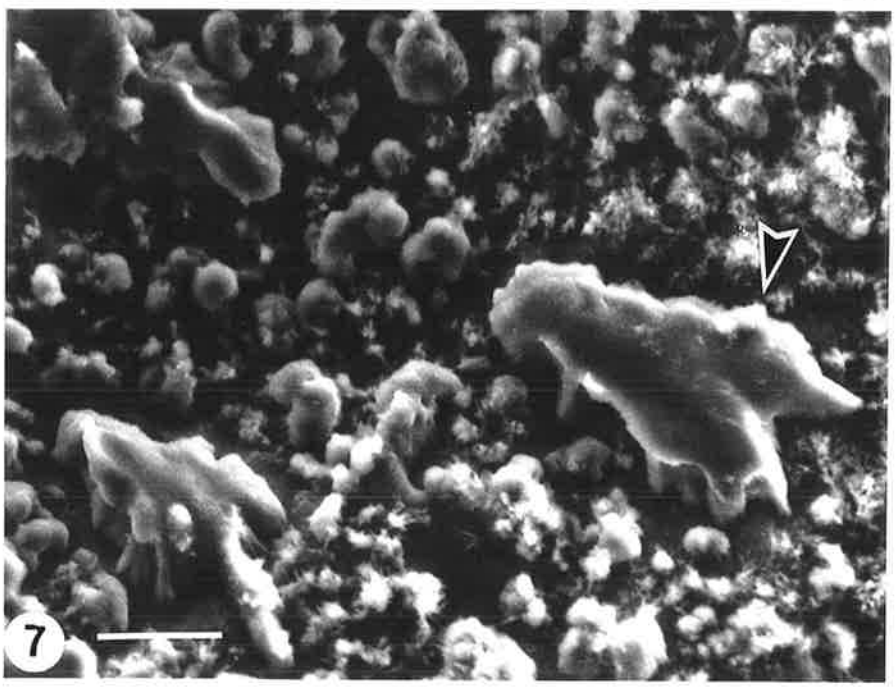
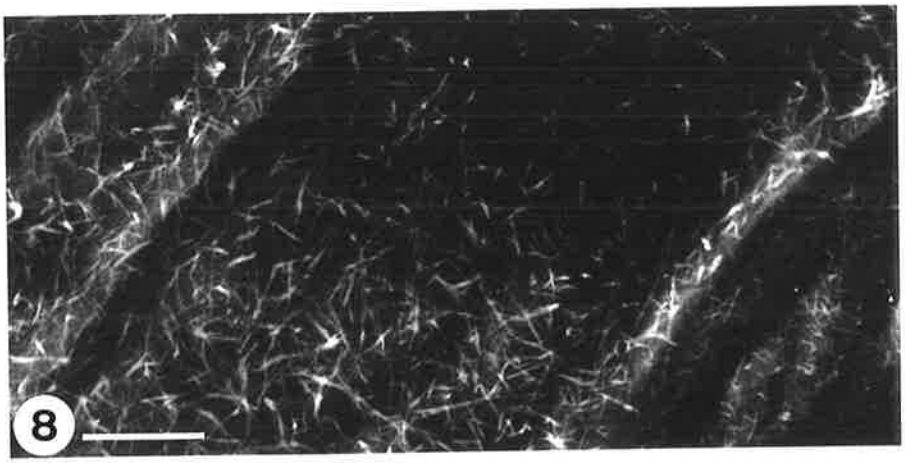

7

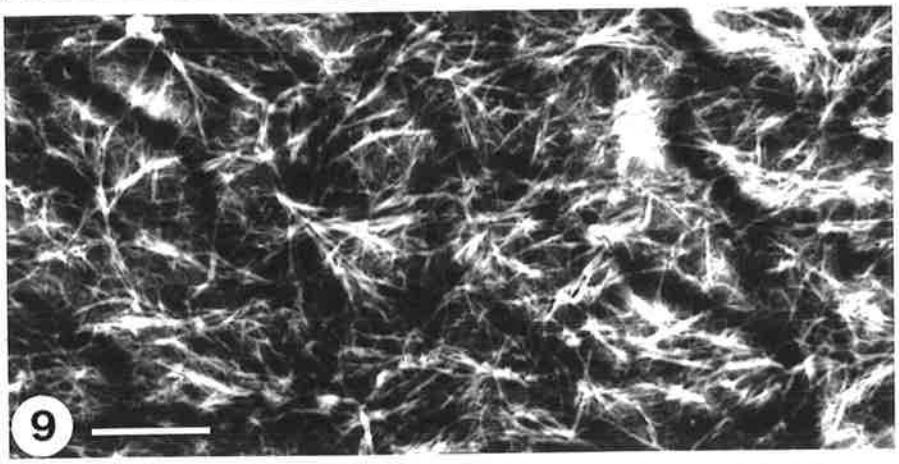
Environmental scanning electron micrographs of wax development of *E. socialis* leaves.

Figure 5.8. Day 0 showing sparse cover of short wax tubes. Scale: 10 µm.

Figure 5.9. Day 16 showing developing wax tubes. Scale: 10 µm.

Figure 5.10. Day 30 showing full cover of wax tubes. Scale: 10 µm.


8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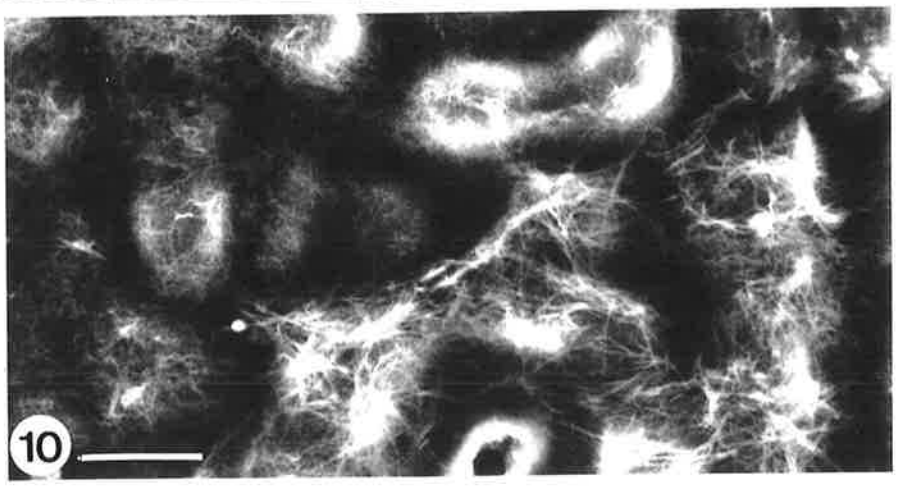

10

Environmental scanning electron micrographs of wax regeneration of *E. cordata*.

Figure 5.11. Immediately following wax removal at day 16. Scale: 10 μm.

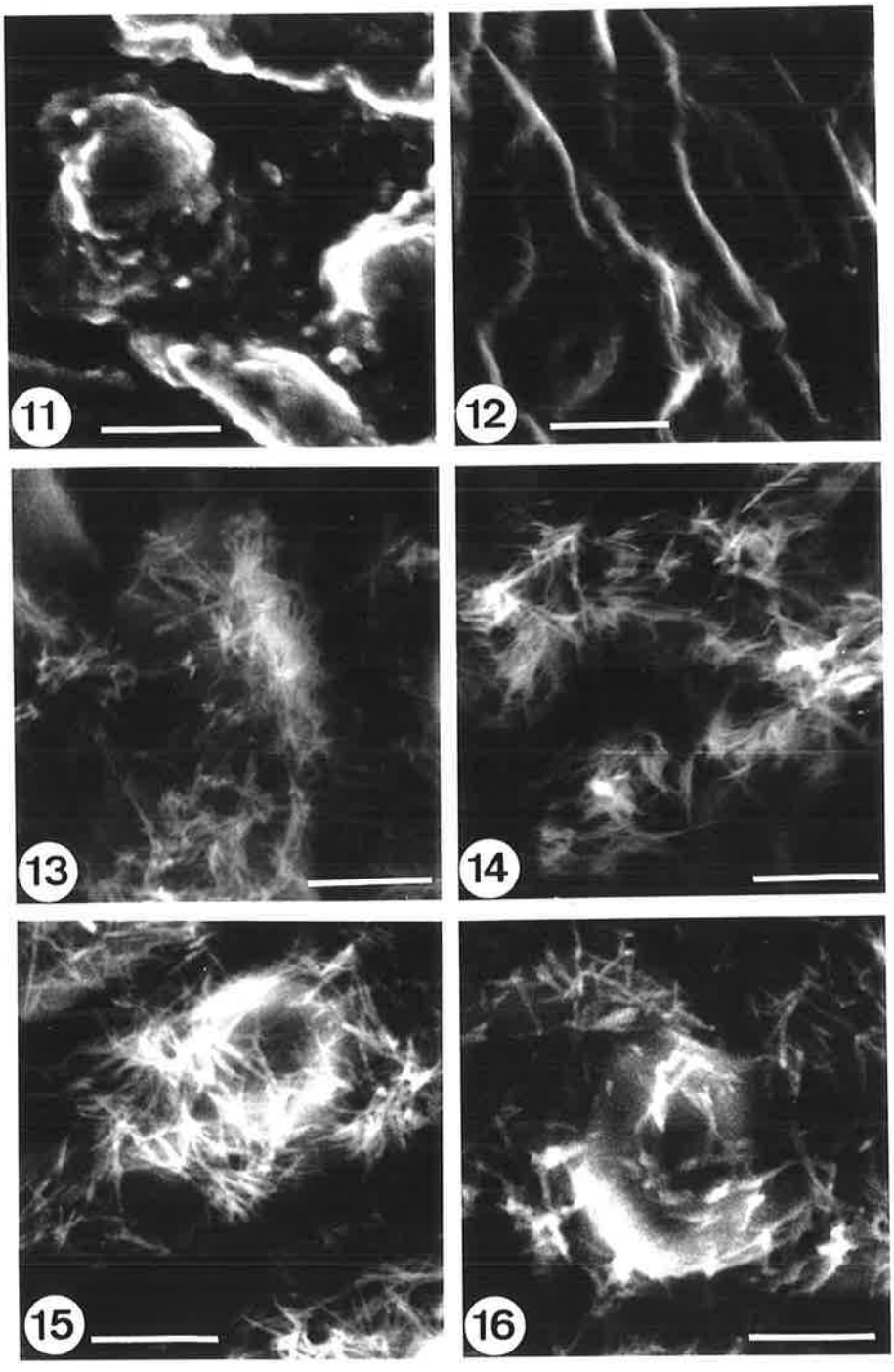
Figure 5.12. One day after wax removal at day 16. Scale: 10 μm.

Figure 5.13. Three days after wax removal at day 16. Scale: 5 μm.

Figure 5.14. Nine days after wax removal at day 16. Scale: 5 μm.

Figure 5.15. Fifteen days after wax removal at day 16. Scale: 5 μm.

Figure 5.16. Nine days after wax removal at day 30. Scale: 5 μm.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16

Table 5.1. Epicuticular wax measurements on juvenile leaves of 18 species of *Eucalyptus*. Data pooled for day 0, 16 and 30 leaves.

Species	Tube length (μm)[z]	Tube diameter (μm)	Percentage adaxial surface covered by wax[z]
Subgenus *Eudesmia*			
Section *Quadria*			
Series *Heteroptera*			
E. tetragona	8.9^{b}	0.26	72.2^{ab}
Subgenus *Monocalyptus*			
Section *Renantheria*			
Series *Eucalyptus*			
E. delegatensis	2.1^{g}	0.42	36.3^{ln}
Subgenus *Symphyomyrtus*			
Section *Bisectaria*			
Series *Kruseanae*			
E. kruseana	5.0^{ce}	0.25	58.8^{bcg}
E. brachyphylla	4.3^{ef}	0.29	30.8^{mn}
Series *Subulatae*			
E. socialis	4.8^{e}	0.24	$40.8^{ghijkln}$
E. gillii	5.1^{ce}	0.26	$47.6^{defghijklm}$
Series *Curviptera*			
E. orbifolia	6.9^{c}	0.26	54.8^{bck}
E. crucis	4.2^{ef}	0.32	61.0^{bcf}
E. macrocarpa	6.8^{cd}	0.28	66.5^{ac}
Series *Porantherae*			
E. albida	12.1^{a}	0.23	81.1^{a}
Section *Maidenaria*			
Series *Viminales*			
E. bridgesiana	5.0^{ce}	0.24	55.2^{bcj}
E. globulus	5.5^{ce}	0.26	62.2^{bcd}
E. gunnii	4.9^{de}	0.25	51.9^{cl}
E. cordata	5.4^{ce}	0.28	61.3^{bce}
E. pulverulenta	5.9^{ce}	0.32	56.5^{bch}
E. cinerea	5.3^{ce}	0.28	55.9^{bci}
Section *Adnataria*			
Series *Striolatae*			
E. pruinosa	2.4^{fg}	0.24	40.5^{hijkn}
Series *Moluccanae*			
E. albens	2.6^{fg}	0.37	28.7^{n}
SEM[y]	0.95		8.89
n	90		9
Significance	***	ns	***

*** $P < 0.001$; ns not significant, n=number of measurements

[z] Within a column, means followed by a different letter are significantly different at $P < 0.05$

[y] SEM= standard error of mean (36 df) based on error mean square from analysis of variance

Table 5.2. Effect of species and position on leaf on epicuticular wax structure from juvenile *Eucalyptus* leaves at Day 0

Species	Tube length (μm)[x]		Tube diameter (μm)[x]		Percentage adaxial surface covered by wax	
	Proximal	Distal	Proximal	Distal	Proximal	Distal
Subgenus *Eudesmia* Section *Quadria* Series *Heteroptera*						
E. tetragona	10.2^{a}	10.4^{a}	0.37^{ab}	0.32^{bd}	59.3	64.6
Subgenus *Monocalyptus* Section *Renantheria* Series *Eucalyptus*						
E. delegatensis	1.6^{r}	2.1^{rs}	0.18^{k}	0.19^{ik}	24.6	33.9
Subgenus *Symphyomyrtus* Section *Bisectaria* Series *Kruseanae*						
E. kruseana	1.8^{u}	3.8^{g}	0.19^{ikl}	0.17^{ln}	68.3	72.3
E. brachyphylla	3.0^{jk}	2.7^{jn}	0.28^{e}	0.35^{c}	46.4	21.7
Series *Subulatae*						
E. socialis	2.4^{q}	4.3^{c}	0.23^{gh}	0.22^{h}	54.1	48.2
E. gillii	2.8^{l}	4.8^{b}	0.18^{km}	0.17^{mn}	34.3	72.1
Series *Curviptera*						
E. orbifolia	1.3^{w}	1.6^{vw}	0.17^{jn}	0.20^{ij}	62.9	71.3
E. crucis	2.8^{lmn}	2.6^{mp}	0.15^{o}	0.38^{a}	70.8	82.1
E. macrocarpa	2.0^{st}	2.9^{kl}	0.17^{jn}	0.20^{ij}	74.7	81.9
Section *Maidenaria* Series *Viminales*						
E. bridgesiana	3.9^{efg}	3.4^{f}	0.23^{g}	0.24^{fg}	69.9	71.1
E. globulus	3.6^{hi}	3.8^{gh}	0.22^{h}	0.22^{h}	88.2	79.2
E. gunnii	1.5^{v}	1.6^{v}	0.22^{h}	0.16^{no}	71.1	75.8
E. cordata	4.2^{c}	4.3^{c}	0.33^{d}	0.25^{f}	59.1	76.5
E. pulverulenta	3.6^{di}	4.0^{de}	0.24^{fg}	0.24^{fg}	74.3	90.8
E. cinerea	2.1^{o}	2.7^{nop}	0.15^{op}	0.20^{ip}	41.3	72.8
Section *Adnataria* Series *Striolatae*						
E. pruinosa	1.6^{v}	2.4^{q}	0.23^{g}	0.24^{f}	27.1	28.2
Series *Moluccanae*						
E. albens	1.1^{x}	1.9^{tu}	0.16^{no}	0.23^{g}	48.9	31.6
SEM[y]	0.32		0.02			
n	10		10			
Significance[z]	***		***			

*** $P < 0.001$, n=number of measurements; [x] Within a measurement parameter, means followed by a different letter are significantly different at $P < 0.05$; [y] SEM= standard error of mean (162 df) based on error mean square from analysis of variance; [z] The interaction of treatment x species was highly significant.

Table 5.3. Effect of leaf age on epicuticular wax structure of juvenile *Eucalyptus* leaves.
Data pooled for 18 species

Age of leaf (days)	Tube length (μm)[z]	Tube diameter (μm)[z]	Percentage adaxial surface covered by wax
0	3.6[b]	0.33[a]	53.3
16	5.4[a]	0.31[a]	54.9
30	5.7[a]	0.20[b]	47.1
SEM[y]	0.32	0.04	
n	540	540	
Significance	***	**	ns

*** $P < 0.001$; ** $P < 0.01$; ns not significant, n=number of measurements

[y] SEM= standard error of mean (72 df) based on error mean square from analysis of variance

[z] Within a column, means followed by a different letter are significantly different at $P < 0.05$

Table 5.4. Effect of leaf age, time and species on regeneration of leaf epicuticular wax after removal. The three way interaction was significant at $P < 0.01$ for tube length and diameter and $P < 0.001$ for percentage surface area covered by wax

Species	Leaf age at wax removal (days) 16						Leaf age at wax removal (days) 30					
	Time after wax removal (days)											
	Control (wax not removed)	0	1	3	9	15	Control (wax not removed)	0	1	3	9	15
	Length of wax tubes (µm)[z]											
E. brachyphylla	4.7	0[f]	2.2[e]	4.3[cd]	5.6[b]	7.1[a]	5.7	0[f]	1.9[e]	2.2[e]	3.7[d]	5.5[bc]
E. cordata	4.8	0[d]	3.1[c]	2.8[c]	4.9[ab]	4.8[ab]	7.4	0[d]	2.2[c]	2.3[c]	4.5[b]	5.8[a]
E. kruseana	5.6	0[d]	2.6[c]	4.9[a]	5.9[a]	5.4[ab]	5.3	0[d]	2.1[c]	4.1[b]	2.3[c]	4.4[b]
	SEM[y] = 0.61 n=40											
	Diameter of wax tubes (µm)[z]											
E. brachyphylla	0.30	0[d]	0.23[bc]	0.24[bc]	0.27[bc]	0.33[a]	0.24	0[d]	0.25[bc]	0.22[c]	0.22[c]	0.29[ab]
E. cordata	0.26	0[d]	0.25[ab]	0.27[a]	0.19[c]	0.18[c]	0.17	0[d]	0.20[bc]	0.20[bc]	0.24[ac]	0.21[ac]
E. kruseana	0.25	0[c]	0.30[a]	0.27[ab]	0.23[b]	0.23[b]	0.25	0[c]	0.21[b]	0.22[b]	0.22[b]	0.27[a]
	SEM[y] = 0.03 n=40											
	Percentage adaxial surface covered by epicuticular wax[z]											
E. brachyphylla	18.8	0[d]	12.4[abc]	9.7[bc]	20.2[a]	19.0[ab]	14.4	0[d]	16.3[ab]	5.4[c]	9.3[bc]	12.5[abc]
E. cordata	68.5	0[f]	23.1[e]	41.2[cd]	63.0[a]	49.0[bc]	58.8	0[f]	21.1[e]	22.6[e]	54.0[ab]	36.5[d]
E. kruseana	55.2	0[e]	15.3[d]	40.6[b]	55.5[a]	60.8[a]	59.3	0[e]	21.7[d]	34.2[c]	18.6[d]	19.8[d]
	SEM[y] = 4.9 n=4											

n = number of measurements

[y] SEM= standard error of mean (66 df) based on error mean square from analysis of variance

[z] Within a species, means followed by a different letter are significantly different at $P < 0.05$

Discussion

Comparison of proximal and distal areas of day 0 *Eucalyptus* leaves has shown a gradation of wax structure from an amorphous state at the base of the leaf to a structured more mature form at the tip. This has not been seen on *Eucalyptus* leaves previously. A similar situation was reported on *Citrus* leaves and fruit (Freeman *et al.*, 1979) on which the amorphous layer was smooth, and the structured wax was viewed only on older leaves, not on the same leaf as was seen here. Jeffree and Sandford (1982) used plant material frozen rapidly in liquid nitrogen to investigate wax structure on several taxa including *E. gunnii*. They considered that amorphous granular coatings observed at low temperatures (-90 °C) on the plant surface was contamination of the cryo-specimen with amorphous water ice. This was not the case here as the specimens were placed into the ESEM fresh with no pretreatment and conditions in the chamber were such that no water was allowed to condense on the leaves. Thus the samples were free from any artefacts due to handling. In most cases wax tubes could be seen crystallising from the amorphous deposits. This supports previous hypotheses (Weisner, 1871; Baker, 1974, 1982) that wax is secreted to the surface in a volatile solvent, which then evaporates to deposit the structured wax. This phenomenon was seen only on very small leaves which were at an early stage of development, and was not observed during regeneration on the much larger 16 and 30 day leaves. The wax morphology of the eighteen species studied agrees with the descriptions given by Hallam and Chambers (1970) except for *E. delegatensis* and *E. bridgesiana*. It was found that *E. delegatensis* subsp. *delegatensis* has tube, not plate wax, and no plate wax was seen on *E. bridgesiana* leaves at any age.

Results from the image analysis of fresh specimens show that the length of tubular wax ranges from 2.1 to 12.1 μm, (0.6 μm minimum, 21.9 μm maximum) and the diameter from 0.23 to 0.42 μm, (0.08 μm minimum, 2.7 μm maximum) somewhat longer and thicker than described by Baker (1982) using preserved material. The age of the leaf has an influence on the structure of the wax. At day 30, eucalypt leaves were at full expansion and the wax tubes were at their longest and narrowest. This may indicate that wax synthesis is still occurring up until day 30, but the results certainly show that the tubes are extending lengthways at the expense of tube diameter. This accounts for

the slight decrease in percentage surface area covered by wax. As the leaf expands, the possibly finite amount of wax is spread over a larger area, the tubes are thinner, and so more leaf surface is visible. Baker and Hunt (1981) also found that ontogenetic variation in wax density was due to the rapidly expanding surface area of the leaf and the changing rate of wax production. Tube waxes are very susceptible to mechanical damage from environmental effects such as wind abrasion, water and pollution (van Gardingen *et al.*, 1991) and thus the measurements on older leaves may vary from younger leaves, but this does not explain the increase in tube length seen here.

When comparing different species, there is wide phenotypic variation seen in the amount and length of wax tubes present on the leaves and the ability of the species to regenerate wax after removal. This high level of variability should be expected due to the large amount of genetic diversity maintained within and between species of *Eucalyptus*. Such variation allows for the selection of superior phenotypes, in this case those with longer tube wax and those with greater capacity to regenerate wax.

The three species with the most surface area covered by wax, *E. albida, E. macrocarpa* and *E. tetragona,* come from shrubland or heathland regions with low rainfall. A high density of epicuticular wax on the leaf surface would be advantageous in minimising water loss from the leaf, protection against solar radiation and warming of leaves in these environments. These three species have the most glaucous juvenile leaves of the 18 species studied, and *E. albida* shows the greatest potential of the three for use in the cut foliage industry because of the white 'bloom' and the small, sessile, ovate-cordate shaped leaves. The other two species have larger leaves and are less attractive for foliage, but have potential for use as foliage with fruit or flowers.

Hallam (1970b) found that wax regeneration on *E. perriniana, E. cephalocarpa, E. alba* and *E. ovata* occurred rapidly on leaves which were in their exponential stage of expansion, and this study confirms that younger leaves regenerate longer tubes earlier than older leaves. Tube diameter on the regenerated wax of all three species was greater than the controls which is in agreement with the findings of Bermadinger-Stabentheiner (1995) working on spruce, who also found that regrowth on needles occurred within 48 hours but was unrelated to needle age. Jones and Sedgley (1993) found that wax did not

regenerate on *E. brachyphylla* or *E. cinerea,* but the leaves were from node five and may not have been in a stage of expansion. The process occurring on the leaves is regeneration of wax, i.e. resynthesis of wax compounds in the epidermal cells, as the leaves were, as much as possible, completely stripped of their wax. It is possible however, that some recrystallisation of the wax is taking place but only a minor part. Furthermore, it was shown in the results that leaves still synthesised wax at day 30, therefore regeneration at day 16 or day 30 is highly likely. Regeneration of wax occurred at different levels for the three different eucalypt species. *Eucalyptus cordata* regenerated longer tubes than the control but the density of wax did not reach control amounts, whereas *E. brachyphylla* regenerated wax beyond control amounts for both measurements, a positive feature from the floriculture industry perspective. This difference between the species can not be attributed to the different types of wax, as was found by Hallam (1970b) from comparison of species with plate and tube wax. In this study the waxes regenerated at a slower rate than was noted by Hallam (1970b) who reported an almost complete cover within 24 hours on leaves from the first four nodes.

Hallam and Chambers (1970) found that the degree of ornamentation on the margins of plates and the branching patterns of tubes and their arrangement could be used as a taxonomic tool. Their studies mainly supported the then current botanical classification of Blakely (1965). They found that the pattern of wax morphology confirmed the homogeneity of the tropical *Corymbia* or Bloodwood group, and that these species have characters in common with the closely allied genus *Angophora.* This work shows consistency across the six species in section *Maidenaria*, series *Viminales* which all show the same characteristic pattern of tubes grouped into fan-shaped clusters. Wax structure may be useful as a taxonomic tool for *Eucalyptus* in conjunction with other morphological traits and molecular analyses.

The use of *Eucalyptus* in the floriculture industry is currently centred on only a few species. Much wider use of the genus will allow growers to diversify and increase their market share. Based on the results of this study of wax structure and regeneration, the following species can be recommended for the cut foliage industry: *Eucalyptus tetragona, E. kruseana, E. brachyphylla, E. gillii, E. orbifolia, E. crucis, E.*

macrocarpa, E. albida, E. globulus, E. gunnii, E. cordata, E. pulverulenta, and *E. cinerea.*

Chapter 6

Epicuticular Wax of Juvenile *Eucalyptus* Leaves and Headspace Analysis of Leaf Volatiles

Introduction

The waxes associated with the leaf cuticle are a mixture of several classes of aliphatic compounds each of which contain homologous series of compounds. The most common classes of compounds found in plant waxes include primary alcohols, alkanes, fatty acids, aldehydes, and wax esters. The site of synthesis of wax esters, alkanes, and cutin is the epidermis (Kolattukudy *et al.*, 1976) but there is some doubt in the literature, regarding the mechanism of wax secretion in eucalypts. The wax may be exuded over and embedded in the surface of the cuticle via canals or ducts, and channels have been seen in *Eucalyptus cinerea* (Hallam, 1964) which traverse the cuticle and could constitute a pathway for the movement of wax or its precursors. Alternatively, Baker (1982), Hallam (1982) and Juniper and Jeffree (1983), suggest that the cuticle acts as a molecular sieve through which wax molecules, which may be in solution in organic solvents, or enclosed in protein or carbohydrate shells, can percolate. This diffusion hypothesis was supported by Anton *et al.* (1994). However, the form of the wax being translocated is not yet known. It could be as precursors, or as the final wax product, dissolved in a solvent to a semi-liquid form, from which the wax crystallises.

Epicuticular wax composition changes with the species or cultivars considered, and is further modified during leaf development, ageing and by the effect of light, temperature, and pollutants (Neinhuis *et al.*, 1994; Shepherd *et al.*, 1995, 1997; Turunen *et al.*, 1997). Genotypic variation in epicuticular wax structure and composition occurs within many species including maize (*Zea mays* L.), oats (*Avena sativa* L.), rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* (L.) Moench.) and wheat (*Triticum aestivum* L.), (O'Toole and Cruz, 1983; Uddin and Marshall, 1988). The inheritance of the presence or absence of epicuticular wax is influenced by several additive genes. In most of the species studied the glaucous form is dominant and the glabrous or 'glossy' form is

recessive. In a study on rice, Hague *et al.* (1992) showed that in a cross between parents with high and low amounts of epicuticular wax, the F_2 population showed a normal distribution between the parents. Within the species *Arabidopsis thaliana*, mutants occur with brighter green stems and siliques than the wild type. These plants are known as eceriferum (*cer*) mutants and the controlling genes have been localised to 21 different loci on the five *Arabidopsis* chromosomes (Koornneef *et al.*, 1989). In a more recent study on *Arabidopsis thaliana cer* mutants McNevin *et al.* (1993) have suggested functions for five of the *cer* genes. These genes regulate different functions in wax biosynthesis, the decarbonylation of fatty aldehydes to alkanes, the elongation of hexacosanoic acid to octacosanoic acid, the reduction of fatty aldehydes to primary alcohols and the production of free aldehydes, and the alteration in the chain length distribution of the different wax classes.

As juvenile *Eucalyptus* leaves unfold and expand, their glaucous appearance may disappear depending on the species. This could be due to changing chemical nature of the wax or a decreasing yield of wax per unit area of leaf. Epicuticular wax is deposited on the leaf surface in, as yet, an unknown form, however it has been postulated that the wax is dissolved in a solvent, is deposited at the surface, the solvent then evaporates leaving the wax to crystallise into various structures according to the wax chemistry. If such a solvent could be discovered it may shed light on the matter of wax movement through the cells and its deposition.

This study seeks to determine the variation in leaf epicuticular wax chemistry and yield between 17 species of *Eucalyptus* with horticultural potential, and to identify a possible solvent for epicuticular wax. The use of solid phase microextraction (SPME) to analyse volatiles emitted into the atmosphere from *Eucalyptus* leaves has not previously been reported. Static headspace sampling using SPME has been used in this study to trap volatiles escaping from freshly opened leaves of six species. The composition of leaf oil has also been analysed from four of these species to compare with the headspace volatiles.

Materials and methods

Sample collection

Plant material used is described in Chapter 2. Thirty two juvenile leaves per species were collected from nodes three to ten, with the oldest leaves fully expanded. Epicuticular wax synthesis still occurs at full expansion, but wax tubes extend lengthways at the expense of tube diameter and this accounts for a slight decrease in percentage surface area covered by wax (Wirthensohn and Sedgley, 1996). As the leaf expands, the possibly finite amount of wax is spread over a larger area, the tubes are thinner, and so more leaf surface is visible .

Epicuticular leaf waxes were obtained by brief immersion (30 sec) of leaves in 40 ml chloroform ($CHCl_3$) at room temperature. The extracts were filtered, transferred into pre-weighed beakers and evaporated to dryness at room temperature. The beakers containing the dried waxes were weighed and the weight of waxes obtained by subtraction. The wax yield for each sample was calculated per unit area (mg/cm^2). Total surface area of leaves was calculated by photocopying the leaves, carefully cutting them out and weighing them and calculating the area from a standard curve constructed from paper cut to known surface areas.

Chemical analysis of wax samples

For qualitative analysis the dried epicuticular wax samples were dissolved in 100 µl $CHCl_3$ and separated into compound classes by thin layer chromatography (TLC) on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) using a solvent system of n-hexane - diethyl ether - acetic acid (80:20:1) (Misra and Ghosh, 1992). Spots were visualised by exposing the TLC plate to UV light 254 nm and to iodine vapour, which was then evaporated by leaving the TLC plate for sometime at room temperature. Compound classes were identified by comparing their R_f-values with authentic samples. The spots were recovered from the plates by elution with $CHCl_3$. The separate compound classes were analysed after the addition of internal standards, and treatment with 100 µl N, O-bis(trimethylsilyl)-acetamide (BSA) and pyridine to convert the oxygenated compounds to their trimethylsilyl derivatives. The samples were then

heated for 1 hour at 70 °C. After heating, silylation reagent was removed by evaporation and the derivatized samples dissolved in 100 µl $CHCl_3$ for gas chromatography (GC) analysis. Internal standards used were n-nonadecane for nonpolar compounds, nonadecanol for polar compounds and methyl heptadecanoate for the ester fractions. Quantification was carried out by comparison with the corresponding standards by peak area integration of the FID signal and expressed as percentage of eluted material.

The analyses were performed with a Gas Chromatograph (Shimadzu 14A) equipped with an AT-35 capillary column (30 m, film thickness 0.25 µm, 0.32 mm i.d., Alltech) with an on column injection system (Shimadzu AOC-17) and autosampler (Shimadzu AOC-1400). GC was carried out with injector temperature of 250 °C, a programmed oven temperature of 160 °C for 2 min then a temperature increase of 6 °C min^{-1} to 320 °C, then held at 320 °C for 20 min, detector at 300 °C, N_2 carrier gas head pressure of 1.25 kg/cm^3, injection volume 5 µl, split ratio 1:50, fitted with a flame ionisation detector (FID). Analysis of *E. tetragona* wax was performed using a gas chromatograph-mass spectrometer (GC-MS) (Finnigan TSQ 70), 70 eV, operating in electron impact (EI) mode under the same conditions as described above with He as carrier gas at 13 p.s.i. head pressure. *Eucalyptus tetragona* was chosen as it was the most representative of all species. The wax constituents of the other species were identified by R_t comparison and GC comparison with authentic compounds, if available, or to mass spectra published in the literature (Stenhagen *et al.*, 1974).

Differences between leaf wax attributes between species were tested by ANOVA using Genstat version 5.1. Wax yield data were log transformed to give a normal distribution prior to ANOVA analysis.

Headspace analysis of developing leaves

SPME is a recently introduced method used in the analysis of air, water and soil. It has many advantages over traditional methods of active sampling and it can be used in applications where fast, sensitive, reproducible, simple to use and reusable sampling devices are required. Six species of *Eucalyptus* were chosen for static headspace sampling using SPME; *E. brachyphylla, E. crucis, E. gunnii, E. kruseana, E.*

macrocarpa and *E. orbifolia.* Trees used were from the Laidlaw plantation at the Waite campus. Four juvenile stems from one tree per species were tagged and leaves removed from nodes 2 – 5, leaving the growing tip intact. Previously, glass headspace vials with septum seals in place (7 cm x 2 cm; Alltech, Australia) were modified by having the bottom of the vial removed. These vials were placed gently over the growing tip of the stem and held in place with non-adsorbent cotton wool around the stem and the bottom of the vial made air-tight with 'Parafilm M' (American National Can) over which a twisty wire was tied for added security. If the stem was too weak to hold up the vial it was secured to a nearby stem. The overall result was a growing tip within an airtight headspace vial which could be left on the tree intact until sampling time.

The absence of air flow through the headspace vial means that the uptake of volatiles is determined by diffusion, therefore sampling should take from several hours to several days. Replicates 1 and 2 were sampled after 14 days, replicates 3 and 4 were sampled after 4 days. At sampling time, appropriate stems were cut, brought back to the lab and a solid phase microextraction needle with attached fibre (polydimethylsiloxane, 100 μm film; Supelco, USA) was inserted through the septum of the vial and left to adsorb volatiles for 5 minutes at room temperature. The fibre was retracted and the needle pulled out of the vial and immediately inserted into the injection port of the GC for analysis. Desorption time in the injection port was 5 minutes, 250 °C, needle depth 2.8 cm. The GC program used the same conditions as above except GC was carried out with a programmed oven temperature of 50 °C for 2 min then a temperature increase of 6 °C min^{-1} to 280 °C, then held at 280 °C for 5 min, detector at 300 °C, vent closed. Empty vials treated in the exact same manner as samples were used as blanks. External standards used were 5 μl each of 1,8-cineole, γ-terpinene and α-pinene (Aldrich). One separate headspace sample from *E. macrocarpa* was subject to GC-MS using the same instrument as the wax analysis and the same program as the headspace analysis. Volatile constituents were identified by GC comparison with authentic compounds, if available, or to mass spectra data published in the literature (Stenhagen *et al.*, 1974).

Leaf oil analysis

Four species of *Eucalyptus* were used for leaf oil analysis, *E. brachyphylla, E. gunnii, E. kruseana,* and *E. macrocarpa.* Ten juvenile leaves from nodes 0 – 3, from the same tree as the headspace analysis were sampled and 1 g lots of fresh leaves were cut into small pieces and extracted for 5 days in 5 ml ethanol containing 200 mg/L tetradecane as internal standard. External standards used were 0.5 µl/ml each of α-pinene, β-pinene, α-phellandrene, α-terpinene, R(+)-limonene, 1,8-cineole, γ-terpinene, p-cymene, linalool, terpinen-4-ol, α-terpineol, geranyl acetate, and tetradecane (Aldrich). GC analysis was the same as in the headspace analysis except injection volume was set at 5 µl, split ratio 1:50. Leaf oil constituents were identified by GC comparison with authentic compounds, where available. The monoterpenes 1,8-cineole and p-cymene co-eluted on the AT-35 column, so all samples were rerun on a BP20 capillary column (50m x 0.32mm, SGE). Differences between leaf oil attributes between species were tested by ANOVA using Genstat version 5.2.

Results

Chemical analysis of wax samples

Total concentration of surface wax extracted from 17 species are presented in Table 6.1. Results of the chemical analyses of leaf waxes from 17 species are shown in Figure 6.1 and Table 6.2. There was a significant difference between species for wax weight ($P < 0.005$) and for wax yield ($P = 0.07$). The largest wax abundance was recovered from *E. globulus* (1.9 mg) and the least from *E. brachyphylla* (0.9 mg). The greatest wax yield was from *E. orbifolia* (4.5 mg/cm^2) and the lowest from *E. delegatensis* (0.77 mg/cm^2).

GC analysis of individual wax samples showed that the major classes of compounds were n-alkanes, n-alcohols, β-diketones, fatty acids, esters and aldehydes. The GC separation of wax components and representative mass spectra of the major compound classes are shown in Appendix 6.

a) The hydrocarbons identified in all species were n-alkanes, the range of homologues was from C_{16} to C_{33}, with odd carbon-numbers dominant. n-Nonacosane

(C_{29}) was the major constituent of n-alkanes in most samples. There were significant differences between species for the amounts of the following homologues; C_{19}, C_{20}, C_{21}, C_{26}, C_{31}, and C_{32}. *Eucalyptus gillii* contained the greatest percentage of alkanes (25.6%) while *E. globulus* contained the least (3.5%).

(b) The primary alcohols identified were all even carbon-numbered ranging from C_{16} to C_{30}. The homologue n-hexacosanol (C_{26}) was the major constituent of most samples. There was a significant difference between species for n-octadecanol (C_{18}). *Eucalyptus bridgesiana* contained the greatest percentage of n-alcohols (11.1%) while *E. cinerea* contained the least (0.6%).

(c) Long chain β-diketones were the major component in all species except *E. delegatensis*. Chain lengths were from C_{29} to C_{37} but no β-diketones with a C_{36} chain length were detected. Odd carbon-numbered homologues were dominant. n-Tritriacontane-14,16-dione (C_{33}) was the principal homologue in all species except *E. albens*, *E. delegatensis* and *E. gillii* where n-pentatriacontane-dione (C_{35}) was the major homologue. Minor homologues were n-hentriacontane-14,16-dione (C_{31}) and n-nonacosane-12,14-dione (C_{29}). There were significant differences between species for amounts of C_{29}, C_{32}, C_{33}, C_{34} and C_{37} homologues. *Eucalyptus globulus* contained the greatest percentage of β-diketones (83.0%) while *E. delegatensis* contained the least at 24.7%.

(d) One homologue was detected in the aldehyde class, n-octadecanal (C_{18}). There was a significant difference between species with *E. albens* containing the greatest amount (9.2%) and *E. brachyphylla* and *E. kruseana* having none detected.

(e) The free fatty acids identified ranged in chain length from C_{14} to C_{34}. The dominant homologue was eicosanoic acid (C20) which was present in all species except *E. cordata*. there were significant differences between species for the homologues C_{17}, C_{19}, and C_{20}. Fatty acid composition varied greatly between species with *E. albens* and *E. tetragona* having the greatest number of acids, while *E. gillii, E. gunnii, E, kruseana* and *E. pruinosa* had the least number of acids. *Eucalyptus orbifolia* contained the greatest amount of fatty acids (15.9%) and *E. globulus* contained the least (3.0%).

(e) Long chain wax esters were a mixture of aromatic esters, and alkyl esters with chain lengths from C_{18} to C_{44}. Alkyl esters consisted of a mixture of acids of even carbon number ranging from 12 to 22 and alcohols of even carbon number ranging from 6 to 22 and odd carbon numbers ranging from 7 to 15. Aromatic esters were benzoic esters which consisted of alcohols of even carbon number ranging from 8 to 10. There was a significant difference between species for the esters C_{19}, C_{26}, C_{30}, C_{32}, and C_{36}. *Eucalyptus cordata* contained the greatest proportion of esters at 26.5% and *E. kruseana* contained the lowest at 6.4%.

Some compounds such as flavonoids and triterpenoids were undetected on this column.

Headspace analysis

The 15 compounds identified in the headspace of six *Eucalyptus* species are presented in Table 6.3. Replicates 2 and 4 of *E. crucis* were omitted from the table as only α-pinene was present in small quantities. No signal was detected from replicate 2 of *E. gunnii* or replicate 1 of *E. orbifolia*. The monoterpene ether 1,8-cineole (3.5–88.6%) was the most consistent compound followed by the bicyclic monoterpene hydrocarbon α-pinene (5.5–68.5%). The monoterpene hydrocarbons sabinene (0.5–4.0%) and limonene (1.1–5.0%) were detected in four of the six species. The greatest range of compounds was detected in *E. macrocarpa* with all but β-pinene observed. There was large variation between species and between replicates.

A low molecular weight compound in the headspace of *E. brachyphylla, E. gunnii, E. macrocarpa* and *E. orbifolia*, (1.0–55.1%) with a short retention time of R_t 1.1 min. was identified as cyano acetic acid. This compound was absent from the oil extract but is unlikely to be the unknown solvent, but perhaps originates from the breakdown of components in the headspace volatiles.

The volatile oil yields ranged from 0.01–1.18 mg/g fresh leaf weight and was greatest in *E. brachyphylla* followed closely by *E. gunnii* and *E. macrocarpa.*

All identified compounds in the headspace analysis except cyano acetic acid, were present in the oil extracts of the same species except for β-pinene which was present in the headspace of *E. gunnii* but absent from the oil extract. This could be due

to the amount in the oil extract which may have been below the detectable threshold or because of obstruction of the β-pinene peak by the extracting solvent.

Leaf oil analysis

Freshly isolated leaf oils extracted by solvent extraction from four *Eucalyptus* species were analysed by GC. The results are presented in Table 6.4. The principal components in each oil were the monoterpenes α-pinene (15.3–32.2%) and 1,8-cineole (20.8–54.7%); there were lesser amounts of the hydrocarbons sabinene (0.2–1.3%), limonene (1.3–5.2%), γ-terpinene (0.1–0.3%), α-selinene (0.1–4.8%) and the alcohol linalool (tr–0.6%). Sesquiterpenes generally occurred as minor components, the main ones were the hydrocarbons alloaromadendrene (0.3–8.2%), aromadendrene (0.1–1.6%) and viridiflorene (0.1–5.8%) and the alcohols ledol (0.1–2.2%) and unidentified alcohol-B (0.2–0.6%) as well as a phenol derivative (0.3–6.1%).

The ratio of oil yields to total volatile yields differ between the species with *E. kruseana* having the greatest ratio at 35.0, followed by *E. brachyphylla, E. gunnii* and *E. macrocarpa* (11.8, 4.1, and 2.1) respectively.

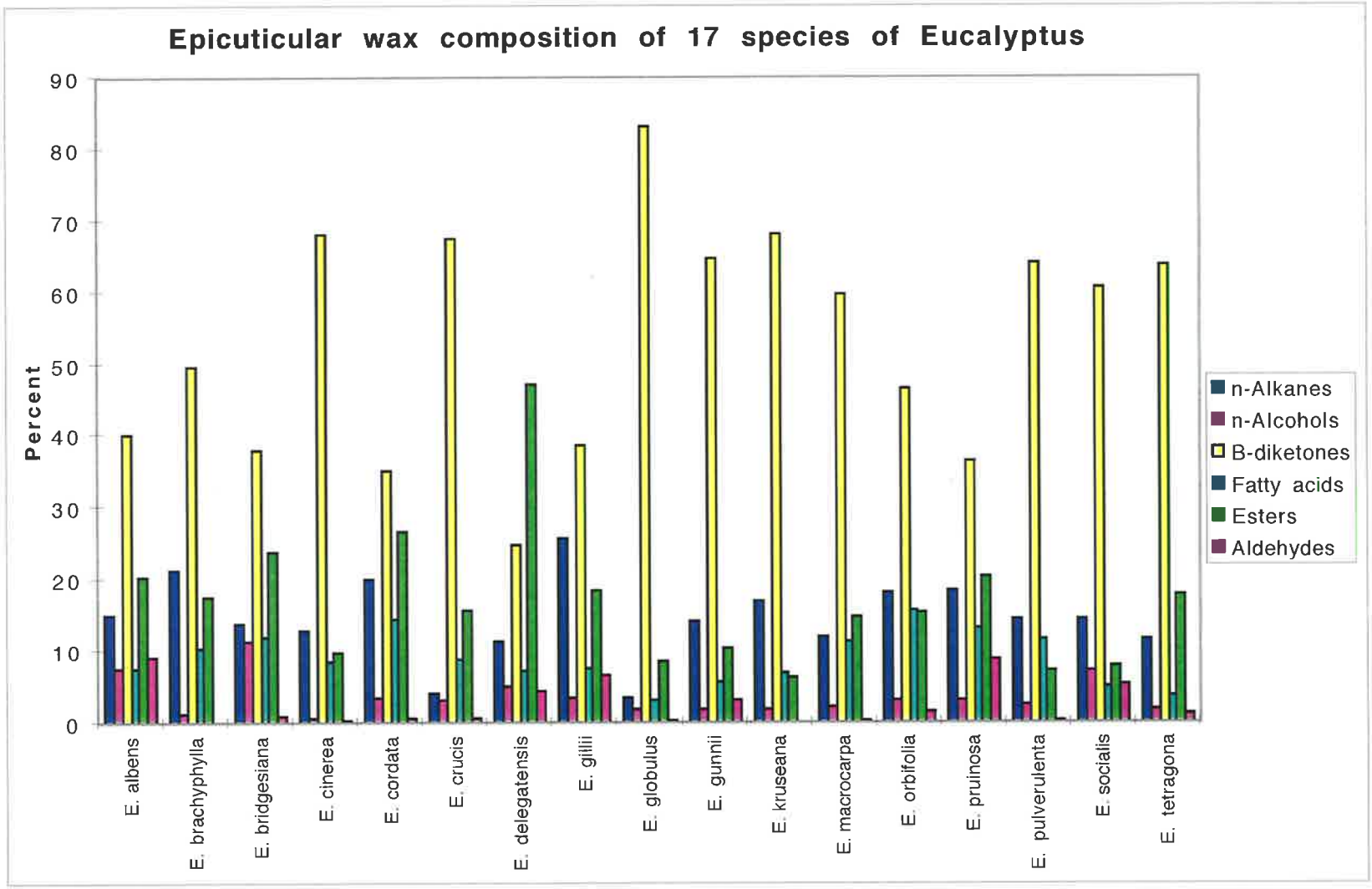


Figure 6.1. Mean percentage contents of compounds of epicuticular leaf wax from 17 *Eucalyptus* species.

Table 6.1 Yields of epicuticular wax from juvenile leaves of 17 *Eucalyptus* species

Species	Wax weight¥ (mg)	Total leaf surface area¥ (cm^2)	Wax yield¥ (mg/cm^2)
E. albens	1.6 ± 0.3	5.81 ± 1.73	1.70 ± 0.66
E. brachyphylla	0.9 ± 0.2	1.18 ± 0.27	3.72 ± 1.72
E. bridgesiana	1.0 ± 0.3	0.95 ± 0.20	4.16 ± 1.72
E. cinerea	1.4 ± 0.3	1.79 ± 0.41	1.94 ± 0.67
E. cordata	1.3 ± 0.2	2.95 ± 0.55	1.55 ± 0.87
E. crucis	1.7 ± 0.2	4.64 ± 1.49	1.40 ± 0.79
E. delegatensis	1.3 ± 0.2	4.97 ± 1.31	0.70 ± 0.25
E. gillii	1.0 ± 0.2	1.90 ± 0.44	2.15 ± 1.21
E. globulus	1.9 ± 0.4	6.45 ± 1.69	0.77 ± 0.27
E. gunnii	1.2 ± 0.2	1.11 ± 0.21	2.22 ± 1.02
E. kruseana	1.0 ± 0.2	1.50 ± 0.30	1.14 ± 0.40
E. macrocarpa	1.1 ± 0.2	1.95 ± 0.83	1.96 ± 0.86
E. orbifolia	1.3 ± 0.3	1.41 ± 0.46	4.50 ± 1.99
E. pruinosa	1.0 ± 0.2	3.05 ± 0.91	2.47 ± 0.89
E. pulverulenta	1.2 ± 0.2	4.64 ± 1.21	0.81 ± 0.33
E. socialis	1.0 ± 0.2	1.52 ± 0.28	2.87 ± 1.61
E. tetragona	1.7 ± 0.2	3.04 ± 1.00	0.95 ± 0.16
Mean	1.3	2.87	2.07
Significance	**	***	*

¥, mean and standard error of 32 leaves per species
***,**,*, $P < 0.001, < 0.005, = 0.07$

Table 6.2 Composition of leaf epicuticular wax from 17 species of *Eucalyptus*, determined by GC and expressed as percent of total eluted material

Compound class	n-Alkanes																	n-Alcohols							
Homologues	16	17	18	19	20	21	22	23	25	26	27	29	30	31	32	33	Total (%)	16	18	20	24	26	28	30	Total (%)
Species																									
E. albens	-	-	-	0.6	-	-	-	0.3	-	0.2	1.1	5.9	0.8	2.0	1.	3.3	15.2	0.3	0.1	0.2	0.1	4.3	2.1	0.4	7.5
E. brachyphylla	2.1	-	-	-	-	2.0	-	-	1.0	1.2	1.2	7.5	0.9	5.6	-	-	21.5	-	-	-	0.4	0.8	-	-	1.2
E. bridgesiana	0.4	-	-	-	-	2.8	0.5	-	-	0.5	1.1	1.1	1.0	2.6	1.1	2.9	14.0.	-	0.4	-	1.1	4.1	1.6	3.9	11.1
E. cinerea	1.0	0.5	-	1.0	0.9	3.3	-	-	-	-	-	6.1	-	-	-	-	12.8	-	-	-	-	0.3	0.3	-	0.6
E. cordata	0.6	-	-	10.0	0.4	6.0	-	-	0.1	0.1	0.7	0.6	0.8	0.4	0.3	0.1	20.1	-	0.1	0.1	1.0	1.0	0.7	0.5	3.4
E. crucis	0.5	-	0.3	-	0.2	1.7	-	-	-	-	0.3	0.4	0.1	0.3	0.3	-	4.1	-	-	0.6	1.3	0.6	0.7	0.1	3.3
E. delegatensis	-	0.6	-	-	-	0.5	-	-	-	0.1	1.3	6.4	0.4	2.1	0.1	-	11.5	-	4.6	-	0.1	0.5	-	-	5.2
E. gillii	3.6	-	-	-	6.7	-	-	-	-	6.3	-	5.8	3.2	-	-	-	25.6	-	0.6	-	-	2.8	-	-	3.4
E. globulus	-	-	0.3	-	0.2	0.4	-	-	tr	0.1	0.5	1.6	-	0.2	0.2	-	3.5	0.1	-	-	0.3	0.4	0.3	0.8	1.9
E. gunnii	-	-	-	0.3	-	4.3	-	0.5	-	0.2	-	8.8	0.2	-	-	-	14.3	0.1	-	-	-	1.1	0.3	0.5	2.0
E. kruseana	0.6	-	-	0.6	0.6	-	-	-	0.6	-	0.9	7.1	2.0	0.6	2.5	0.8	16.3	-	-	-	0.2	0.3	-	1.3	1.8
E. macrocarpa	-	-	-	0.3	0.3	1.8	-	-	-	0.3	-	6.5	2.4	0.4	-	-	12.0	0.2	0.2	-	0.8	0.2	-	0.7	2.1
E. orbifolia	0.2	-	-	-	-	-	-	-	4.9	-	-	8.5	2.0	1.8	0.8	-	18.2	-	-	-	0.2	1.5	0.7	0.8	3.2
E. pruinosa	0.3	-	-	7.7	-	-	-	-	-	0.7	-	4.8	-	4.9	-	-	18.4	-	-	-	0.5	1.5	0.1	1.0	3.1
E. pulverulenta	0.1	0.1	-	-	0.3	3.1	0.1	3.1	2.7	-	0.2	4.5	-	0.1	-	0.1	14.4	-	-	0.1	0.8	1.1	0.1	0.4	2.5
E. socialis	-	-	-	0.5	0.5	3.5	-	1.9	-	-	-	6.2	0.2	1.3	0.2	-	14.3	-	0.3	-	-	6.7	-	0.3	7.3
E. tetragona	-	-	-	-	1.0	6.0	-	1.4	0.2	-	-	2.9	0.1	-	0.1	-	11.7	0.1	0.1	-	0.2	0.3	1.0	0.3	2.0
Mean	0.6	0.1	tr	1.2	0.7	2.1	tr	0.4	0.6	0.6	0.4	4.9	0.9	1.3	0.4	0.4		0.1	0.4	0.1	0.4	1.6	0.5	0.6	
Significance	ns	ns	ns	**	***	***	ns	ns	ns	*	ns	ns	ns	***	***	ns		ns	*	ns	ns	ns	ns	ns	

-, not detected; ***, **, *, $P < 0.001, < 0.005, < 0.05$; ns, not significant.

Table 6.2 continued.

Compound class	β-diketones									Aldehyde
Homologues	29	30	31	32	33	34	35	37	Total (%)	18
Species										
E. albens	6.1	3.5	2.7	2.5	10.3	3.6	11.4	-	40.1	9.2
E. brachyphylla	1.4	-	-	-	29.4	6.0	12.9	-	49.7	-
E. bridgesiana	2.2	2.3	2.5	1.1	14.9	4.4	10.8	-	38.2	1.0
E. cinerea	0.3	1.6	5.9	1.8	37.9	4.0	16.6	-	68.1	0.3
E. cordata	0.1	1.4	1.8	0.4	22.0	0.4	8.8	-	34.9	0.6
E. crucis	0.3	2.6	5.8	1.0	39.7	1.3	15.1	1.6	67.4	0.5
E. delegatensis	-	1.0	1.8	5.1	2.9	0.8	11.3	1.8	24.7	4.3
E. gillii	-	4.1	-	-	12.2	1.4	20.7	-	38.4	6.7
E. globulus	0.3	2.7	9.2	1.7	55.9	2.3	10.8	0.1	83.0	0.2
E. gunnii	-	1.9	1.9	4.2	37.1	1.9	13.2	4.2	64.4	3.2
E. kruseana	-	-	3.4	0.3	47.5	0.3	16.4	-	67.9	-
E. macrocarpa	0.3	2.9	5.0	2.4	35.3	1.7	12.2	-	59.8	0.2
E. orbifolia	1.5	1.5	1.6	6.5	20.1	2.1	12.3	0.5	46.1	1.4
E. pruinosa	2.7	0.4	6.1	-	15.4	-	11.7	-	36.3	8.9
E. pulverulenta	4.8	0.8	4.0	1.0	39.3	1.9	11.9	0.5	64.2	0.3
E. socialis	1.2	0.3	1.5	0.4	37.4	3.3	16.4	-	60.5	5.5
E. tetragona	0.4	1.4	1.7	0.6	49.4	0.3	9.8	0.4	64.0	1.2
Mean	1.3	1.7	3.2	1.7	29.8	2.1	13.1	0.5		2.6
Significance	*	ns	ns	*	*	***	ns	**		**

-, not detected; ***, **, *, $P < 0.001, < 0.01, < 0.05$; ns, not significant.

Table 6.2 continued.

Compound class	Fatty acids																		
Homologues	14	15	16	17	18	19	20	21	22	24	27	28	29	30	31	32	33	34	Total (%)
Species																			
E. albens	-	2.8	0.2	0.8	-	0.2	1.0	0.1	0.3	1.4	-	-	-	0.3	0.1	-	-	0.2	7.4
E. brachyphylla	1.3	-	-	-	-	-	7.4	-	-	1.6	-	-	-	-	-	-	-	-	10.3
E. bridgesiana	-	2.5	-	-	-	-	6.3	-	-	0.6	-	-	0.4	0.4	1.7	-	-	-	11.9
E. cinerea	-	0.2	-	6.6	-	-	1.6	-	-	-	-	-	-	-	-	-	-	-	8.4
E. cordata	-	2.0	-	5.6	-	0.1	-	-	-	0.2	2.7	1.0	0.3	2.0	0.2	0.3	-	-	14.4
E. crucis	0.5	0.2	-	0.7	0.5	-	6.9	-	-	-	-	-	-	-	-	-	-	-	8.8
E. delegatensis	-	1.3	-	-	0.3	-	2.9	-	0.8	-	-	-	0.2	0.6	0.3	-	0.3	0.8	7.5
E. gillii	-	-	-	6.5	-	-	-	-	-	-	-	-	-	-	-	-	1.1	-	7.6
E. globulus	-	-	0.3	0.3	2.0	-	0.3	-	-	-	-	-	-	-	0.1	-	-	-	3.0
E. gunnii	-	-	-	-	-	-	5.3	-	-	-	-	-	-	0.3	-	-	-	-	5.6
E. kruseana	-	-	-	-	-	-	6.6	-	-	-	-	0.4	-	-	-	-	-	-	7.0
E. macrocarpa	0.1	0.2	-	0.2	0.4	-	5.1	-	-	3.2	0.5	1.6	-	-	0.1	-	-	-	11.4
E. orbifolia	-	-	0.5	-	1.0	-	12.7	0.1	-	-	-	-	0.1	0.2	-	0.2	1.1	-	15.9
E. pruinosa	-	-	-	-	-	0.2	12.8	-	-	-	-	-	-	-	-	-	-	-	13.0
E. pulverulenta	0.5	-	0.4	3.6	-	0.6	6.0	-	-	-	0.3	-	-	tr	-	-	-	0.2	11.6
E. socialis	0.2	-	0.2	0.5	-	-	4.0	-	-	-	-	-	-	-	-	-	-	-	4.9
E. tetragona	0.3	0.1	0.3	1.5	0.2	0.2	0.7	-	-	0.1	0.3	0.1	-	-	0.1	-	-	-	3.9
Mean	0.2	0.5	0.1	1.5	0.3	0.1	4.7	tr	0.1	0.4	0.2	0.2	0.1	0.2	0.2	tr	0.2	0.1	
Significance	ns	ns	ns	*	ns	*	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	

-, not detected; tr, indicates trace amounts less than 0.05% detectable; *, $P < 0.05$; ns, not significant.

Table 6.2 continued.

Compound class	Esters																			
Homologues	18	19	20	22	25	26	27	28	29	30	31	32	33	34	35	36	37	38	44	Total (%)
Species																				
E. albens	0.8	2.5	0.6	1.5	4.2	0.6	0.2	0.7	0.2	-	0.4	0.4	0.4	1.5	0.1	3.7	-	0.8	1.9	20.5
E. brachyphylla	-	11.3	1.7	-	-	1.4	0.2	-	-	0.6	-	-	-	-	-	2.1	-	0.3	-	17.6
E. bridgesiana	-	8.4	-	-	-	0.7	1.0	3.0	0.3	5.7	-	0.3	-	0.8	-	0.5	-	1.0	2.5	24.1
E. cinerea	-	3.9	-	-	-	0.1	-	-	-	5.1	-	-	-	0.3	-	-	-	-	0.1	9.7
E. cordata	-	2.9	-	-	1.6	1.2	0.7	0.9	0.5	4.1	0.4	2.3	1.6	3.6	1.4	1.6	-	1.4	2.3	26.5
E. crucis	0.7	2.4	0.4	-	-	0.2	0.3	1.0	0.1	2.3	0.1	2.3	0.4	2.9	1.3	0.5	0.3	-	0.3	15.6
E. delegatensis	2.8	21.8	2.8	1.0	-	3.1	-	-	-	0.7	0.7	1.0	1.4	5.5	1.0	3.5	-	1.4	-	46.6
E. gillii	-	6.3	-	-	-	0.4	-	-	-	-	0.1	3.9	-	2.0	-	2.3	3.0	0.3	-	18.4
E. globulus	-	1.7	0.1	0.1	-	0.2	0.1	1.0	0.3	1.3	0.1	1.5	-	1.2	-	0.8	-	0.2	-	8.4
E. gunnii	0.8	1.2	-	-	1.3	0.9	-	-	-	-	0.5	2.3	-	0.4	2.3	0.5	-	0.3	0.2	10.6
E. kruseana	0.3	1.1	0.5	-	0.7	0.2	-	-	-	0.7	-	0.1	0.1	1.7	-	1.1	-	-	-	6.4
E. macrocarpa	1.1	1.3	0.4	0.4	1.0	0.2	1.1	1.5	0.4	2.3	0.3	0.6	-	0.5	-	1.9	0.6	0.4	0.7	14.7
E. orbifolia	0.7	2.0	-	0.2	3.2	0.8	0.1	-	0.1	0.3	-	-	-	0.6	-	2.4	4.7	0.1	0.2	15.4
E. pruinosa	0.8	1.6	1.8	-	2.8	0.8	-	-	0.3	0.9	-	-	0.3	1.3	1.1	7.0	0.8	0.8	-	20.4
E. pulverulenta	0.4	1.2	0.5	-	1.6	0.2	-	0.5	0.3	1.0	-	0.8	-	0.2	-	0.2	-	0.1	tr	7.1
E. socialis	0.4	0.9	1.1	-	1.3	0.6	-	0.9	0.1	0.1	-	-	0.1	0.6	0.1	1.3	-	0.4	-	7.9
E. tetragona	-	0.6	0.2	0.9	1.2	0.1	-	7.3	tr	0.6	0.3	1.2	0.1	3.1	-	1.6	tr	0.4	0.2	17.8
Mean	0.5	4.2	0.6	0.2	1.1	0.7	0.2	1.0	0.2	1.5	0.2	1.0	0.3	1.6	0.4	1.8	0.6	0.5	0.5	
Significance	ns	**	ns	ns	ns	**	ns	ns	ns	***	ns	*	ns	ns	ns	**	ns	ns	ns	

-, not detected; tr, indicates trace amounts less than 0.05% detectable; ***, **, *, $P < 0.001, < 0.01, < 0.05$; ns, not significant.

Table 6.3 Percentage peak areas of compounds identified in the headspace of six *Eucalyptus* species sampled by headspace analysis using SPME

Headspace	*E. brachyphylla*				*E. crucis* †		*E. gunnii* §			*E. kruseana*				*E. macrocarpa*				*E. orbifolia* §		
Components	rep 1	rep 2	rep 3	rep 4	rep 1	rep 3	rep 1	rep 3	rep 4	rep 1	rep 2	rep 3	rep 4	rep 1	rep 2	rep 3	rep 4	rep 2	rep 3	rep 4
α–thujene	-	-	-	-	-	-	-	-	-	-	-	-	-	9.2	-	-	-	-	-	-
α–pinene	41.1	13.8	32.6	26.7	41.8	66.6	11.4	-	5.5	68.5	11.4	13.1	44.8	57.4	36.0	46.5	55.1	12.0	-	35.8
sabinene	1.6	-	-	-	-	-	0.5	-	-	-	-	-	-	1.2	1.0	-	-	-	-	4.0
β–pinene	-	-	-	-	-	-	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-
α–phellandrene	3.3	-	-	-	-	-	-	-	-	-	-	-	-	5.8	6.1	3.2	-	-	-	-
limonene	1.1	-	-	-	-	-	5.0	-	-	-	-	-	-	1.5	1.1	1.6	-	-	-	2.2
1,8-cineole	48.5	35.0	67.4	41.1	22.9	6.1	79.2	69.9	72.2	31.5	88.6	3.2	3.5	19.5	18.1	16.2	8.0	32.9	47.1	46.4
γ–terpinene	-	-	-	-	-	-	1.3	-	-	-	-	-	-	-	-	-	-	-	-	5.4
α–selinene	-	-	-	-	-	-	-	-	-	-	-	-	-	0.7	3.6	-	-	-	-	-
alloaromadendrene	-	-	-	-	-	-	-	-	-	-	-	-	-	2.7	12.9	29.2	31.8	-	-	-
aromadendrene	-	-	-	-	-	-	-	-	-	-	-	1.0	-	0.2	1.7	3.2	3.6	-	-	-
phenol deriv	-	-	-	-	-	-	-	-	-	-	-	-	-	0.7	2.9	-	-	-	-	-
viridiflorene	-	-	-	-	-	12.2	-	-	-	-	-	-	-	0.5	3.7	-	-	-	-	-
sesquiterpene alcohol-B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6	-	-	-	-	-
ledol	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6	3.0	-	-	-	-	-
unidentified compounds¥	4.4	51.2	-	32.2	35.3	15.1	2.2	30.1	22.3	-	-	82.6	51.7	-	9.3	-	1.5	55.1	52.9	6.2
Volatile oil yield‡	1.18	0.03	0.05	0.05	0.10	0.01	1.16	0.02	0.60	0.04	0.11	0.15	0.04	0.27	1.16	0.10	0.11	0.04	0.02	0.48

-, not detected; †, replicates 2 and 4 were omitted as the volatile oil composition was very small with only α-pinene detected in trace quantities.
§, replicate 2 in *E. gunnii* and replicate 1 in *E. orbifolia* were omitted as they showed no detectable signals.
‡, oil yield expressed as 1,8-cineole equivalents mg/g fwt.
¥, including cyano acetic acid R_t 1.1 min.

Table 6.4 Mean and standard error of leaf oil components extracted by solvent extraction using 99.6% ethanol and expressed as percent peak area from four species of *Eucalyptus*

Leaf oil Components	*E. brachyphylla* n=7	*E. gunnii* n=10	*E. kruseana* n=10	*E. macrocarpa* n=9	Mean & Significance	
α–thujene	0.1 ± 0.0	-	0.1 ± 0.0	0.2 ± 0.0	0.1	***
α–pinene	24.1 ± 0.3	15.3 ± 0.4	30.1 ± 0.6	32.2 ± 1.3	25.3	***
sabinene	0.8 ± 0.1	0.2 ± 0.0	1.3 ± 0.0	0.6 ± 0.1	0.7	***
β–pinene	0.5 ± 0.1	-	0.8 ± 0.0	0.6 ± 0.0	0.5	***
α–phellandrene	0.9 ± 0.1	-	4.3 ± 0.2	8.1 ± 1.2	3.4	***
α–terpinene	-	-	-	0.4 ± 0.2	0.1	**
limonene	2.0 ± 0.0	5.2 ± 0.2	2.4 ± 0.0	1.3 ± 0.2	2.8	***
β–phellandrene	0.1 ± 0.0	-	0.1 ± 0.0	-	0.1	***
1,8-cineole	26.5 ± 0.7	54.7 ± 0.5	34.8 ± 0.6	20.8 ± 0.6	35.2	***
γ–terpinene	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2	***
p-cymene	tr	-	tr	1.8 ± 0.3	0.5	***
linalool	0.6 ± 0.0	0.2 ± 0.0	tr	0.1 ± 0.0	0.2	***
terpinen-4-ol	0.2 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	-	0.3	***
α–terpineol	1.2 ± 0.1	3.3 ± 0.1	0.5 ± 0.0	-	1.3	***
6 methyl hepten-2-one	0.1 ± 0.0	tr	0.1 ± 0.0	0.1 ± 0.0	0.1	*
α–selinene	0.9 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	4.8 ± 0.2	1.4	***
alloaromadendrene	3.0 ± 0.2	1.2 ± 0.1	0.3 ± 0.0	8.2 ± 0.4	3.1	***
sesquiterpene hydrocarbon	0.2 ± 0.0	-	0.1 ± 0.0	0.2 ± 0.0	0.1	***
aromadendrene	0.7 ± 0.1	0.5 ± 0.0	0.1 ± 0.0	1.6 ± 0.1	0.7	***
phenol derivative	1.2 ± 0.2	6.1 ± 0.4	0.3 ± 0.0	1.8 ± 0.3	2.5	***
viridiflorene	5.8 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	2.1 ± 0.1	1.7	***
sesquiterpene alcohol-A	tr	0.2 ± 0.0	-	-	0.1	***
sesquiterpene alcohol-B	0.6 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.5 ± 0.1	0.4	***
ledol	1.1 ± 0.1	0.5 ± 0.0	0.1 ± 0.0	2.2 ± 0.2	0.9	***
C_{26} *alkane*	-	-	-	tr	tr	**
C_{23} *ketone*	0.1 ± 0.0	0.1 ± 0.0	-	0.1 ± 0.0	0.1	***
unidentified R_t *21.2*	13.3 ± 0.3	-	11.8 ± 0.2	-	5.9	***
unidentified compounds	16.0 ± 0.8	11.2 ± 0.7	11.7 ± 0.7	12.4 ± 0.7	12.6	***
Total oil yield‡	15.5± 0.5	7.3 ± 0.4	11.9 ± 0.7	3.4 ± 0.3	9.2	***

- , not detected.
tr, indicates trace amounts less than 0.1% detectable.
***, **, *, $P < 0.001, < 0.01, < 0.05$.
‡, expressed as 1,8-cineole equivalents mg/g fwt.

Discussion

Leaf waxes of 17 *Eucalyptus* species consist of alkanes, alcohols, fatty acids, wax esters, aldehydes and β-diketones. In general the β-diketones were the major wax constituent. This is in agreement with other workers (Hallam and Chambers, 1970; Horn and Lamberton, 1962; Horn *et al.*, 1964; Tulloch, 1976). Only *E. delegatensis,* which is known to be glaucous due to its cell structure (Barber, 1955), contained wax esters as the major component. Glaucousness has been variously correlated with the presence of high concentrations of β-diketones, C_{29} and C_{31} hydrocarbons, C_{29} ketones and to n-alcohols (Tulloch, 1976; Vioque *et al.*, 1994, 1996). Stevens *et al.* (1994) compared wax composition and ultrastructure in glaucous and glossy species of *Sedum* and concluded that different triterpenoid combinations were primarily responsible for glaucousness and that the amount of wax deposit was relatively high on glaucous species compared to green. They concluded that this may be an intensifying effect. Belding *et al.* (1998) found that wax yield of apple cultivars and the environment where the fruit is grown contributed more to wax ultrastructure than epicuticular wax composition. In this study, all species had tube wax on juvenile leaves but there were differences between the species, in the length and conformation of tubes and percentage surface area covered by wax (Chapter 5).

The large variation in wax yield and wax composition among the species studied is expected due to the great genetic diversity of the genera. The greatest differences between the species is seen in the percentage composition of the β-diketones, esters and n-alkanes. Variation in epicuticular wax composition among species has also been shown in mangrove (Rafii *et al.*, 1996), Solanaceae (Zygadlo *et al.*, 1994), Lamiaceae (Maffei, 1994) and Gramineae (Maffei, 1996). This work shows no relationship between the wax yield and the yield of volatiles. As the wax yields increase there is no associated increase in volatile yields. Of the six species studied for volatile analysis, the species with the highest wax yields, *E. orbifolia* and *E. brachyphylla*, did not have the highest yield of volatiles. Wax yield is increased by environmental factors such as high light intensity (Baker, 1974, 1982; Banks and Whitecross, 1971; Hallam, 1970b; Shepherd *et al.*, 1995) and low substrate water content (Letchamo and Gosselin, 1996).

All of the species in the present study were grown in the same environment under the same irrigation and fertilisation regime and therefore any differences in wax yield or composition are due to genetic differences.

Alkane synthesis occurs in the epidermal layer of cells and fatty acid elongation and wax ester synthesis occur in the epidermis (Kolattukudy *et al.*, 1976). The wax components must then be transported through the cell wall and cuticle to crystallise on the leaf surface. Using gel electrophoresis, Hallam and Juniper (1992) separated three different molecular weight proteins from eucalypt leaf wax, which they believe act as lipid transporters. Insects are known to use a high density lipoprotein (Lp) to effect lipid transport through aqueous mediums to the cuticle (Schal *et al.*, 1998). Jenks *et al.* (1994) have found a similar situation in *Sorghum*, where vesicles containing wax precursors, release their contents at cork cell secretion sites. Whether the wax is carried by lipoproteins or by other means, it is still uncertain in what form the wax is carried. Using headspace-SPME this study has attempted to find evidence of a highly volatile solvent which may act as the medium in which epicuticular wax is dissolved. One compound, cyano acetic acid was detected in the headspace and was absent from the oil extract but is more likely to be an artefact rather than a solvent.

Headspace analysis of several species have been published previously, for example *Eucalyptus globulus* (Mateus *et al.*, 1995; Street *et al.*, 1997), *Pinus sylvestris* (Sadof and Grant, 1997), *Prunus* (Visai and Vanoli, 1997), *Pyrus* (Scutareanu *et al.*, 1997), *Fagus sylvatica* (Tollsten and Müller, 1996), *Lycopersicon* sp. (Smith *et al.*, 1996), and *Ranunculus acris* (Bergström *et al.*, 1995), but relatively few using SPME, for example Matich *et al.* (1996) on apple volatiles, Schafer *et al.* (1995) on *Pinus* sp. and Ulrich *et al.* (1995) on strawberry aromas.

Headspace techniques are more sensitive to air-borne volatiles than other extraction methods such as solvent extraction and are nondestructive to the plant material. Volatile collection by headspace sorption onto the SPME fibre is enhanced as the accumulation of volatiles is enriched over the time of sampling. The key components of both the headspace volatiles and leaf oils of the species analysed were α-pinene and 1,8-cineole. The proportions of α-pinene and 1,8-cineole in headspace

replicates 1 and 2 of *E. kruseana* were reversed. However, all standard samples replicated well showing the same retention times and relative peak areas before and after the samples were run, therefore, any differences between replicates 1 and 2 or 3 and 4 on a single tree in the headspace analysis must be due to environmental reasons such as position on the tree and being subject to shading or direct sunlight. Other sources of variation can be ruled out, such as genetic, type and age of leaf, and techniques of extraction and analysis. The maximum temperature experienced by reps 1 and 2 was 31.1 °C whereas maximum temperature for reps 3 and 4 was cooler at 22.6 °C. This may explain the lower volatile yield in reps 3 and 4 in most of the species. This was also the case with *Fagus*, Tollsten and Müller (1996) found that there was an increase in volatile emission as ambient temperature increased and increased emission may also be related to solar radiation.

Different proportions of volatiles were recovered from headspace than found in the oils. This was also the case for the headspace volatiles and oils of *Boronia* (Mactavish and Menary, 1997). Spence and Tucknott (1983) found that volatiles produced within the plant are subsequently trapped in the epicuticular wax where they are released as the plant deteriorates. This may explain the differences seen in this analysis as the epicuticular wax cover on the juvenile leaves is a dense network of tubes covering up to 81% of the leaf surface (Chapter 5). Thus any trapped volatiles would not be released for a few months.

Eucalyptus species are well known to exhibit variation at population and specific levels for attributes such as essential oil terpenoids (Bignell *et al.*, 1994, 1996a,b,c, 1997a,b; Boland *et al.*, 1991; Li *et al.*, 1995, 1996; Wang *et al.*, 1997). Variations in oil composition and yield among clones of *E. camaldulensis* also occurs (P. Dunlop, pers. comm.). Within species chemical types or variants show marked differences in the chemical composition of their essential oils. Oil yield also varies from season to season, with winter/spring yields higher than summer yields in *E. globulus* (Wang *et al.*, 1997; Youseff *et al.*, 1991) however Li *et al.* (1995) stated that variation within species outweighed any seasonal effect on oil yield. Variation in oil yield

between leaf types is common among Symphyomyrtus species with juvenile leaves producing more oil than adult leaves (Li *et al.*, 1994, 1996).

Eucalyptus brachyphylla is considered to be a hybrid of *E. kruseana* and *E. loxophleba* Benth. Grayling and Brooker (1996) compared the leaf oils of the three and concluded that the hybrid leaf oil composition was intermediate of the parental plants, thus providing evidence for the hybrid status of *E. brachyphylla*. This study found similar percentages for *E. kruseana* and *E. brachyphylla* oil extracts except for a lower 1,8-cineole value for the latter. Means of the headspace volatiles gives similar results to the oil extract except the 1,8-cineole value is higher for *E. brachyphylla*, making it the same as Grayling and Brooker's (1996) oil value. They found two chemotypes of *E. kruseana, E. loxophleba* and *E. brachyphylla* containing varying amounts of 1,8-cineole, α-pinene and α-phellandrene. Chemical variants within several *Eucalyptus* species have been reported previously (Boland *et al.*, 1991). When their epicuticular wax composition is compared, *E. brachyphylla* had a higher n-alkane, fatty acid and ester composition than *E. kruseana*.

This study has looked at 17 *Eucalyptus* species, some of which have potential to be used for essential oil production as well as for cut foliage production. The following species have had their leaf oils analysed previously and contain high 1,8-cineole content; *E. pulverulenta* [82.5% (Brophy *et al.*, 1985)], *E. bridgesiana* [80.0% (Singh *et al.*, 1991)], *E. globulus* [60-85% (Lassak, 1988)], *E. kruseana* (51%), *E. brachyphylla* (58%), *E. orbifolia* (65.9%) and *E. crucis* (62.3%)(Bignell *et al.*, 1996a), *E. pruinosa* [63.2% (Bignell *et al.*, 1997a)], *E. cinerea* (54%), *E. cordata* (55%)(Baker and Smith, 1920) and *E. gunnii* [54.7% (this chapter)].

Chapter 7

Variability in Waxiness of Juvenile *Eucalyptus gunnii* Foliage for Floriculture

Introduction

The juvenile stems of *Eucalyptus gunnii* are one of the most popular of the foliages because of their attractive small, round leaves which are normally very glaucous. The species is cultivated for floriculture in Italy, France, USA and Australia. This glaucousness or waxiness gives the leaves a grey green bloom. In a population of cultivated *E. gunnii* there appear up to 10% of trees whose leaves have a green phenotype which is not as desirable in the cut foliage market. This phenomenon is well known in several Tasmanian species of *Eucalyptus* (Barber, 1955) and is due to clinal variation within a species where the green phenotypes are found in the more sheltered environments and the glaucous phenotypes are more frequent at exposed, higher altitudes. There is little information, however, on how the wax structure or composition varies between the phenotypes. Furthermore if a DNA marker could be found to identify genotypes as green or glaucous, this could be used in a breeding program to select superior grey cultivars.

The technique of identifying molecular markers using randomly amplified polymorphic DNA (RAPD) was developed by Welsh and McClelland (1990) and Williams *et al.* (1990). This assay uses a polymerase chain reaction (PCR) which is capable of analysing a large number of loci inherited in a Mendelian fashion. The technique of bulk segregant analysis (Michelmore *et al.*, 1991) examines specific genomic regions against a background of random genetic background of unlinked loci. It detects polymorphisms generated by RAPD markers between two bulk DNA samples derived from a population segregating for a gene of interest. Bulked segregant analysis has been used to analyse the genetically complex trait of wax synthesis by screening bulks of informative individuals. Comparison of bulks of extreme individuals with green or glaucous leaf colour may identify markers linked to this qualitative trait. The

aim of this work is to correlate genetic markers, chemical composition and ultrastructure of epicuticular wax with glaucousness of *Eucalyptus gunnii*.

Materials and methods

Plant material

Fresh leaf material (Figure 7.1) of *Eucalyptus gunnii* was collected from trees in a commercial plantation at Forest Range, South Australia. Trees were chosen on the basis of extremes of leaf colour, with 15 glaucous trees and 13 green trees chosen. Young leaves (nodes 1-5) were sealed in plastic bags and kept cold until returned to the laboratory. The samples were processed immediately for chemical analysis and frozen at -20 °C for DNA extraction. Fresh leaves were also used for electron microscopy.

Scanning electron microscopy

Microscopy was conducted as described in Chapter 5 (Wirthensohn and Sedgley, 1996). Two juvenile leaves from the third node of each tree were examined fresh on the day of sampling. Sections, 5 x 5 mm, were carefully cut from the middle of the leaves, and placed on a cold stage, adaxial surface uppermost, of an Electroscan environmental scanning electron microscope (ESEM) at 2.4 Torr, 6-8 mm working distance, 20 kV, 4-10 °C and 100 µm aperture. Digital images were taken of the leaves and image analysis was performed on a Macintosh LC 475 computer using the public domain NIH Image program (written by Wayne Rasband at the US National Institute of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov). Measurements were taken of tube length and tube diameter at ten randomly selected areas on the image, measurement of percentage of the adaxial leaf surface covered by wax utilised the whole image, and observations were made on the gross morphology of the wax. Data were analysed using ANOVA.

Wax extraction

Epicuticular wax from fifty leaves from five green trees and ten leaves from one grey tree, from nodes three to five, was extracted by immersing leaves in glass beakers containing 40 ml chloroform ($CHCl_3$) for 30 seconds. The extracts were filtered, transferred into pre-weighed beakers and evaporated to dryness at room temperature.

The beakers containing the dried waxes were weighed and the weight of waxes obtained by subtraction. The wax yield for each sample was calculated per unit area ($\mu g/cm^2$). Total surface area of leaves was calculated by photocopying the leaves, carefully cutting them out and weighing them and calculating the area from a standard curve constructed from paper cut to known surface areas.

Wax analysis

Wax analysis was conducted as described in Chapter 6. For qualitative analysis the epicuticular wax samples of *E. gunnii* leaves were separated into compound classes by thin layer chromatography (TLC) on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) using a solvent system of n-hexane–diethyl ether–acetic acid (80:20:1) (Misra and Ghosh, 1992). Spots were visualised by exposing the TLC plate to UV light 254 nm and to iodine vapour. The spots were removed from the plates and eluted with $CHCl_3$. The wax samples were analysed, after the addition of internal standards. Internal standards used were n-nonadecane for nonpolar compounds, nonadecanol for polar compounds and methyl heptadecanoate for the ester fractions. Quantification was carried out by comparison with the corresponding standards by peak area integration of the FID signal and expressed as percentage of whole wax.

The analyses were performed with a Gas Chromatograph (GC) (Shimadzu 14A) equipped with an AT-35 capillary column (30 m, film thickness 0.25 µm, 0.32 mm i.d., Alltech) with an on column injection system (Shimadzu AOC-17) and autosampler (Shimadzu AOC-1400). GC was carried out with injector at 250 °C, oven temperature program of 2 min at 160 °C, 6 °C min^{-1} to 320 °C, then held at 320 °C for 20 min, detector at 300 °C, N_2 carrier gas head pressure of 1.25 kg/cm^3, injection volume 5µl, split ratio 1:50, fitted with a flame ionisation detector (FID). Wax constituents were identified by GC comparison with authentic compounds run under identical conditions of analysis and by GC-MS analysis as described in Chapter 6.

DNA isolation

DNA was extracted using the CTAB isolation method (Doyle and Doyle, 1990) modified as follows to improve yields and remove contaminants: 0.5 to 0.6 g frozen leaf tissue was ground in a mortar and pestle with liquid nitrogen and scraped into a 14 ml

centrifuge tube containing 6 ml of preheated CTAB isolation buffer. After incubation at 60 °C the samples were extracted twice with chloroform-isoamyl alcohol (24:1, v:v). The aqueous phase was removed to a clean centrifuge tube and 1/10 volume of 3M sodium acetate and 1 volume of cold isopropanol was added and gently mixed and placed in a -20 °C freezer for one to two hours to precipitate the DNA. After centrifugation the DNA pellet was washed twice with 70 % ethanol, dried and resuspended in 400 µl TE buffer (Sambrook *et al.*, 1989) and transferred to eppendorf tubes. To remove contaminants 7.5M ammonium acetate was added to the TE buffer and DNA suspension to a final concentration of 2.5M and placed in a -20 °C freezer for 15 minutes. The mixture was centrifuged at 12,000 g for 20 minutes. The supernatant was transferred to fresh eppendorf tubes and the DNA was precipitated again as above. The DNA was resuspended in 400 µl TE buffer with 1 µl RNAse.

DNA was subjected to gel electrophoresis on 1 % agarose gels in TBE buffer (Sambrook *et al.*, 1989), and stained with ethidium bromide. DNA concentration was estimated by visual assessment of band intensities, compared to known genomic DNA standards. The concentration of DNA extracted varied from approximately 5ng/µl to 100 ng/µl. The concentration of all DNA samples used for RAPD analysis was adjusted to approximately 5 ng/µl prior to bulking. Aliquots (50ng of DNA) of each individual with green or glaucous leaves were bulked together to form a 'green' and 'glaucous' bulk.

PCR amplification

The optimised PCR reactions were carried out in 25-µl volumes containing 1 x *Taq* buffer (Gibco-BRL), 3 mM $MgCl_2$, 200µM of dGTP, dATP, dCTP, dTTP (Promega), 1µM 10-mer primer (Operon Technologies), 1 unit *Taq* polymerase (Gibco-BRL), 0.5 µl Gene 32 Protein (Pharmacia Biotech), and 20 ng of genomic DNA for both bulked and individual tree reactions. Each reaction mix was overlaid with PCR- grade paraffin oil. Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) programmed for an initial denaturation step at 94 °C for 2 min., followed by 41 cycles of 94 °C for 1 min., 36 °C for 1 min., 72 °C for 2 min. and terminated with a final extension step at 72 °C for 5 min. DNA amplification fragments were electrophoresed in 1.75% agarose gels (SeaKem GTG, FMC

BioProducts) in 1 x TBE buffer. A negative control was added in each run to test for contamination. A 100 bp ladder molecular-weight marker (Gibco-BRL) was used on each gel to aid interpretation of band identity between gels. Polymorphisms between bulks was confirmed by repeating the amplification three times. DNA fragments were visualised by ethidium bromide staining and fluorescence, and fragment patterns were photographed under UV light with Polaroid 667 film.

Screening of RAPD markers

Eighty primers were evaluated for polymorphisms between the green and glaucous bulk DNA samples (series OPA, OPB, OPC and OPD, Operon Technologies). The presence of a RAPD fragment in one bulk and absence in the other provided evidence for a putatively linked marker. To exclude false positives, assays were repeated on the individual DNAs from each bulk using the appropriate primer.

Results

Scanning electron microscopy

Both green and glaucous *E. gunnii* had tube waxes and there were significant differences between the two types in tube length and diameter and in percentage surface area covered by wax (Table 7.1; Figure 7.2). Glaucous types had significantly longer tubes than green types at 5.4 μm and 3.3 μm respectively ($P < 0.001$). Glaucous types had significantly larger diameter tubes than green types at 0.27 μm and 0.19 μm respectively ($P < 0.001$). Glaucous types had significantly larger surface area covered by wax than green types at 53.6 % and 16.7 % respectively ($P < 0.001$).

Table 7.1 Epicuticular wax measurements on green and glaucous *E. gunnii* leaves

E. gunnii type	Tube length (μm)	Tube diameter (μm)	Percentage adaxial surface covered by wax
glaucous	5.4	0.27	53.6
green	3.3	0.19	16.7
S.E.	0.33	0.02	6.6
n	60	60	6
Significance	***	***	***

*** $P < 0.001$; S.E., standard error of the mean; n, number of measurements

Wax analysis

Wax yield was 23 % greater in the glaucous leaves at 25.2 μg/cm^2 compared to the green leaves at 19.4 μg/cm^2. This was due in part to the greater amount of wax extracted from the glaucous leaves and also the smaller surface area of the glaucous leaves (Table 7.2).

Table 7.2 Wax yield and surface area of green and glaucous *E. gunnii* leaves

E. gunnii type	Mean wax yield (mg)	Mean surface area (cm^2)	Wax yield per cm^2 (μg/cm^2)
glaucous	1.90	75.44	25.19
green	1.54	80.56	19.37
S.E.	0.07	4.7	—

S.E., standard error of the mean.

GC of individual wax samples showed that the major classes of compounds of leaf waxes of *E. gunnii* were long chain hydrocarbons, free primary alcohols, long chain β-diketones, free fatty acids and long chain esters (Figures 7.3 & 7.4).

The β-diketones were the major class of compounds identified in both the glaucous and green epicuticular leaf wax, amounting to 53% and 65% of the wax respectively (Figure 7.3). The homologue range was from C_{31} to C_{37} however C_{35} and C_{36} were not detected in either type and C_{32}, C_{34} and C_{37} were not detected in the glaucous type. n-Hentriacontan-14,16-dione (C_{31}) was the major homologue in glaucous and green *E. gunnii* types at 49% and 51% respectively, followed by n-tritriacontan-16,18-dione (C_{33}) at 4% and 13% respectively.

The next largest compound group detected were the long chain esters which ranged from C_{26} to C_{38} and accounted for 19.7% and 23.5% of the whole wax of glaucous and green types respectively (Figure 7.4). The major esters identified in the glaucous types were methyl esters of odd carbon number ranging from C_{27} to C_{31}. The green types showed a greater range of esters with the major ones being 1,2-benzenedicarboxylic acid decyl octyl ester (C_{26}) and an alkyl ester (C_{38}).

Alkanes comprised 12.7 % of the glaucous wax compared to 4.5 % of the green wax. Alkanes detected were C_{18}, C_{22} and C_{23}, however C_{22} was not detected in the glaucous wax. Primary alcohols made up 5.4 % of the glaucous wax compared to 3.4 %

of the green wax. The alcohol homologues detected were C_{20}, C_{24}, C28, and C_{30}, with C_{20} and C_{24} not detected in glaucous wax. Free fatty acids accounted for 8.9 % of the glaucous wax and 3.4 % of the green wax, with the range including C_{19}, C_{25}, C_{29} and C_{31}, with C_{25} and C_{29} not detectable in the glaucous wax (Figure 7.4).

Screening of RAPD markers

Eighty random primers were screened against the two DNA bulks from the 'green' and 'glaucous' *E. gunnii* populations. The presence of a band in one bulk and absence in the other is putative evidence for a marker linked to the waxiness trait. An average of 11 to 12 bands were amplified per primer with two primers giving no amplification products. The fragments ranged in size from 300 to 2100 bp. The primers OPA-7 (GAAACGGGTG), OPA-19 (CAAACGTCGG), OPB-3 (CATCCCCCTG), OPB-11 (GTAGACCCGT), and OPD-20 (ACCCGGTCAC) provided reproducible polymorphic fragments between the green and glaucous bulks. Polymorphic bands present in the glaucous bulk and absent in the green bulk were OPA-19_1700, OPB-3_700, OPB-11_650, OPB-11_720, OPD-20_575 and OPD-20_850 (Figure 7.5). Polymorphic bands present in the green bulk and absent in the grey bulk were OPA-7_650, OPA-7_1150, OPA-19_750, and OPB-11_480 (Figure 7.5). These primers were selected for detailed comparison of the 13 green and 15 glaucous individuals.

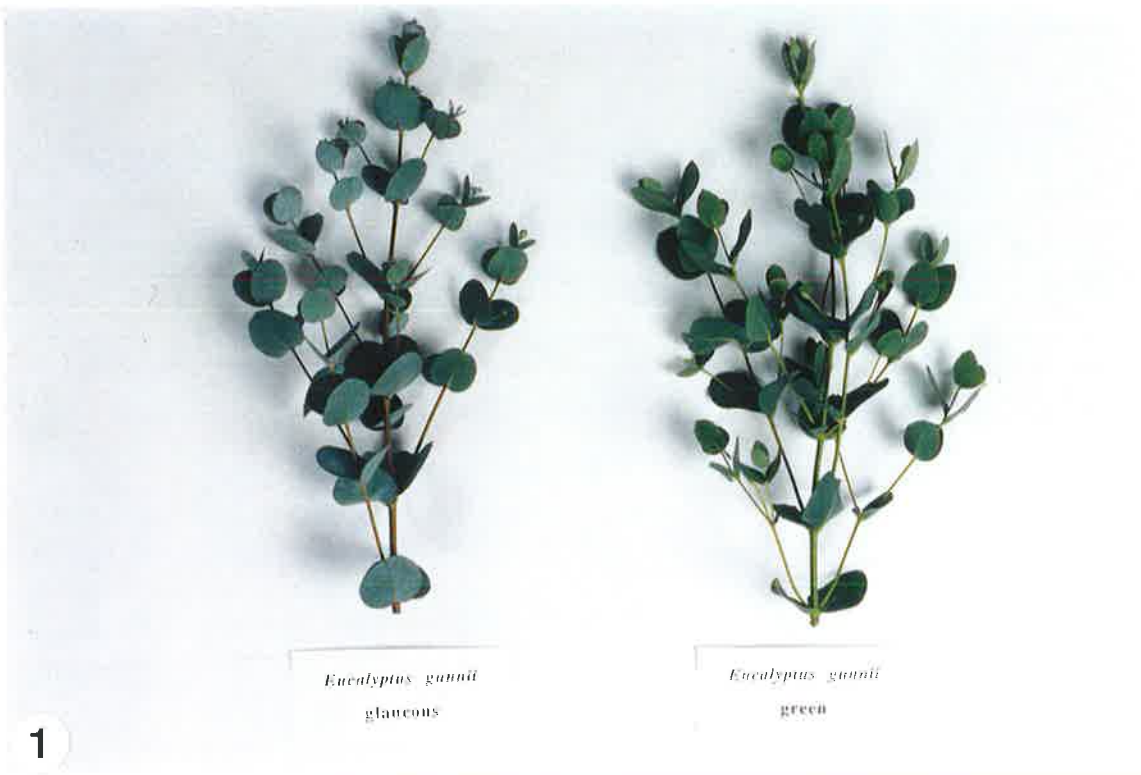
Analysis of individuals revealed that fragments OPA-7_1150, OPA-19_750, OPB-11_480, OPB-11_650, and OPD-20_575 were false positives. Fragment OPA-7_650 was absent in all glaucous individuals and present in all except four green individuals (Figure 7.6). Fragments OPA-19_1700 and OPB-11_720 (Figures 7.7 & 7.9) were present in all but four glaucous plants and absent in all but one and two green plants respectively; OPB-3_700 fragment was present in all but one glaucous plant and absent in all but one green plant (Figure 7.8); OPD-20_850 was present in all glaucous plants and absent in all but two green plants (Figure 7.10). Two additional fragments OPD-20_1380 and OPD-20_1250 were good markers, even though they did not appear polymorphic in the bulk comparison. Fragment OPD-20_1380 was present in all glaucous plants and absent in all but two green plants and fragment OPD-20_1250 was

present in all but two glaucous plants and absent in all but two green plants (Figure 7.10).

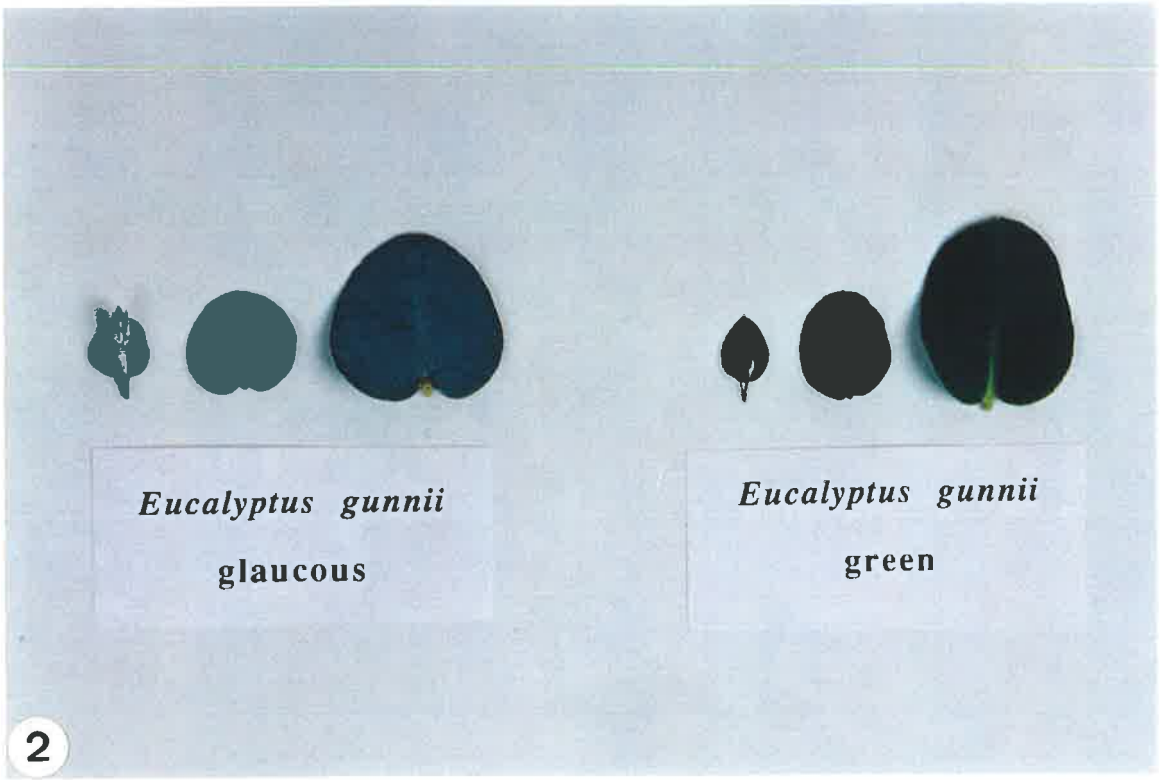
Figure 7.1

1. Stems of glaucous and green *Eucalyptus gunnii.*

2. Terminal leaves, young juvenile and fully expanded juvenile leaves from glaucous and green *Eucalyptus gunnii.*


Eucalyptus gunnii
glaucous
Eucalyptus gunnii
green

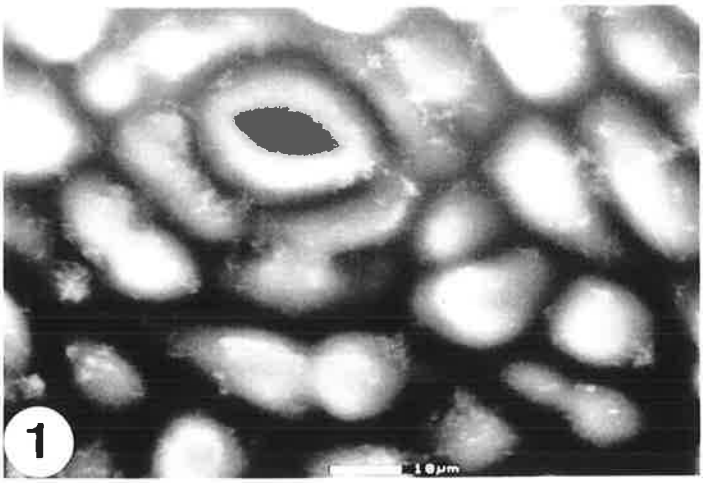
1

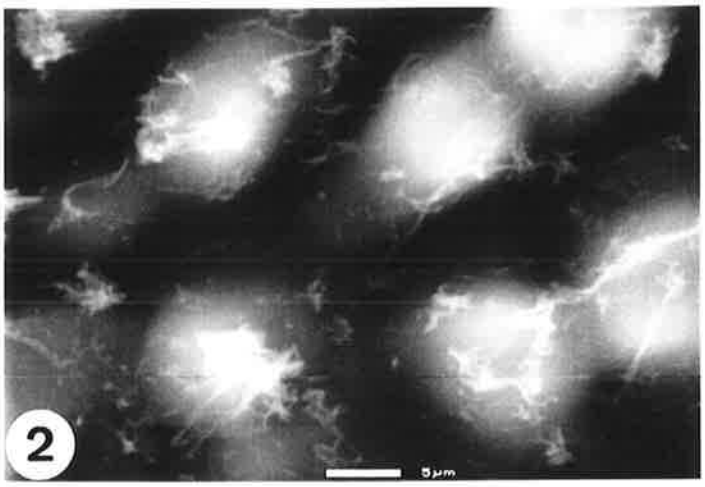

Eucalyptus gunnii
glaucous
Eucalyptus gunnii
green

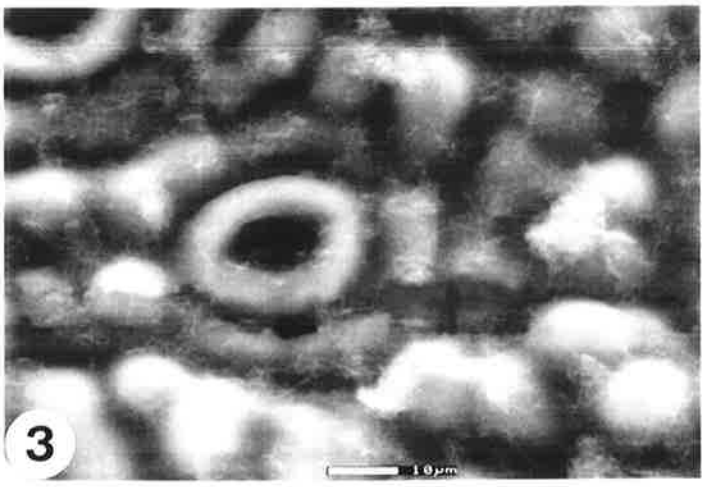
2

Figure 7.2

1. Environmental scanning electron micrograph of adaxial leaf surface of green *Eucalyptus gunnii* showing sparse cover of short wax tubes. Scale: 10 µm.
2. Environmental scanning electron micrograph of adaxial leaf surface of green *Eucalyptus gunnii* showing sparse cover of short wax tubes. Scale: 5 µm.
3. Environmental scanning electron micrograph of adaxial leaf surface of glaucous *Eucalyptus gunnii* showing cover of wax tubes. Scale: 10 µm.
4. Environmental scanning electron micrograph of adaxial leaf surface of glaucous *Eucalyptus gunnii* showing cover of wax tubes. Scale: 5 µm.


1
10µm


2
5µm


3
10µm

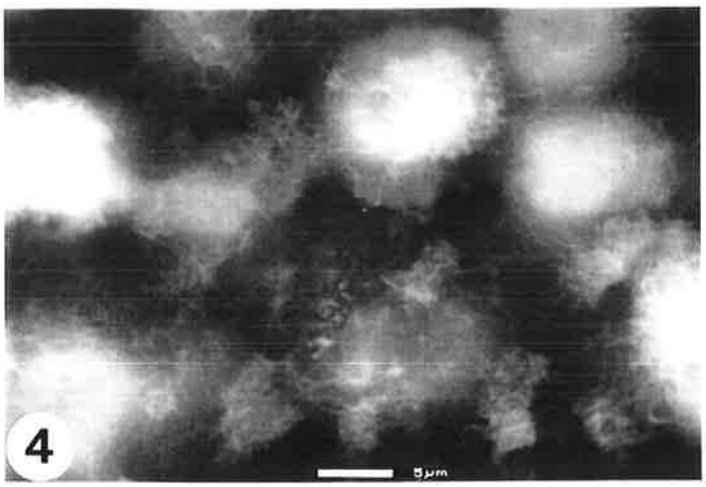

4
5µm

Figure 7.3
Percentage composition (%) of β-diketones from leaf waxes of green and glaucous *Eucalyptus gunnii*. The numbers on the x-axis denote carbon number of β-diketone.

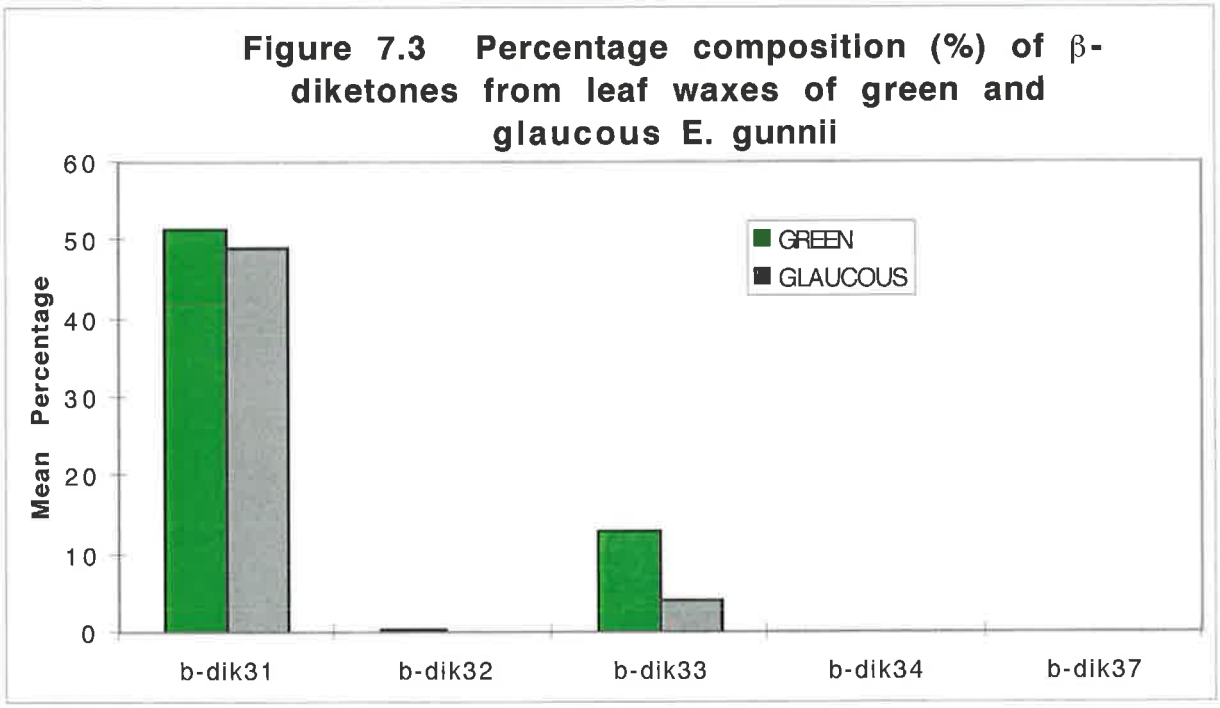


Figure 7.4
Percentage composition (%) of major compound classes from leaf waxes of green and glaucous *Eucalyptus gunnii*.
X-axis legend: alk18-23 = C_{18}-C_{23} alkane; alc18-30 = C_{18}-C_{30} primary alcohol; ffa19-31 = C_{19}-C_{31} free fatty acid; est22-38 = C_{22}-C_{38} ester.

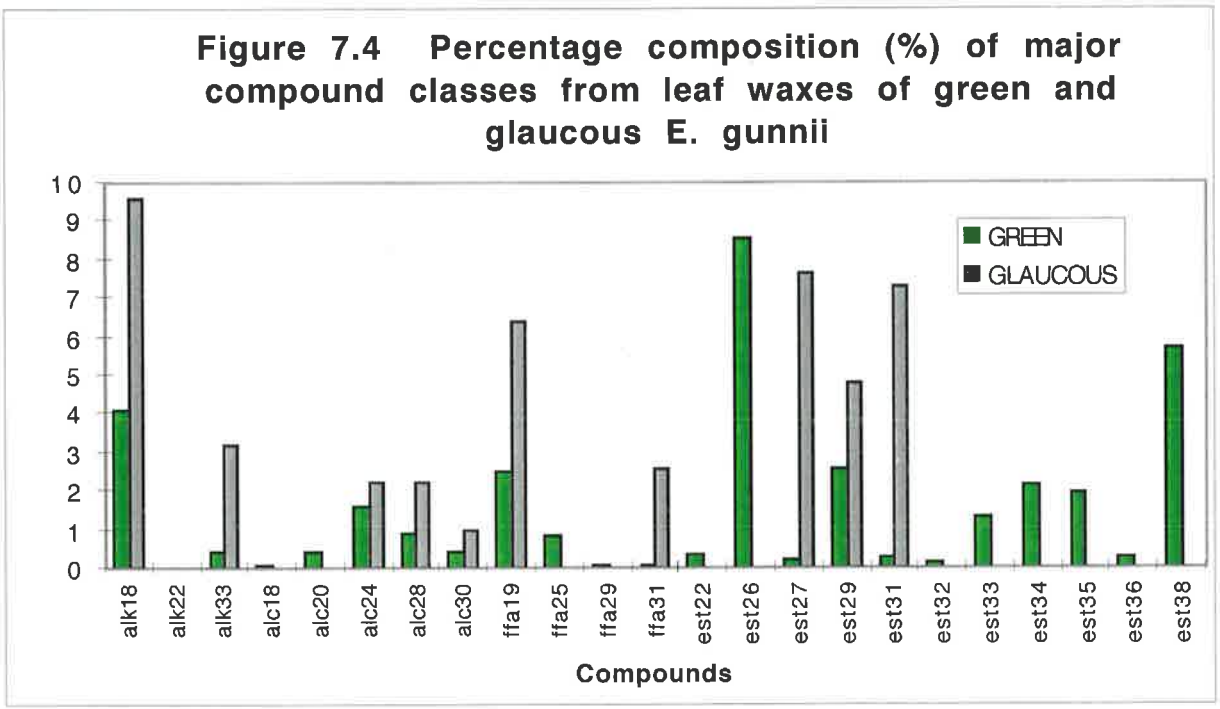


Figure 7.5

Shown here are amplification products from five primers, OPA-7 (lanes 2 & 3), OPA-19 (lanes 5 & 6), OPB-3 (lanes 8 & 9), OPB-11 (lanes 11 & 12), and OPD-20 (lanes 14 & 15). Any difference between the two samples identifies a putatively linked marker, such as with OPA-7, where one fragment (650 bp) is present in the green bulk sample (lane 3) but absent in the glaucous sample (lane 2). Molecular-weight markers (100-bp ladder, Gibco-BRL) are present in lanes 1 and 16.

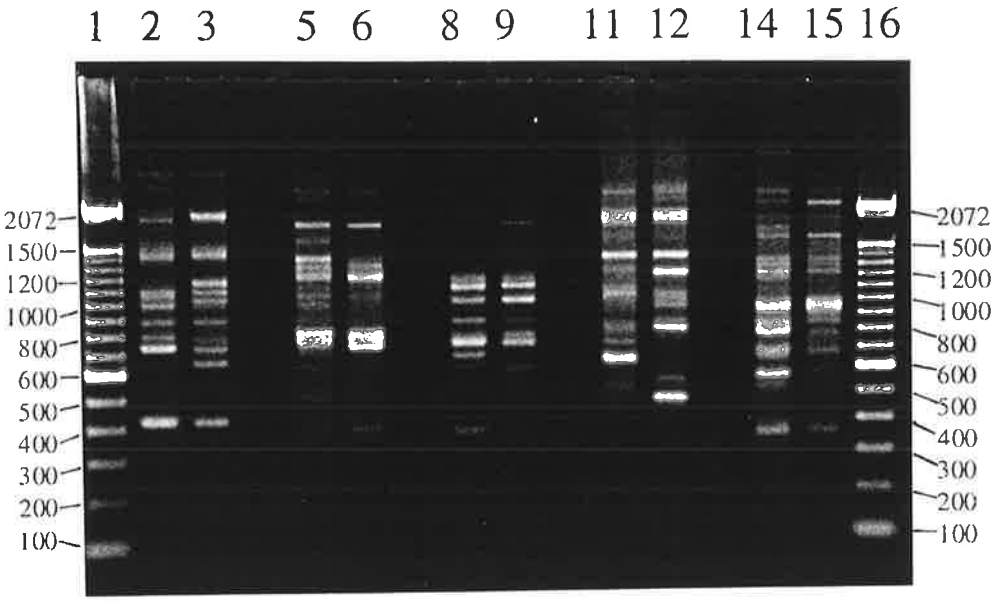
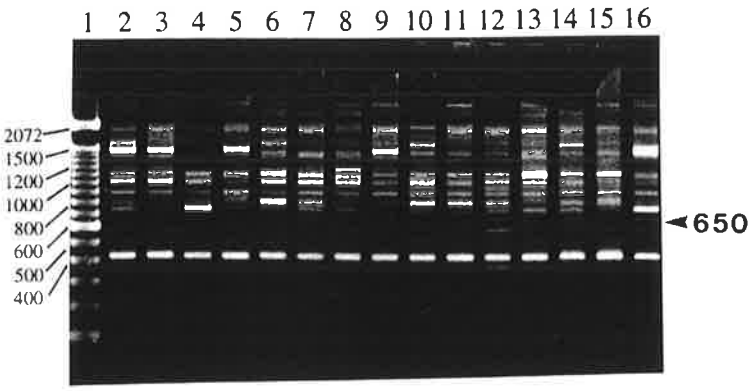

1 2 3 5 6 8 9 11 12 14 15 16
2072
1500
1200
1000
800
600
500
400
300
200
100
2072
1500
1200
1000
800
600
500
400
300
200
100

Figure 7.6

Segregation analysis with primer OPA-7 and DNA from 15 individuals of glaucous *E. gunnii* (lane 2-16) and 13 individuals of green *E. gunnii* (lane 17-19, 22-31). The solid arrowhead indicates the green-linked RAPD fragment OPA-7_650. Molecular-weight markers (100-bp ladder, Gibco-BRL) are present in lanes 1, 20 and 21.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
2072
1500
1200
1000
800
600
500
400
650

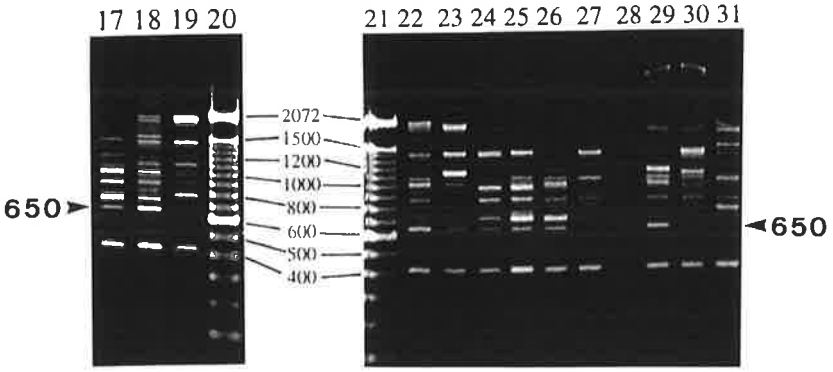
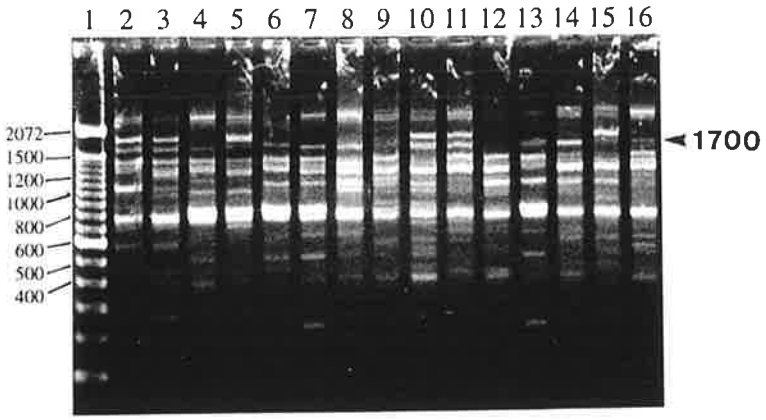

17 18 19 20
21 22 23 24 25 26 27 28 29 30 31
650
2072
1500
1200
1000
800
600
500
400
650

Figure 7.7

Segregation analysis with primer OPA-19 and DNA from 15 individuals of glaucous *E. gunnii* (lane 2-16) and 13 individuals of green *E. gunnii* (lane 17-19, 22-31). The solid arrowhead indicates the glaucous-linked RAPD fragment OPA-19_1700. Molecular-weight markers (100-bp ladder, Gibco-BRL) are present in lanes 1, 20 and 21.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
2072
1500
1200
1000
800
600
500
400
1700

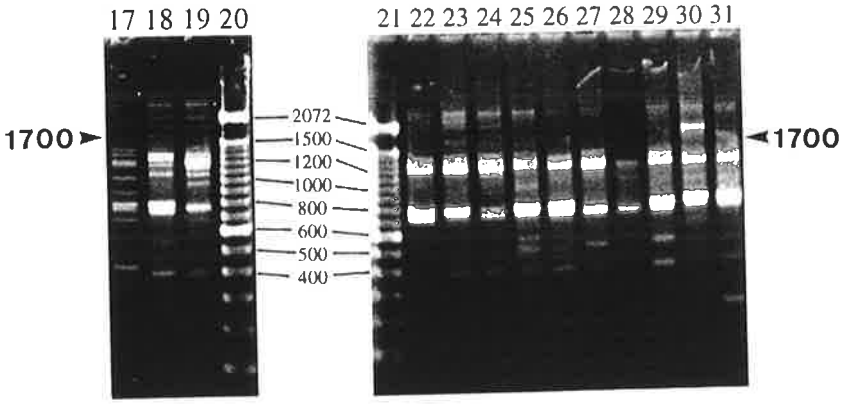
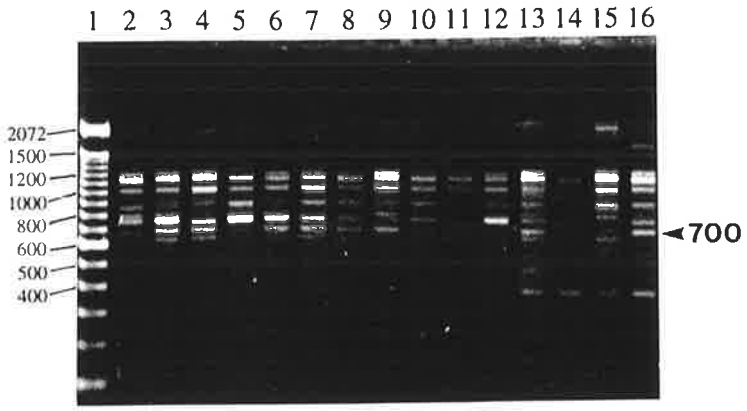

17 18 19 20
21 22 23 24 25 26 27 28 29 30 31
1700
2072
1500
1200
1000
800
600
500
400
1700

Figure 7.8

Segregation analysis with primer OPB-3 and DNA from 15 individuals of glaucous *E. gunnii* (lane 2-16) and 13 individuals of green *E. gunnii* (lane 17-19, 22-31). The solid arrowhead indicates the glaucous-linked RAPD fragment OPB-3_700. Molecular-weight markers (100-bp ladder, Gibco-BRL) are present in lanes 1, 20 and 21.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2072
1500
1200
1000
800
600
500
400
700

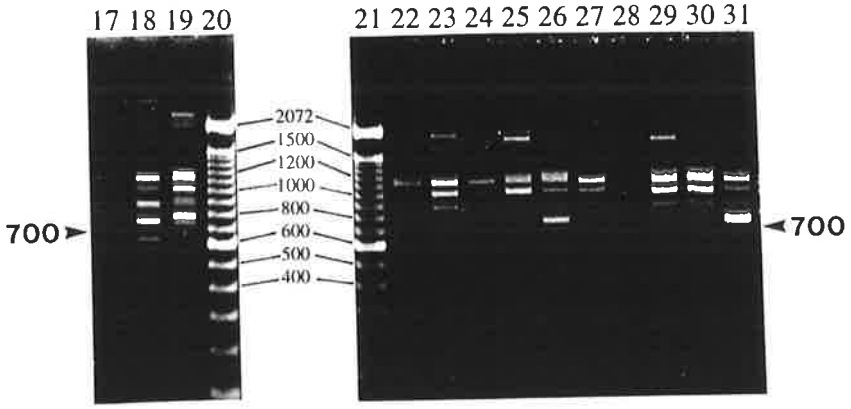
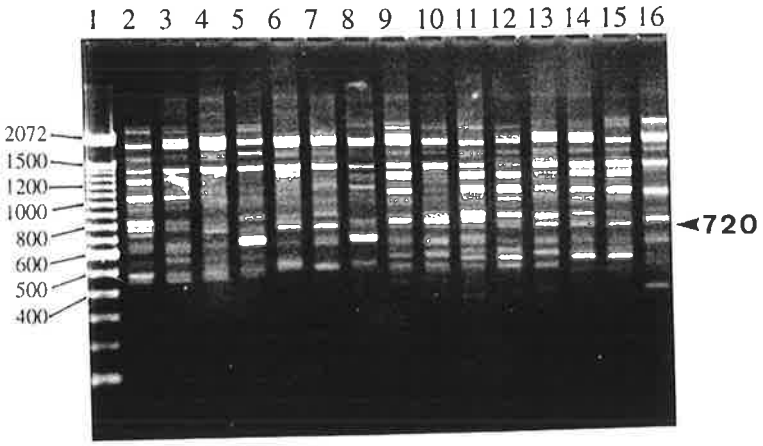

17 18 19 20
21 22 23 24 25 26 27 28 29 30 31
2072
1500
1200
1000
800
600
500
400
700
700

Figure 7.9

Segregation analysis with primer OPB-11 and DNA from 15 individuals of glaucous *E. gunnii* (lane 2-16) and 13 individuals of green *E. gunnii* (lane 17-19, 22-31). The solid arrowhead indicates the glaucous-linked RAPD fragment OPB-11_720. Molecular-weight markers (100-bp ladder, Gibco-BRL) are present in lanes 1, 20 and 21.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
2072
1500
1200
1000
800
600
500
400
720

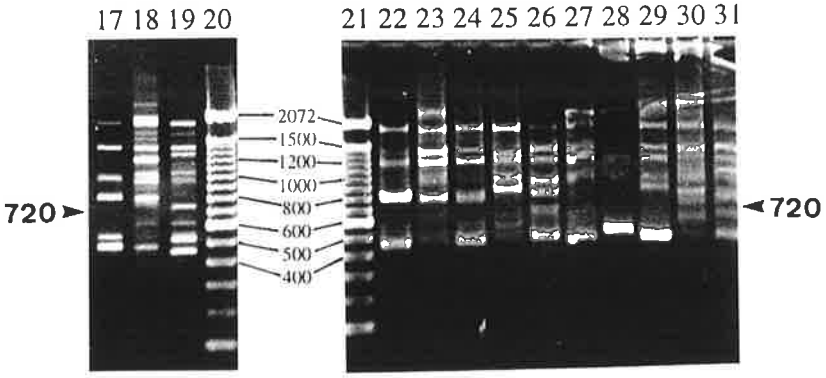
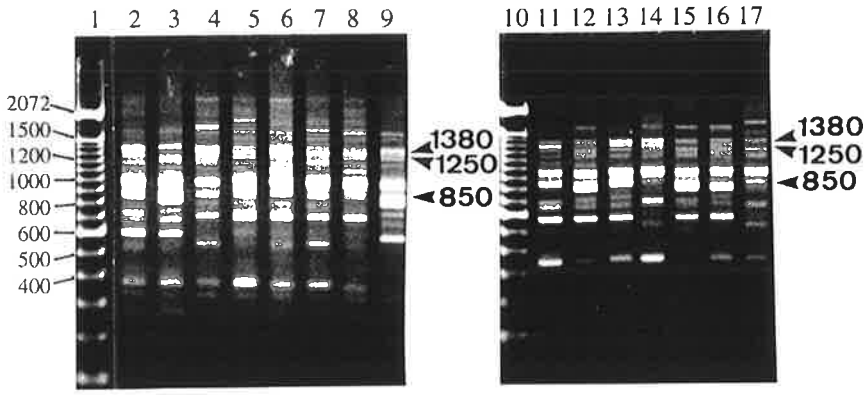
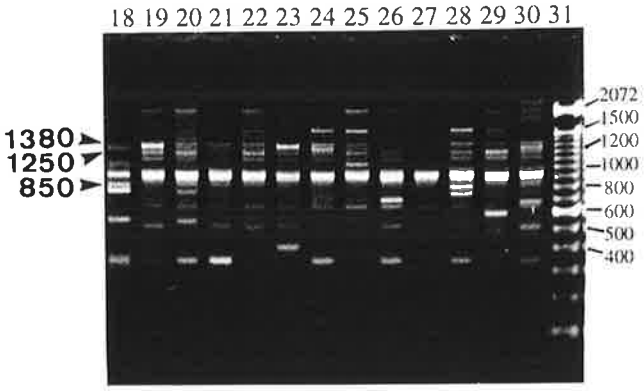

17 18 19 20
21 22 23 24 25 26 27 28 29 30 31
720
2072
1500
1200
1000
800
600
500
400
720

Figure 7.10

Segregation analysis with primer OPD-20 and DNA from 15 individuals of glaucous *E. gunnii* (lane 2-9, 11-17) and 13 individuals of green *E. gunnii* (lane 18-30). The solid arrowheads indicate the glaucous-linked RAPD fragments OPD-20_850, OPD-20_1250, and OPD-20_1250. Molecular-weight markers (100-bp ladder, Gibco-BRL) are present in lanes 1, 10 and 31.


1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17
2072
1500
1200
1000
800
600
500
400
1380
1250
850
1380
1250
850


18 19 20 21 22 23 24 25 26 27 28 29 30 31
1380
1250
850
2072
1500
1200
1000
800
600
500
400

Discussion

Comparison of the green and glaucous *Eucalyptus gunnii* leaf wax showed differences in morphology, chemical composition and genomic DNA. Morphologically the glaucous types had significantly longer and thicker wax tubes and covered a greater surface area of leaf than green types. Both the green and glaucous leaves had tube waxes, with the visible difference in appearance attributable to a difference in structure and density of tubes. Some eucalypt species have plate waxes, which diffract the light differently from tube waxes, to give a greener visible appearance (Hallam, 1970b), but plate waxes were not observed on the green *E. gunnii* leaves. Wax yield was greater in glaucous types and the percentage of alkanes, primary alcohols and free fatty acids was higher, while β-diketones and esters were lower than green types. Using RAPD and bulked segregant analysis, DNA markers were found that would distinguish most of the green and glaucous trees.

The PCR based RAPD technique is used for the accurate identification of individuals which is important in plant breeding. The technique produces many loci and is inexpensive to set up and operate. RAPDs have been used successfully to identify genotypes, clones and cultivars in several genera (Hu and Quiros, 1991; Keil and Griffin, 1994; Mailer *et al.*, 1994; Yu and Nguyen, 1994; Sedgley *et al.*, 1996; López-Valenzuela *et al.*, 1997). Nesbitt *et al.* (1997) used RAPDs to confirm clonal fidelity and distinguish individuals in *Eucalyptus globulus.*

Bulked segregant analysis (BSA) is a method which facilitates the identification of markers that are tightly linked to the locus of interest, in this case, epicuticular wax production. RAPD patterns that are characteristic of plant populations or cultivars can be obtained by using bulks of genomic DNA from several individuals of a heterogeneous population in PCR reactions with random primers. The BSA technique has wide applications and has been used successfully in tree breeding in *Populus* (Cervera *et al.*, 1996; Villar *et al.*, 1996), *Prunus* (Chaparro *et al.*, 1994; Warburton *et al.*, 1996), *Pinus* (Emebiri *et al.*, 1997), *Malus* (Koller *et al.*, 1994; Markussen *et al.*, 1995; Cheng *et al.*, 1996), *Picea* (Lehner *et al.*, 1995), *Ulmus* (Benet *et al.*, 1995) and *Pistacia* (Hormaza *et al.*, 1994).

In this study a total of seven markers using five primers were found that would distinguish some green and glaucous types of *E. gunnii.* This relatively large number of markers and the fact that none distinguished all green and glaucous trees may be due to the fact that the loci of interest may occupy a large region of the genome, therefore the probability of tagging is large, and in species of *Eucalyptus*, which preferentially outcross, the high levels of heterozygosity in the majority of the loci result in large amounts of DNA polymorphism. This however makes it harder to detect a dominant marker gene linked to the target gene. The most useful markers produced by bulked segregant analysis in this study were OPA-7_650, OPB-3_700, OPD-20_850 and OPD-20_1380. A combination of the following markers would distinguish all green individuals from glaucous individuals: OPA-7 with either OPA-19, OPB-3 or OPD-20; OPA-19 with OPB-11 or OPD-20; and OPD-20 with either OPB-3 or OPB-11. Most traits of interest in forest tree improvement such as yield are controlled by more than one gene.

The amount of wax on the glaucous leaves was significantly more than on the green leaves and covered a larger surface area of leaf. This may be due to a down regulation of wax biosynthesis such as C_{16} and C_{18} fatty acid precursor synthesis in the green types. Li *et al.* (1997) found that *E. gunnii* showed much variation in morphological components between localities, including an increase in wax yield along the cline as well as a change in wax composition. Wax yield tended to be lower in the green species of that study. Morphological variation such as glaucousness may occur within and between the species. Clinal intergradation is common within subgenera. Pryor (1976) states that clinal variation occurs when morphological and physiological differences within a species are correlated with particular changes in the physical environment. Clines occur along environmental gradients such as altitude, latitude or other factors such as water availability or frost sensitivity. The cline of *E. gunnii* is associated with increasing exposure to alpine environment (Potts and Reid, 1985a), and has been shown to be genetically based (Potts and Reid, 1985b). *Eucalyptus gunnii* is one of the most frost tolerant eucalypt species (Barber, 1955; Pryor, 1957) and

maximum glaucousness occurs on trees which border frost hollows and grow in high light intensities (Potts and Reid, 1985a).

This study found that the green *E. gunnii* contained more β-diketones and less alkanes than the glaucous types which is in contradiction to Li *et al.* (1997) who found that green populations of *E. urnigera, E. gunnii* and *E. archeri* had less β-diketones and more alkanes than the glaucous phenotypes. This would suggest that glaucousness is not entirely related to β-diketone content which is confirmed by the fact that green *E. viminalis* has a high β-diketone content and adult *E. globulus* leaf wax contains greater amounts of β-diketone than glaucous juvenile leaves (Li *et al.*, 1997). Rather, glaucousness seems to be related more to wax yield as evidenced in this study.

There are likely to be many genes regulating wax biosynthesis due to the diversity of wax components. Using wax deficient mutants such as the *glossy* or *eceriferum* (*cer*) mutants many workers have begun to isolate the genes responsible for the production of epicuticular wax in barley, maize, *Arabidopsis*, and *Brassica* spp., (Baker, 1974; von Wettstein-Knowles, 1979; Bianchi *et al.,* 1985; Koornneef *et al.*, 1989; McNevin *et al.*, 1993; Schnable *et al.*, 1994; Jenks *et al.*, 1995, 1996; Xia *et al.*, 1996). Many of these mutants display glossy green stems and fruits as well as leaves. Wax biosynthesis is a series of enzymatic steps in a number of pathways, any one of which could be altered, thus changing the final composition of the epicuticular wax.

Bulked segregant analysis helped identify molecular markers linked to glaucousness in *E. gunnii*. These markers may facilitate the management of *E. gunnii* breeding and selection for the cut foliage industry, by providing an initial screen for glaucousness. More BSA needs to be done on populations of known parentage and progenies to identify and map the genetic linkage between the markers and the trait of interest.

Chapter 8

General Discussion

The aim of this work has been to study a range of *Eucalyptus* species that have potential as cut foliage producers for the floriculture market. The horticultural management of these species and factors which influence their productivity have been assessed. This will help improve the management of existing plantations and the continuity of supply to established markets.

As floriculture is a competitive industry with constant demand for new species or cultivars, there is a need for selection for improved quality and improved yield. Selections of superior plants are made on the basis of plant form and colour. However, quality is subjective and relies on consumer preferences. Of the 18 species studied in this work, 12 have potential as cut foliage on aesthetic grounds. They are: *E. tetragona, E. kruseana, E. socialis, E. gillii, E. crucis, E. bridgesiana, E. globulus, E. gunnii, E. cordata, E. pulverulenta, E. cinerea* and *E. albida*. All have small, rounded attractive leaves and/or glaucous leaves which gives them a blue-green or grey-green colouring.

While commercial plantations are often established using seedlings, propagation from seeds of selected superior plants can lead to undesirable variation in the progeny. Vegetative propagation is therefore ideal for the clonal production of superior plants. When plants are difficult to multiply by cuttings, micropropagation can be used successfully and while it is the more expensive method it can result in more rapid multiplication and reductions in disease. Micropropagation has been used to propagate nodes of *E. glaucescens* with a success rate of 67% (Douglas and Egan, 1998). In *Eucalyptus*, natural putative hybrids are F1 progeny and these novel plants may be another source of material for the floriculture market. For example, the hybrid known as 'Little Boy Blue' is a putative hybrid between *E. kruseana* and *E. pulverulenta* and is used as cut foliage due to its delicate shape and the blue hue of the leaves.

The discovery of molecular markers which are linked to desirable traits will vastly improve plant selection. Vegetative propagation is not at a commercial stage for

Eucalyptus gunnii, therefore there is a need to identify undesirable individuals. Comparison of bulks of extreme individuals with green or glaucous leaf colour among *E. gunnii* has identified seven markers linked to this qualitative trait. These markers may be linked to a biosynthetic step in the wax production pathway. They will facilitate the breeding and selection of *E. gunnii* for the floriculture industry, by providing an initial screen for glaucousness. Further work needs to be done using populations of known parentage and progenies to identify and map the genetic linkage between the markers and the trait of interest. Comparison of the green and glaucous *E. gunnii* leaf wax also showed differences in morphology, yield, and chemical composition.

Maximum stem production from each tree is one of the main aims of the grower. This aspect has been examined in Chapter 3 which deals with the effect of pruning to different heights and season of pruning on the production of cut stems. Most eucalypts respond well to pruning, they regenerate new juvenile stems from buds located either in the lignotuber, if one is present, or from adventitious buds beneath the bark. Many species will change leaf phase from juvenile to adult relatively quickly and these species must be pruned regularly to maintain the desired juvenile foliage. Flower markets would prefer a constant flow of foliage stems – as opposed to a feast or famine situation. Species such as *E. pulverulenta* can be heavily pruned and the resulting shoots can be picked all year with highs in late summer and lows in mid to late winter. It is necessary to prune species such as *E. pulverulenta* , *E. cinerea* and *E. cordata* annually, even though they remain in the juvenile phase, because the previous years leaves will have lost much of their glaucous bloom due to removal of the leaf epicuticular wax by wind and abrasion.

The timing of pruning is important because poorly timed budburst may result in damage to young tender shoots. Some species are, however more tolerant of frost than others e.g. *E. gunnii* and *E. globulus* (Barnard, 1968). The pruning trial involving *E. gunnii* showed that delaying the pruning of *E. gunnii* until July or October resulted in the production of more marketable stems than earlier pruning. The pruning trials on the remaining species in the study showed positive regressions between the number of regrowth stems and trunk diameter and lignotuber diameter. Measurement of the trunk

diameter or lignotuber could therefore be used for prediction of yield. Thus as the tree matures and the trunk and lignotuber diameters increase, stem yield should steadily increase. The vigour of regeneration also depends on the species. All species studied have the ability to coppice, however other variables can affect coppicing ability such as shoot origin, season of cutting, diameter, height of stump, age and spacing (Blake 1983).

Native plants make up the bulk of Australia's floriculture exports (Lake, 1991) but there is little information on postharvest requirements. A long vase life of cut stems is however a desirable trait. Once the foliage is cut it must be placed in a solution containing an antibacterial agent and also a source of carbohydrate to minimise wilting. Work has been conducted on several native species regarding carbohydrate status, storage and effects of ethylene (Joyce, 1988). Most information on postharvest techniques, however relate only to flowering stems (Halevy and Mayak, 1979, 1981; Barth, 1990; Teagle *et al.*, 1991). This study demonstrated that vase life of *E. cinerea* and *E. globulus* can be extended by the addition of up to 2% sucrose. Sucrose solutions greater than 5% were not beneficial to the stems and were associated with browning and wilting of the leaves. This is the first study to investigate sucrose pulsing before dry storage of eucalypt stems, and is also the first to assess new hybrids. There was no advantage of sucrose pulsing to extend vase life, or to improve vase life following dry storage, except for the hybrid *E. spathulata* x *E. platypus*.

Juvenile *Eucalyptus* stems are principally desirable as cut foliage because of the wax 'bloom' or glaucousness of their leaves along with their shape. The glaucousness is related to wax morphology which is in turn related to wax yield and chemistry. The removal of wax due to wind or rough handling is a problem in the floriculture industry as it detracts from the appearance of the foliage. Tube wax which was present on all the species studied, is very easily removed from the leaf thereby downgrading the quality of the product (Jones and Sedgley, 1993). The high level of variation in the morphology of wax tubes present on the leaves and the ability of the species to regenerate wax is related to the large amount of genetic diversity within and between species of *Eucalyptus*. Such variation is beneficial because it provides a greater selection of

superior phenotypes, in this case those with longer tube wax and those with greater capacity to regenerate wax.

The ESEM allows the use of fresh plant material with no pretreatment. Comparison of proximal and distal areas of very young *Eucalyptus* leaves has shown a gradation of wax structure from an amorphous state at the base of the leaf to a structured more mature form at the tip. This has not been seen on *Eucalyptus* leaves previously. In this study wax tubes could be seen crystallising from amorphous deposits. This supports previous hypotheses that wax is secreted to the surface in a volatile solvent, which then evaporates leaving the wax to crystallise. The β-diketones were the major wax constituent of all species studied except *E. delegatensis*. This is in agreement with other workers (Hallam and Chambers, 1970; Horn and Lamberton, 1962; Horn *et al.*, 1964; Tulloch, 1976).

In conclusion this study has assessed eighteen species of *Eucalyptus* of which twelve are suitable for the cut foliage market based on their leaf morphology and glaucousness, their coppicing ability, and their postharvest vase life. Tube wax was common to all species and the main constituents of the epicuticular wax were similar in all species. An unknown solvent was discovered in the headspace of newly opened leaves which may act as the carrier for the epicuticular wax. Molecular markers were found which would distinguish green from glaucous *E. gunnii* which will aid the selection process of desirable plants.

Further work needs to be carried out on the postharvest requirements of more species and hybrids. Pruning trials over several seasons would be advantageous to gauge the impact of repeated pruning on stem production. Future work to map and sequence the molecular markers for *E. gunnii* needs to be carried out. There is also a need to examine more species using the headspace technique to help identify the unknown solvent.

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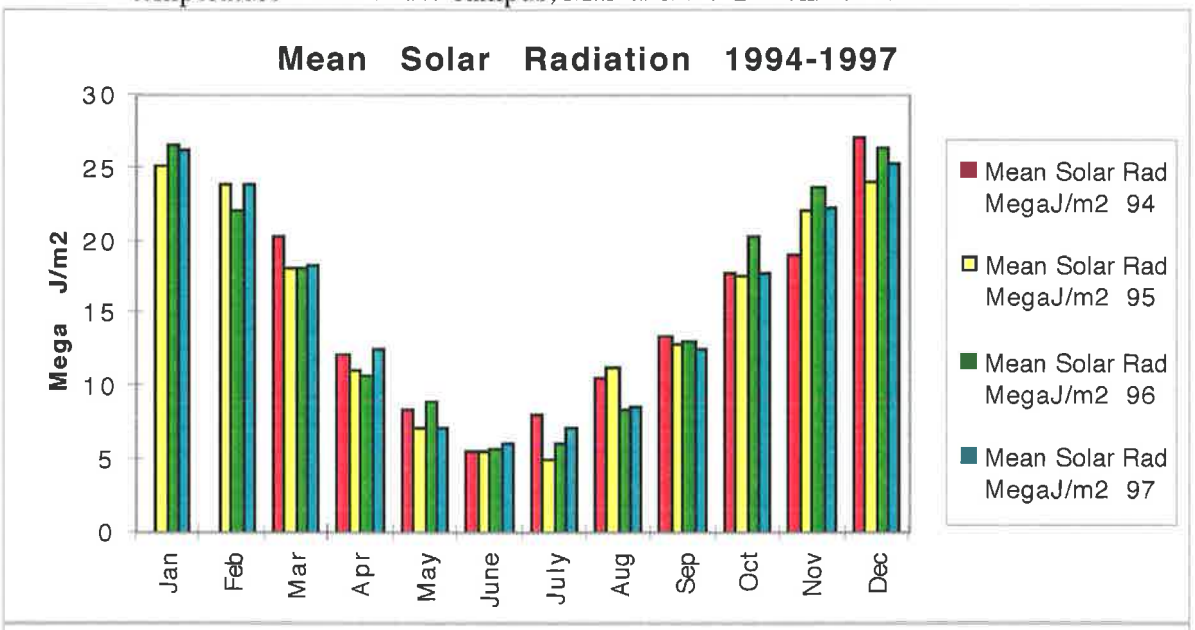
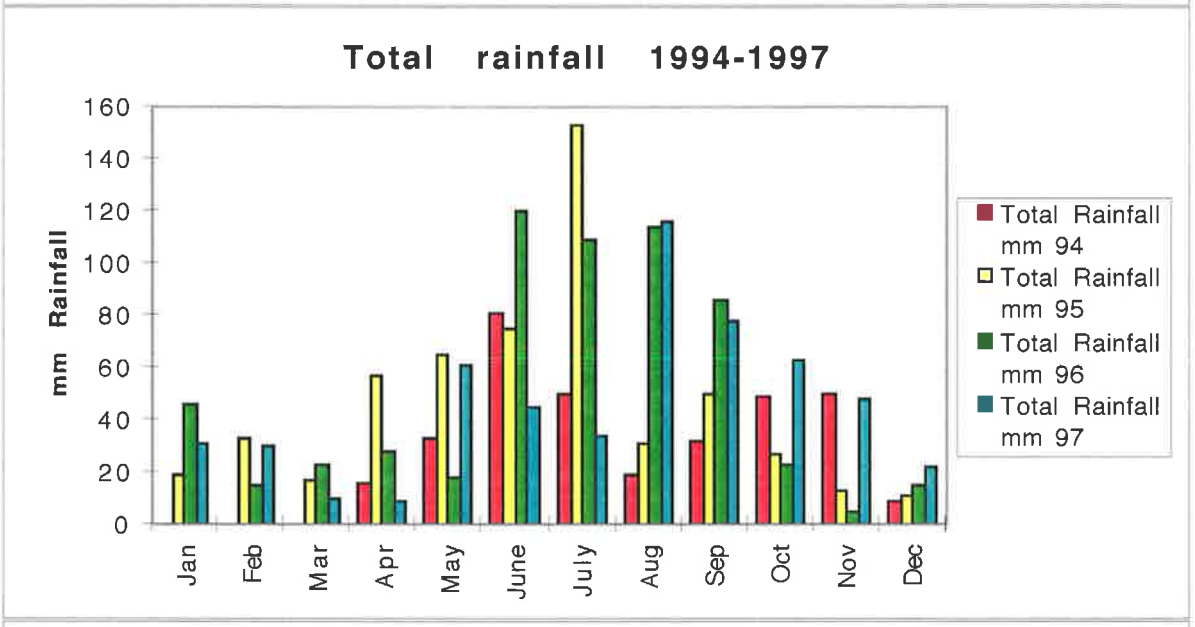
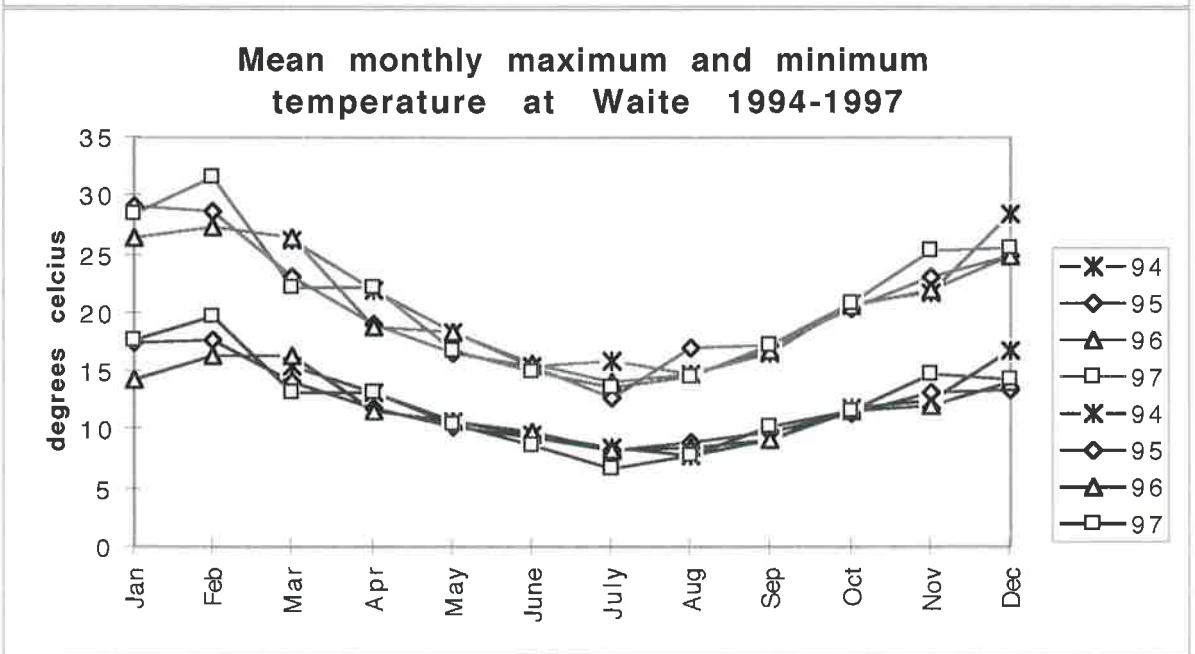
Appendix 2. Mean solar radiation, total rainfall, and mean monthly maximum and minimum temperature at the Waite campus, March 1994–December 1997.







Appendix 3

Wirthensohn, M. G. and Sedgley, M. (1998). Effect of pruning on regrowth of cut foliage stems of seventeen Eucalyptus species. *Australian Journal of Experimental Agriculture* **38**, 631-636.

Wirthensohn, M. G. & Sedgley, M. (1998). Effect of pruning on regrowth of cut foliage stems of seventeen Eucalyptus species. *Australian Journal of Experimental Agriculture, 38*(6), 631-636.

Appendix 4

Wirthensohn, M. G., Ehmer, R. and Sedgley, M. (1996). Production and Postharvest Treatment of Cut Stems of *Eucalyptus* L'Hér. Foliage. *HortScience* **31,** 1007-1009.

Wirthensohn M.G., Sedgley M. & Ehmer, R. (1996). Production and postharvest treatment of cut stems of Eucalyptus L. Hér. foliage. *HortScience*, *31*(6), 1007-1009.

Appendix 5

Wirthensohn, M. G., and Sedgley, M. (1996). Epicuticular wax structure and regeneration on developing juvenile *Eucalyptus* leaves. *Australian Journal of Botany* **44,** 691-704.

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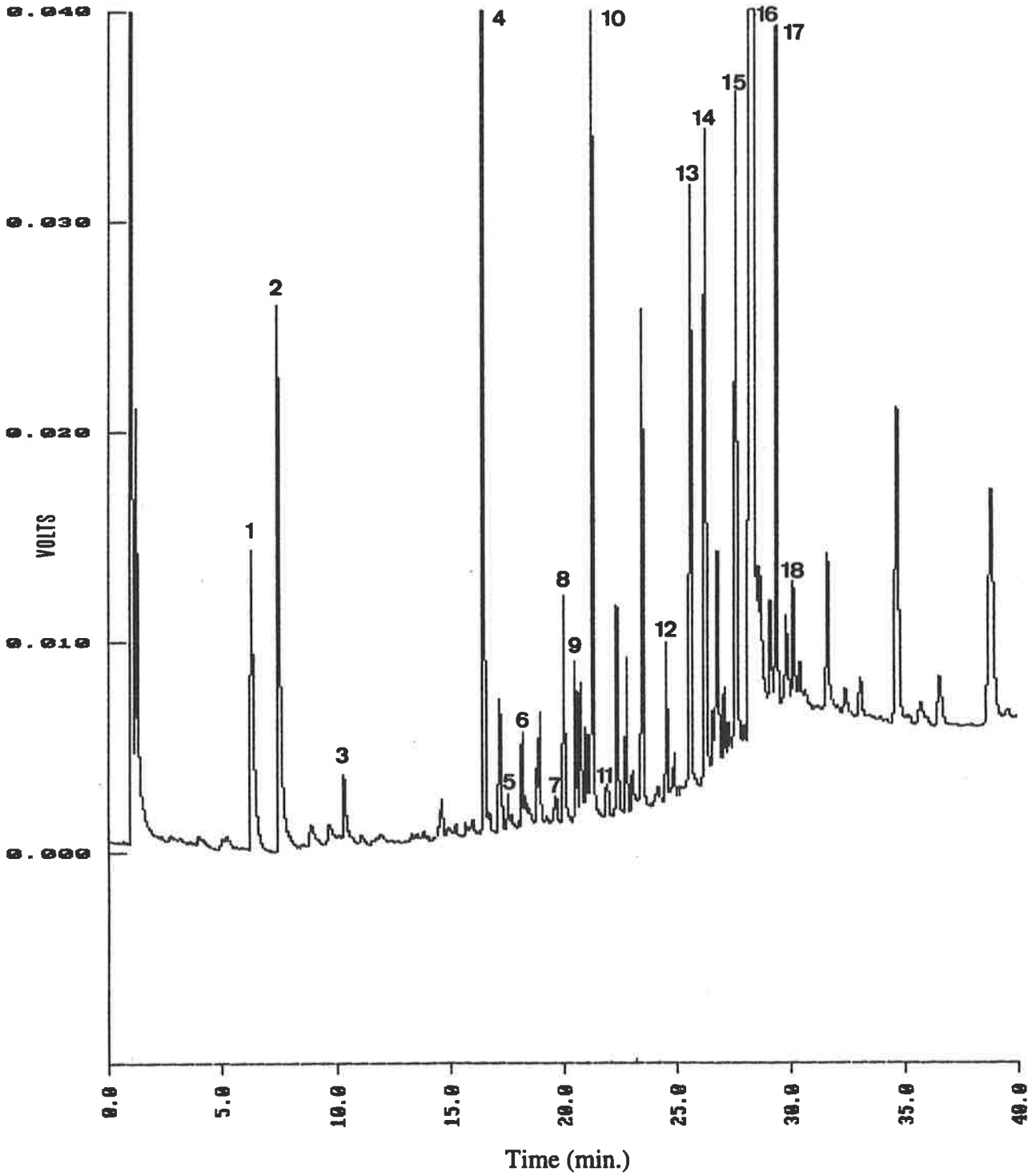
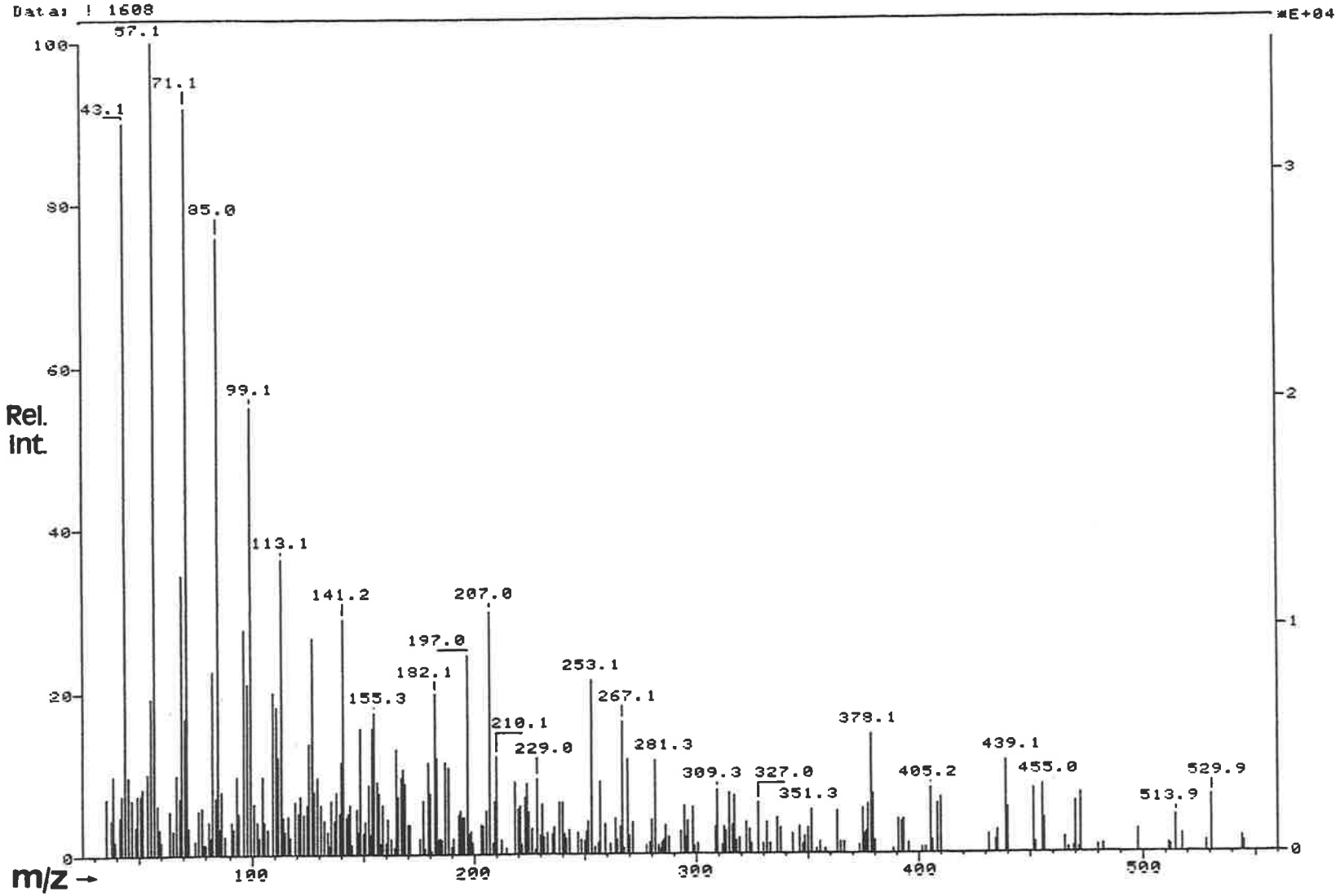
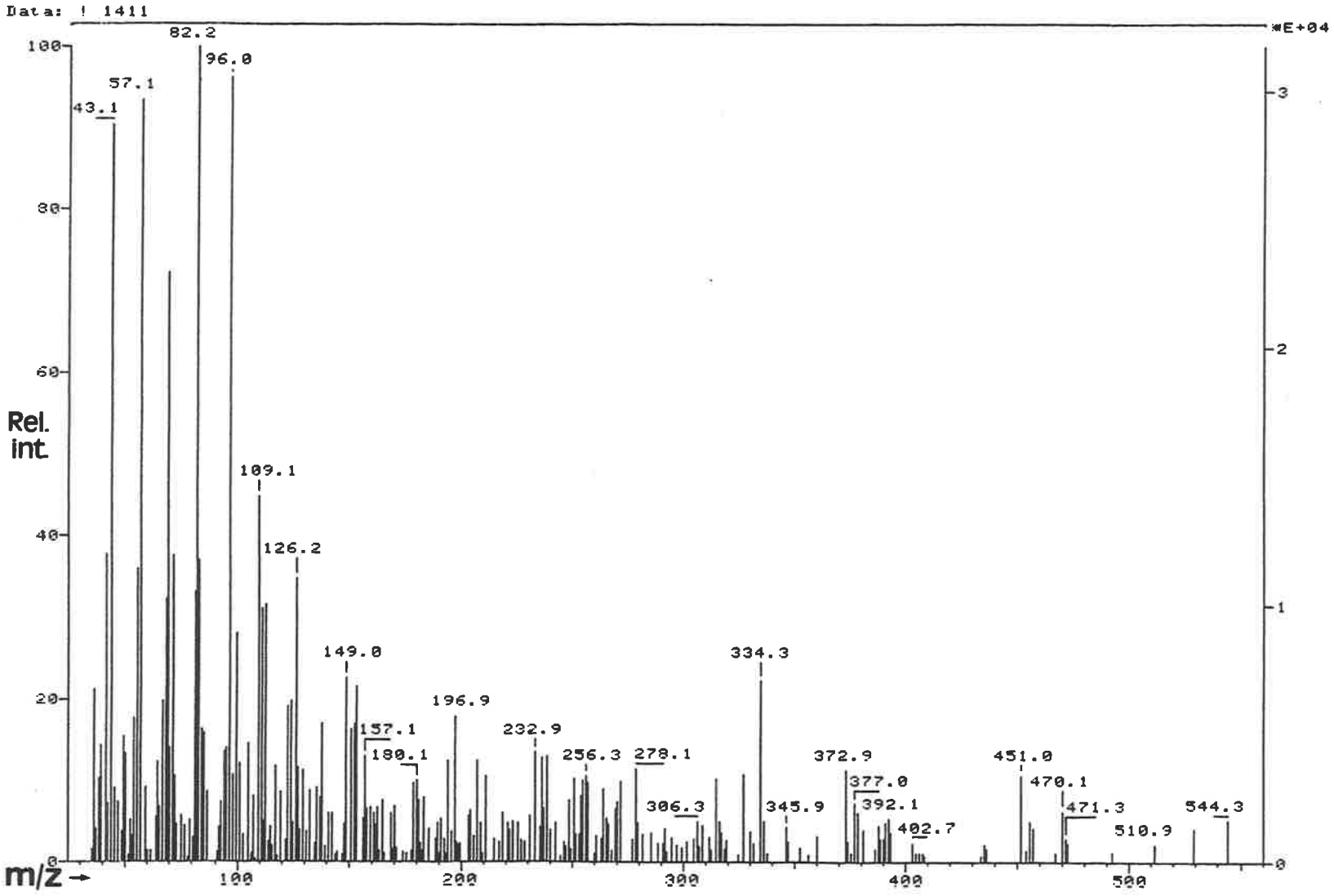


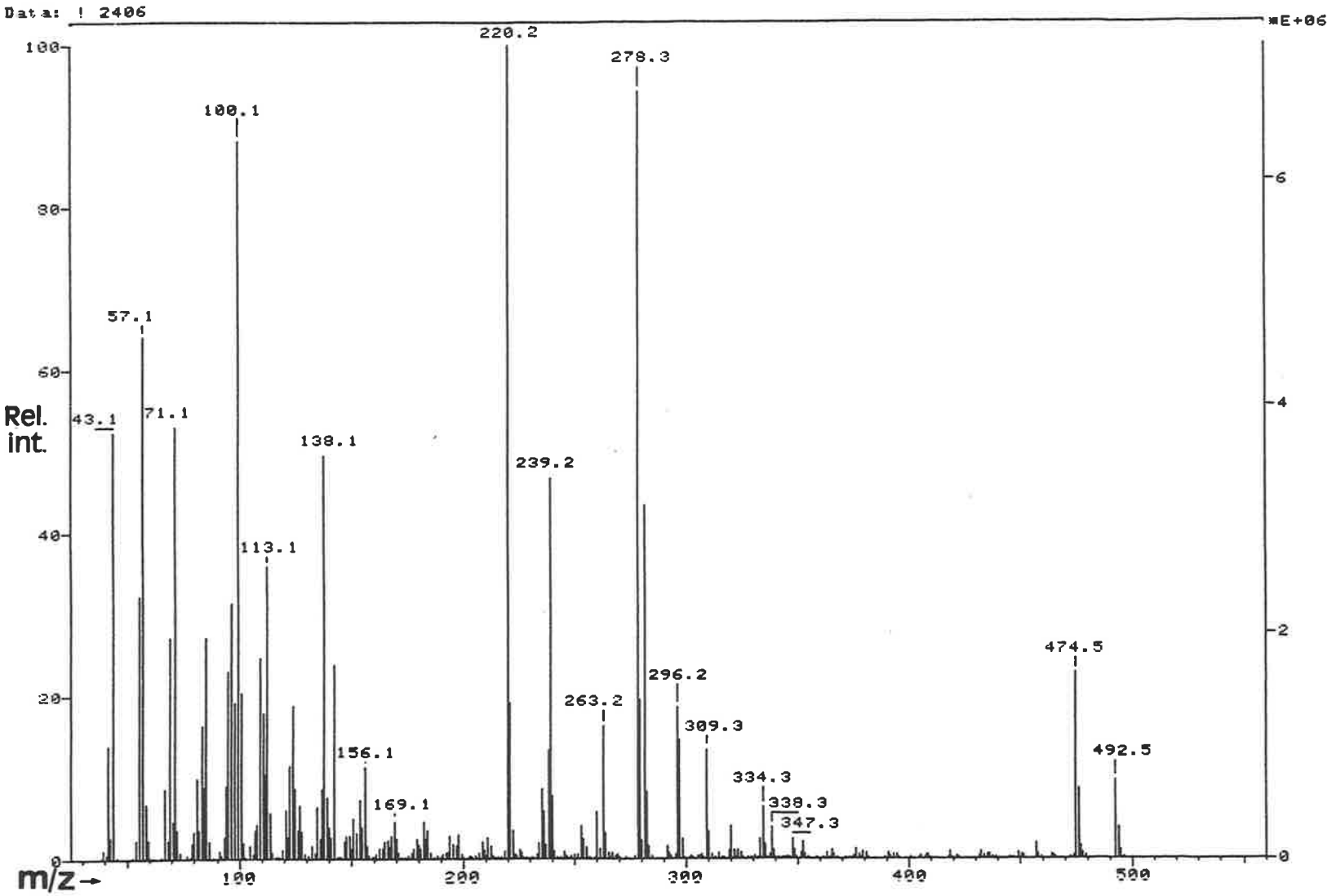
Appendix 6.1. GC separation of the components from the wax of *E. tetragona* juvenile leaves. Compound peaks: **1.** n-C_{19} alkane, **2.** n-C_{20} alkane, **3.** C_{19} fatty acid, **4.** C_{22} ester, **5.** C_{17} alcohol, **6.** n-C_{29} alkane, **7.** n-C_{30} alkane, **8.** C_{18} aldehyde, **9.** C_{26} ester, **10.** C_{23} ester, **11.** n-C_{32} alkane, **12.** C_{34} ester, **13.** C_{32} ester, **14.** C_{31} β-diketone, **15.** C_{34} ester, **16.** C_{33} β-diketone, **17.** C_{36} ester, **18.** C_{35} β-diketone.



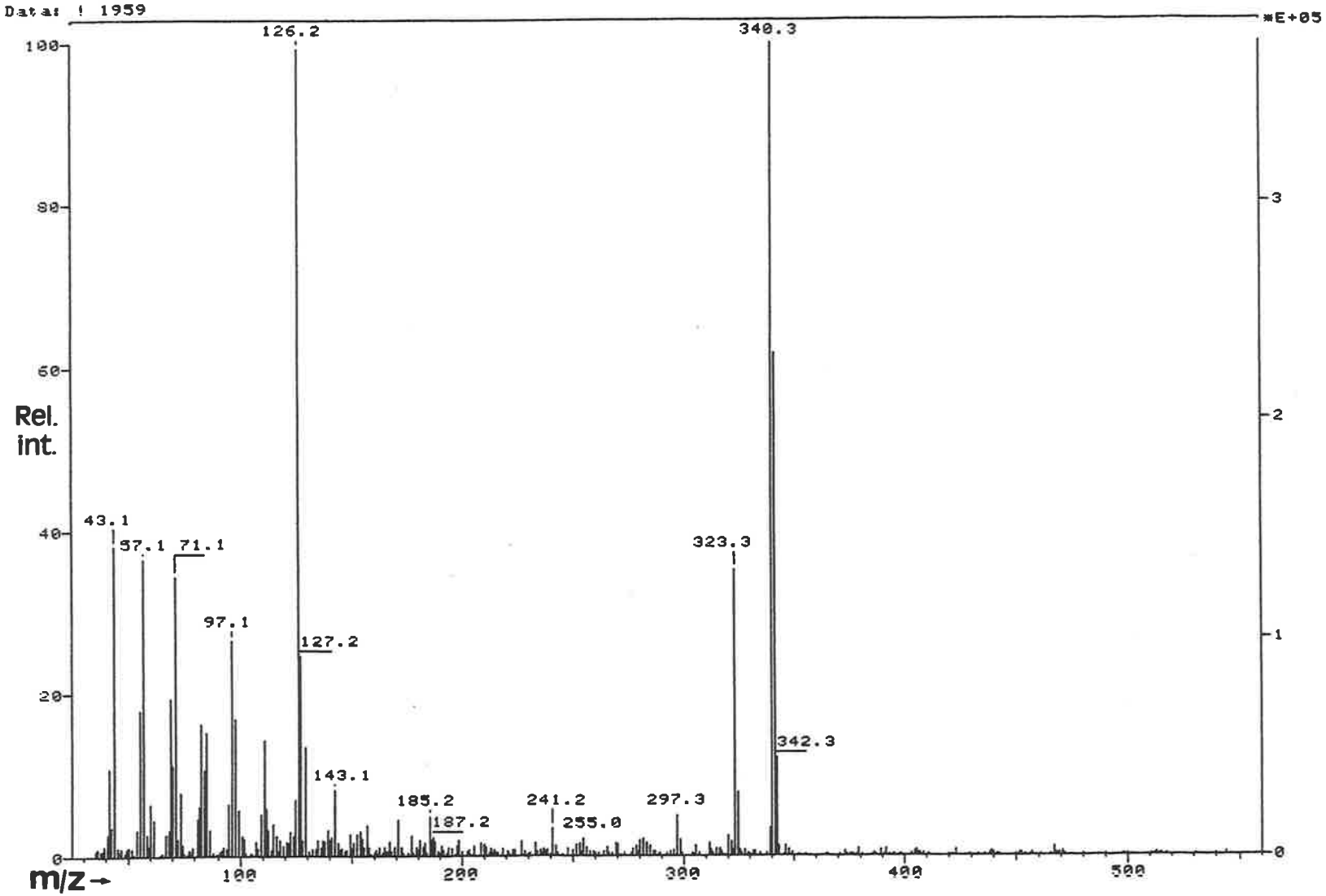
Appendix 6.2. Mass spectra of the n-C_{30} alkane from the wax of *E. tetragona* juvenile leaves.



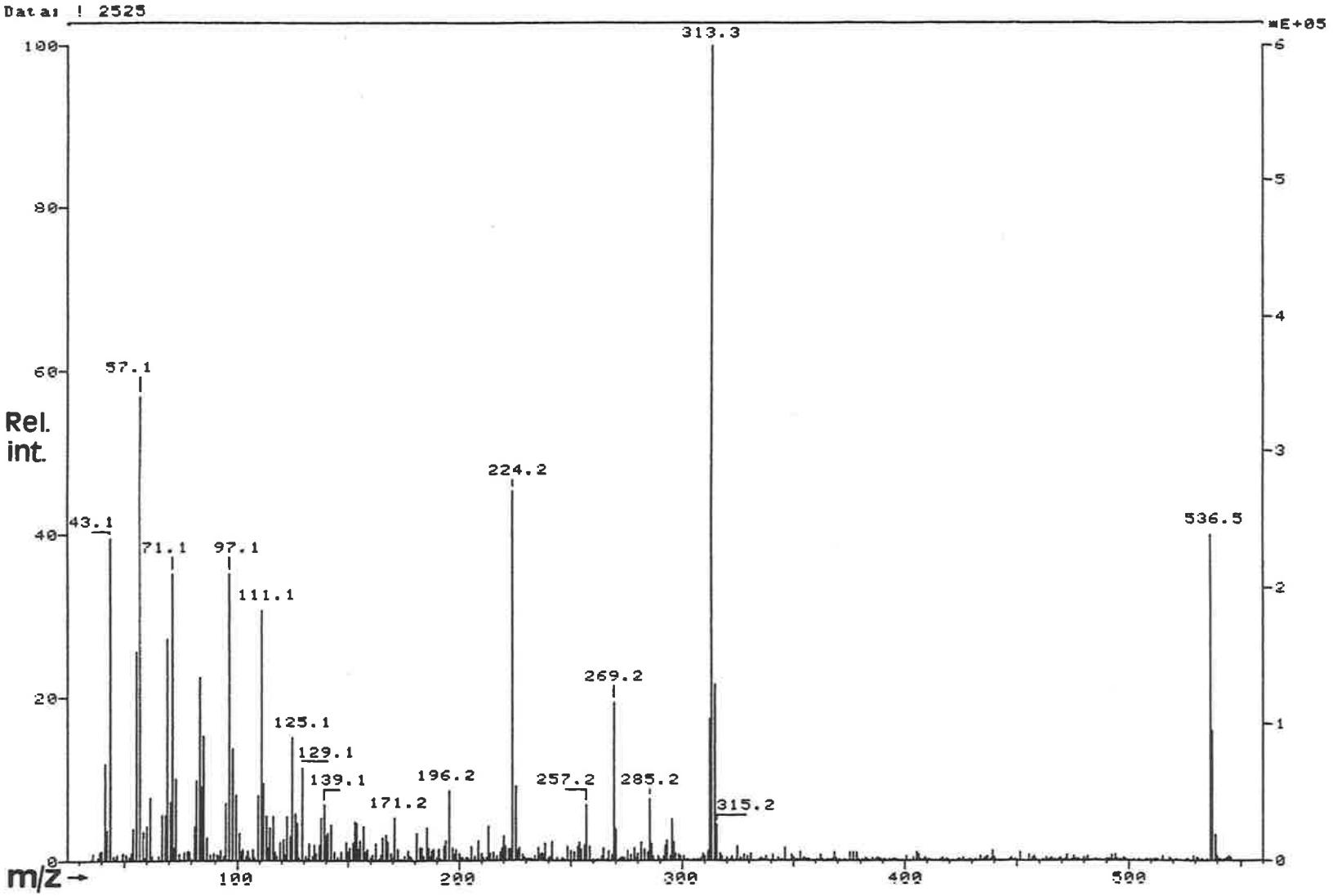
Appendix 6.3. Mass spectra of the C_{17} alcohol from the wax of *E. tetragona* juvenile leaves.



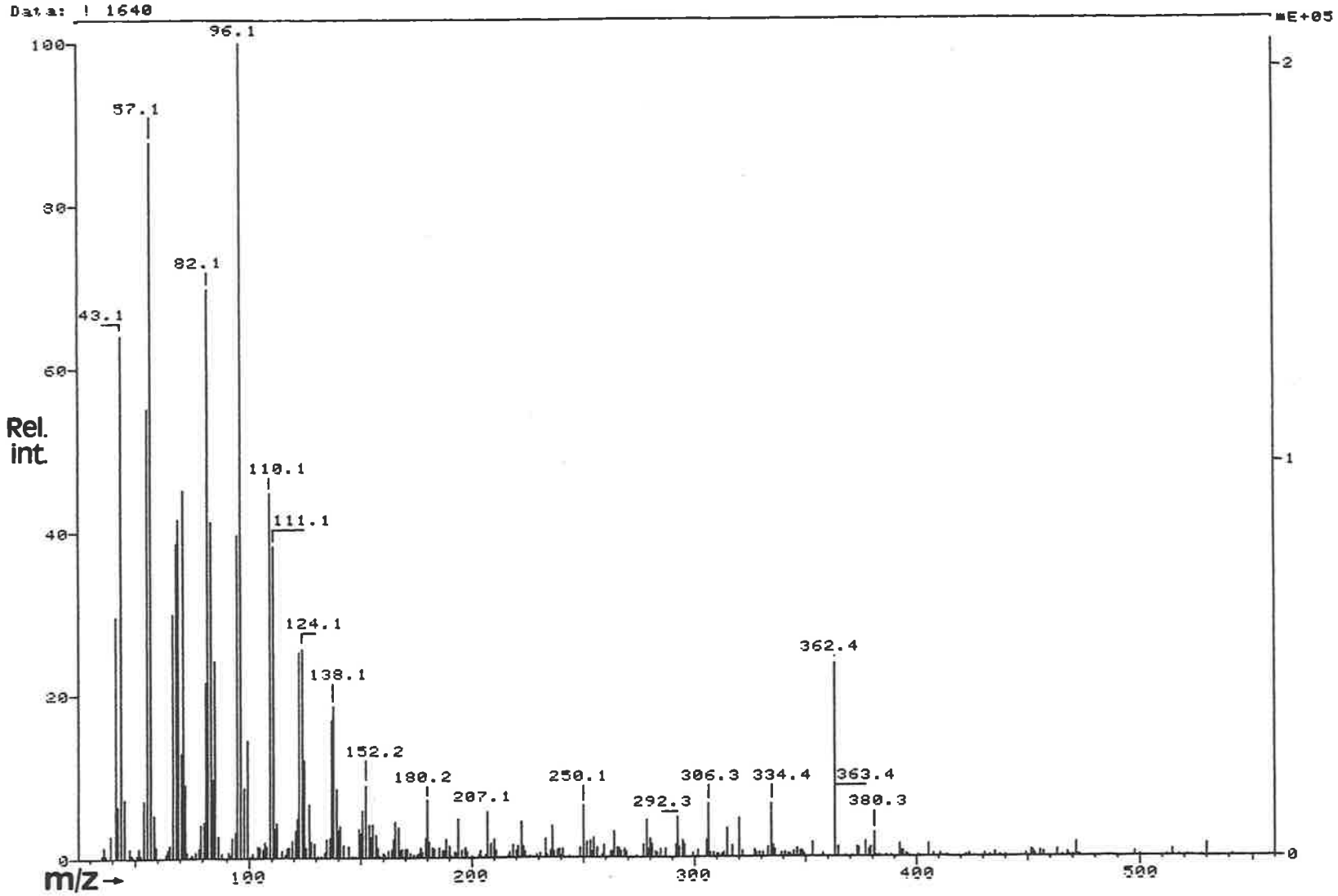
Appendix 6.4. Mass spectra of the C_{33} β-diketone tritriacontan-16,18-dione from the wax of *E. tetragona* juvenile leaves.



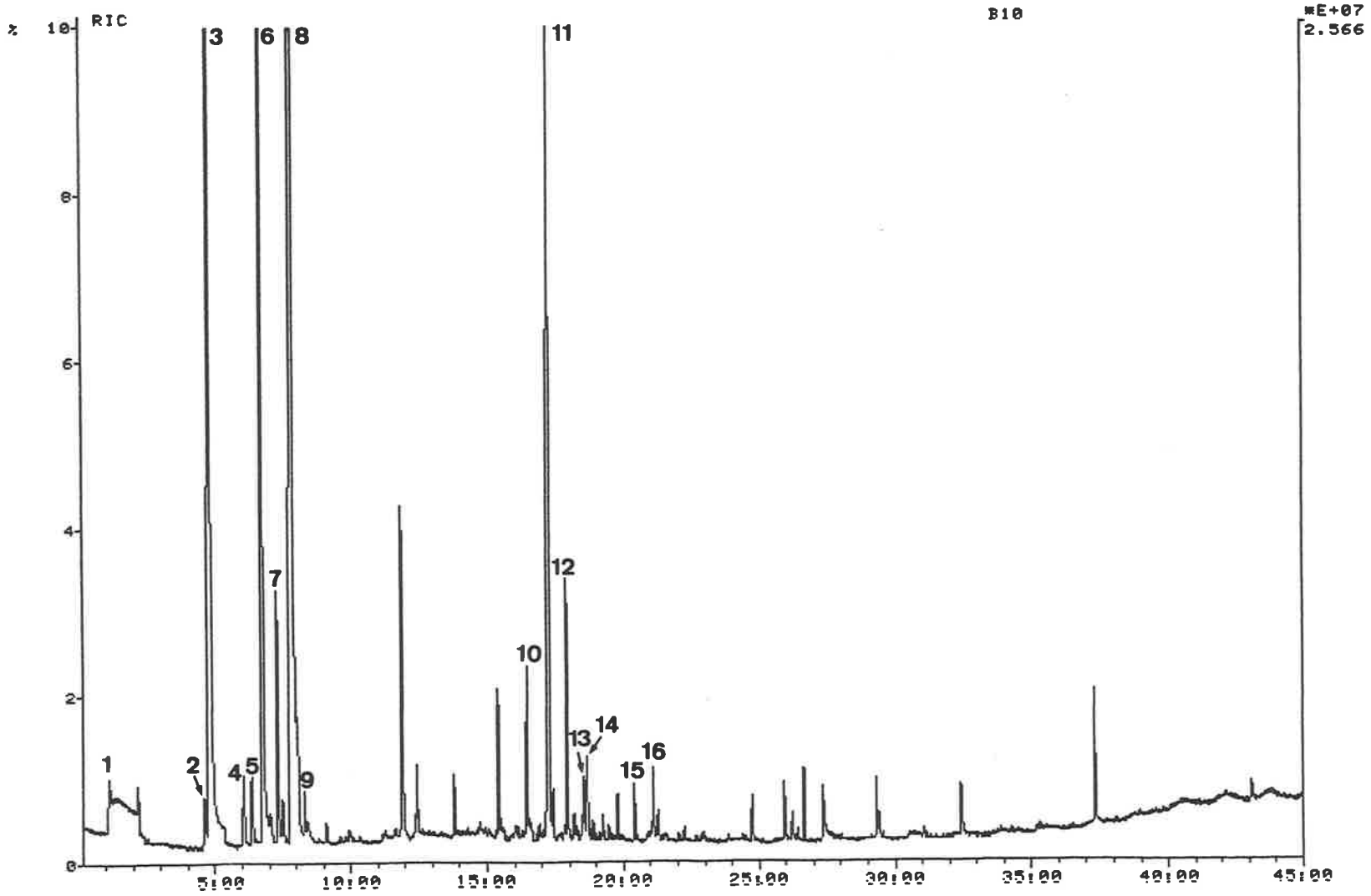
Appendix 6.5. Mass spectra of the C_{22} fatty acid from the wax of *E. tetragona* juvenile leaves.



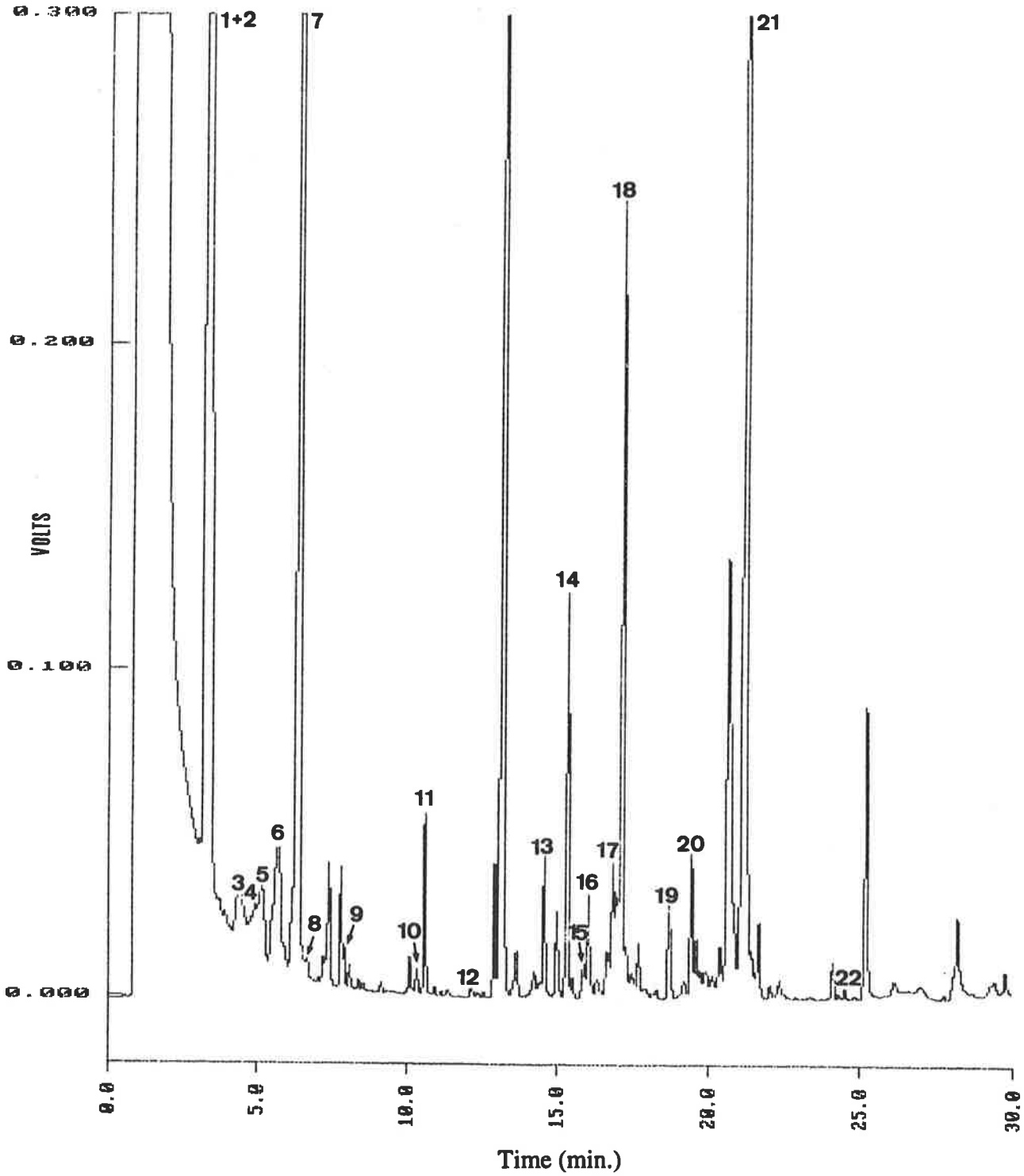
Appendix 6.6. Mass spectra of the C_{36} ester, eicosanoic acid hexadecyl ester from the wax of *E. tetragona* juvenile leaves.



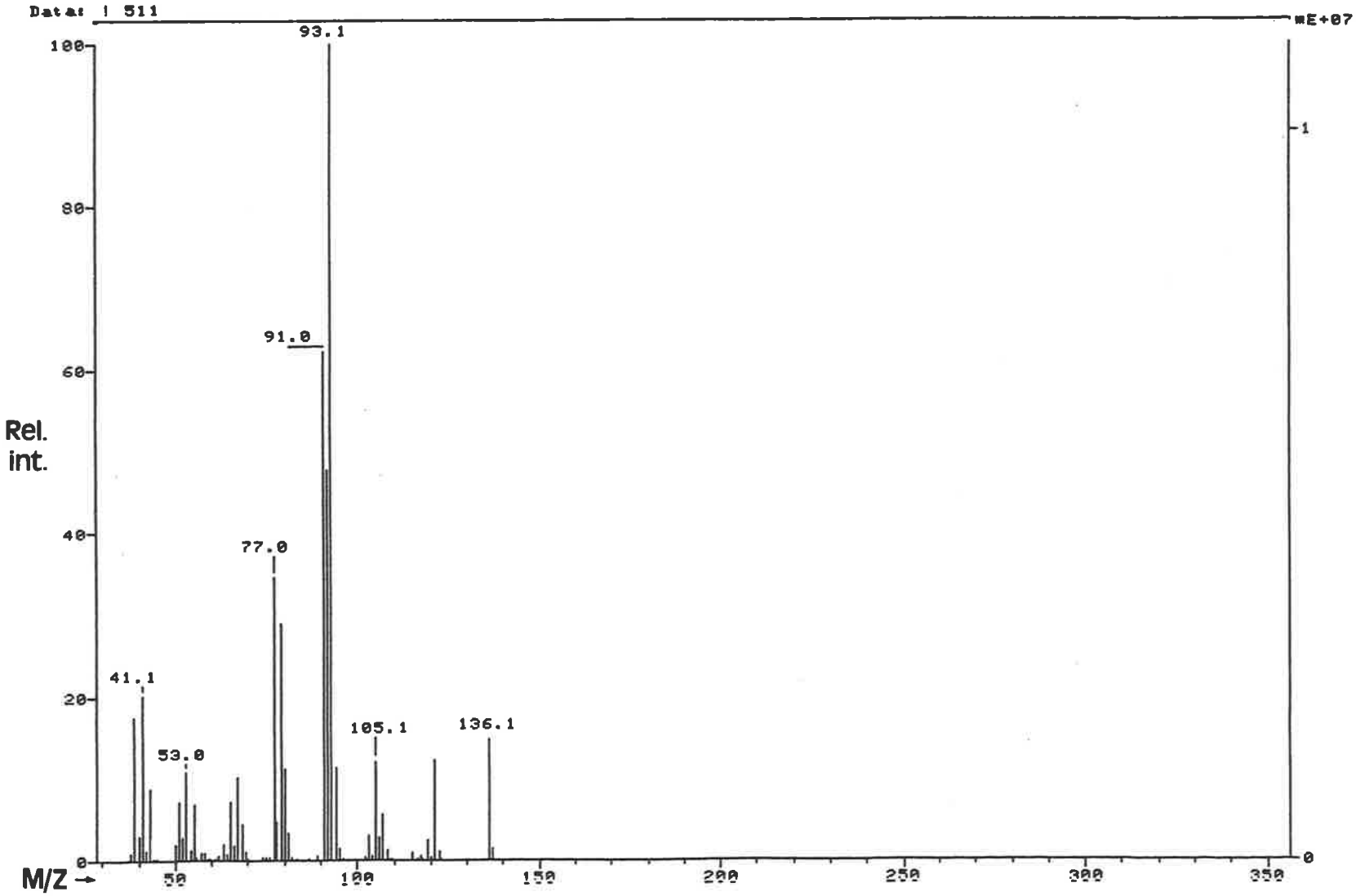
Appendix 6.7. Mass spectra of the C_{18} aldehyde from the wax of *E. tetragona* juvenile leaves.



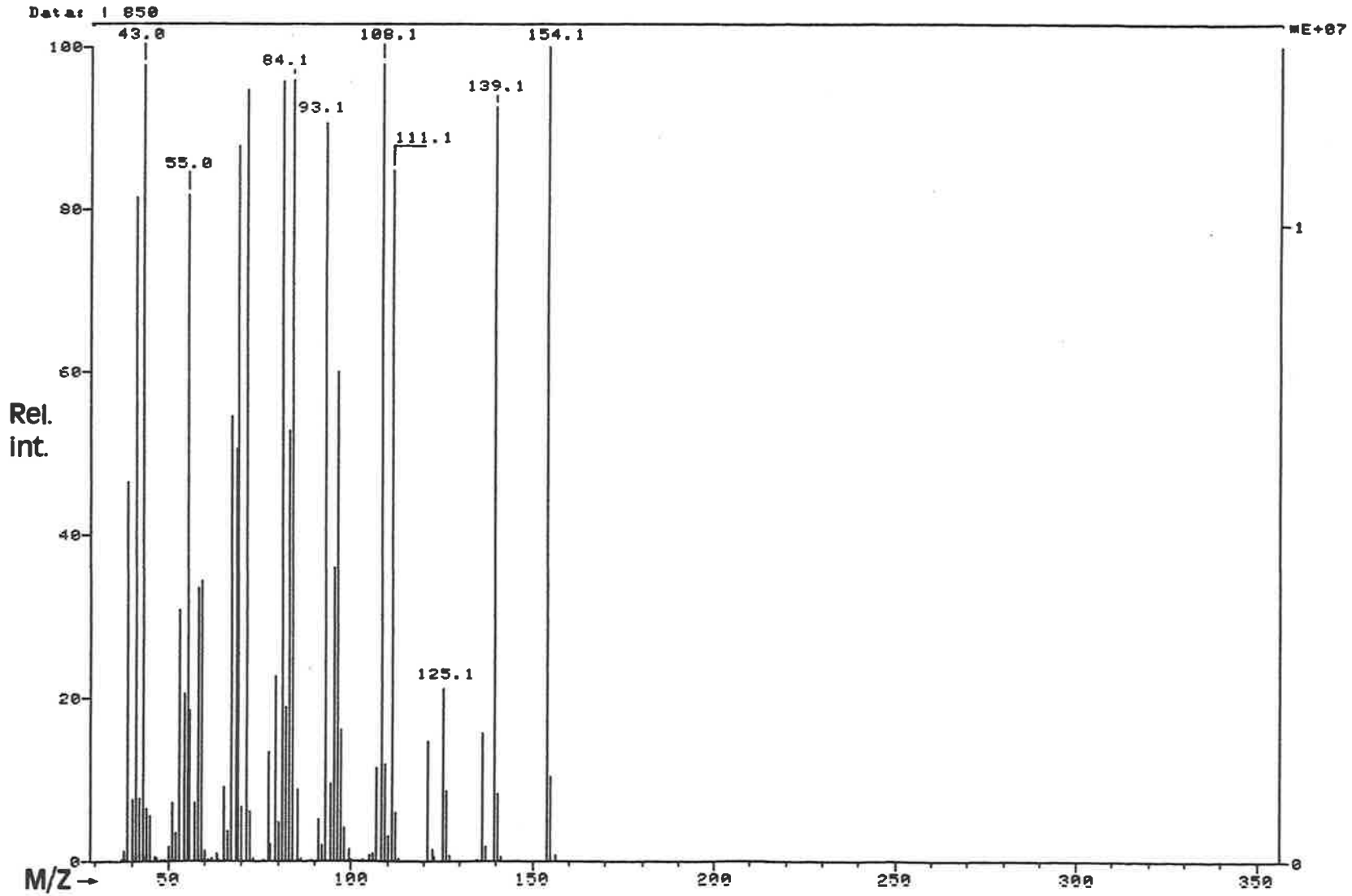
Appendix 6.8. GC separation of the components from SPME of the headspace surrounding newly opened *E. macrocarpa* juvenile leaves. Compound peaks: **1.** cyano acetic acid, **2.** α-thujene, **3.** α-pinene, **4.** sabinene, **5.** β-pinene, **6.** α-phellandrene, **7.** limonene, **8.** 1,8-cineole, **9.** γ-terpinene, **10.** α-selinene, **11.** alloaromadendrene, **12.** aromadendrene, 13. phenol derivative, **14.** viridiflorene, **15.** sesquiterpene alcohol-B, **16.** ledol.



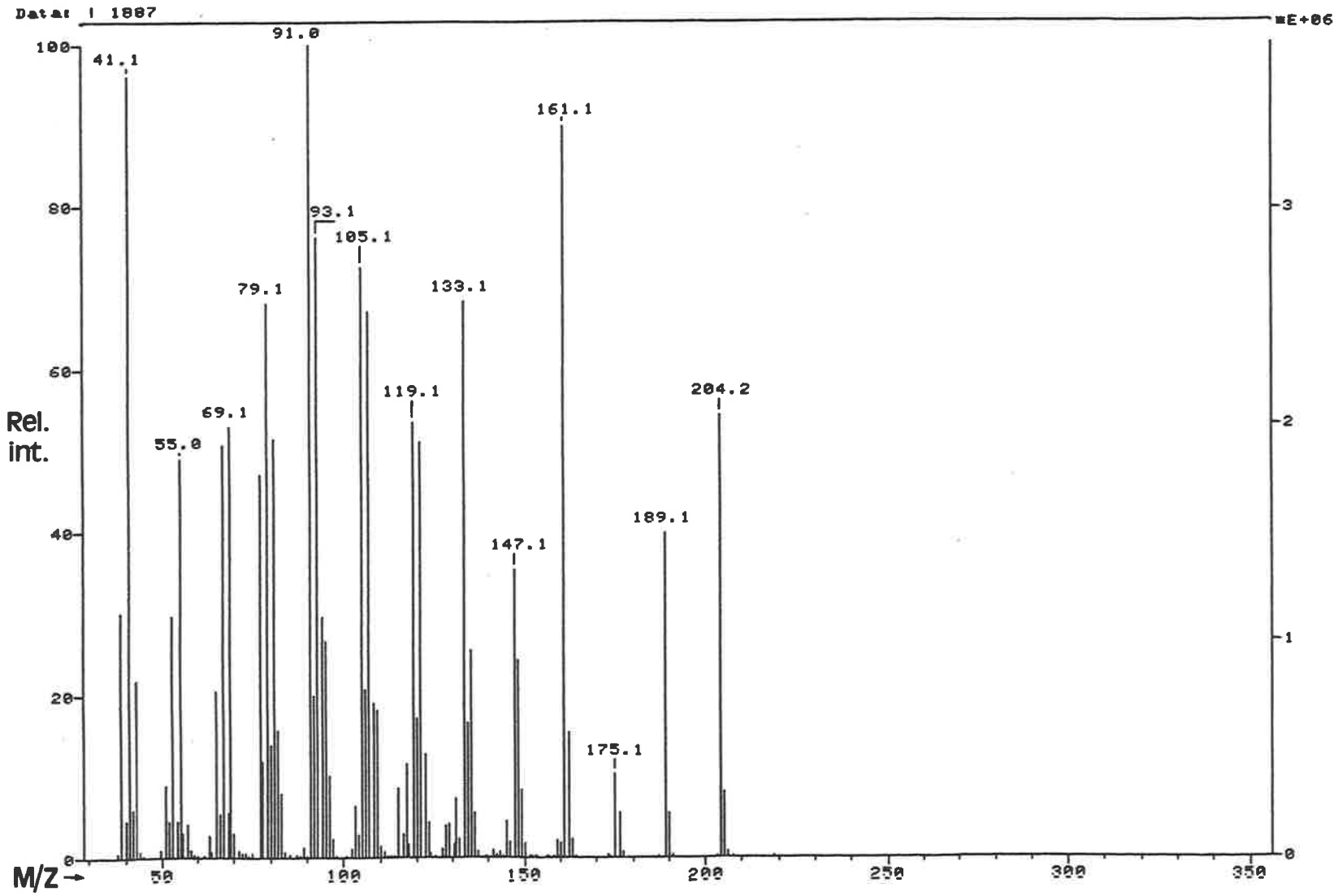
Appendix 6.9. GC separation of the components from the oil of *E. brachyphylla* juvenile leaves. Compound peaks: **1.** α-thujene, **2.** α-pinene, **3.** sabinene, **4.** β-pinene, **5.** α-phellandrene, **6.** limonene, **7.** 1,8-cineole, **8.** γ-terpinene, **9.** linalool, **10.** terpinen-4-ol, **11.** α-terpineol, **12.** 6 methyl hepten-2-one, **13.** α-selinene, **14.** alloaromadendrene, **15.** sesquiterpene hydrocarbon, **16.** aromadendrene **17.** phenol derivative, **18.** viridiflorene, **19.** sesquiterpene alcohol-B, **20.** ledol, **21.** unidentified R_t 21.2, **22.** C_{23} ketone.



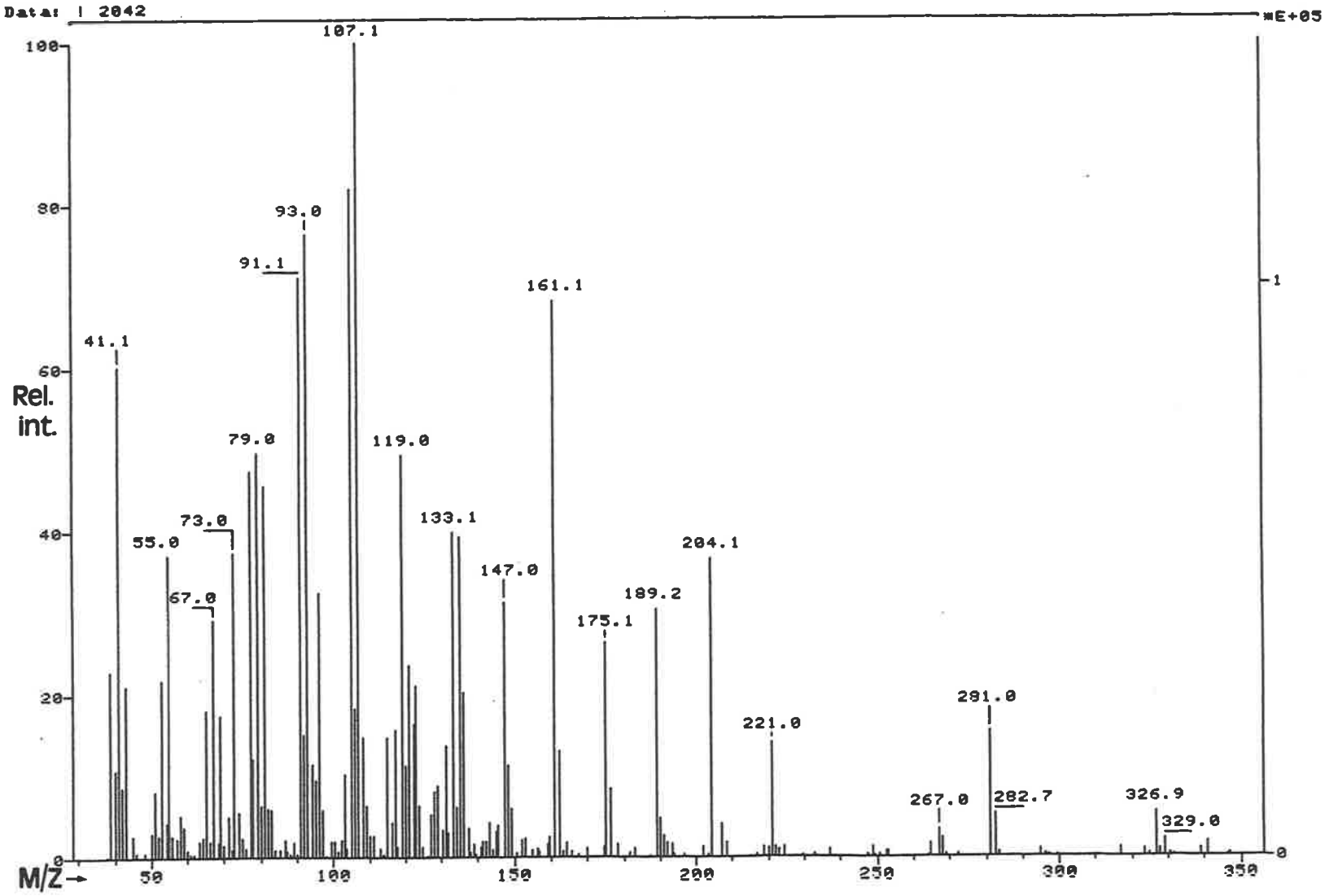
Appendix 6.10. Mass spectra of α-pinene from the headspace sample of *E. macrocarpa.*



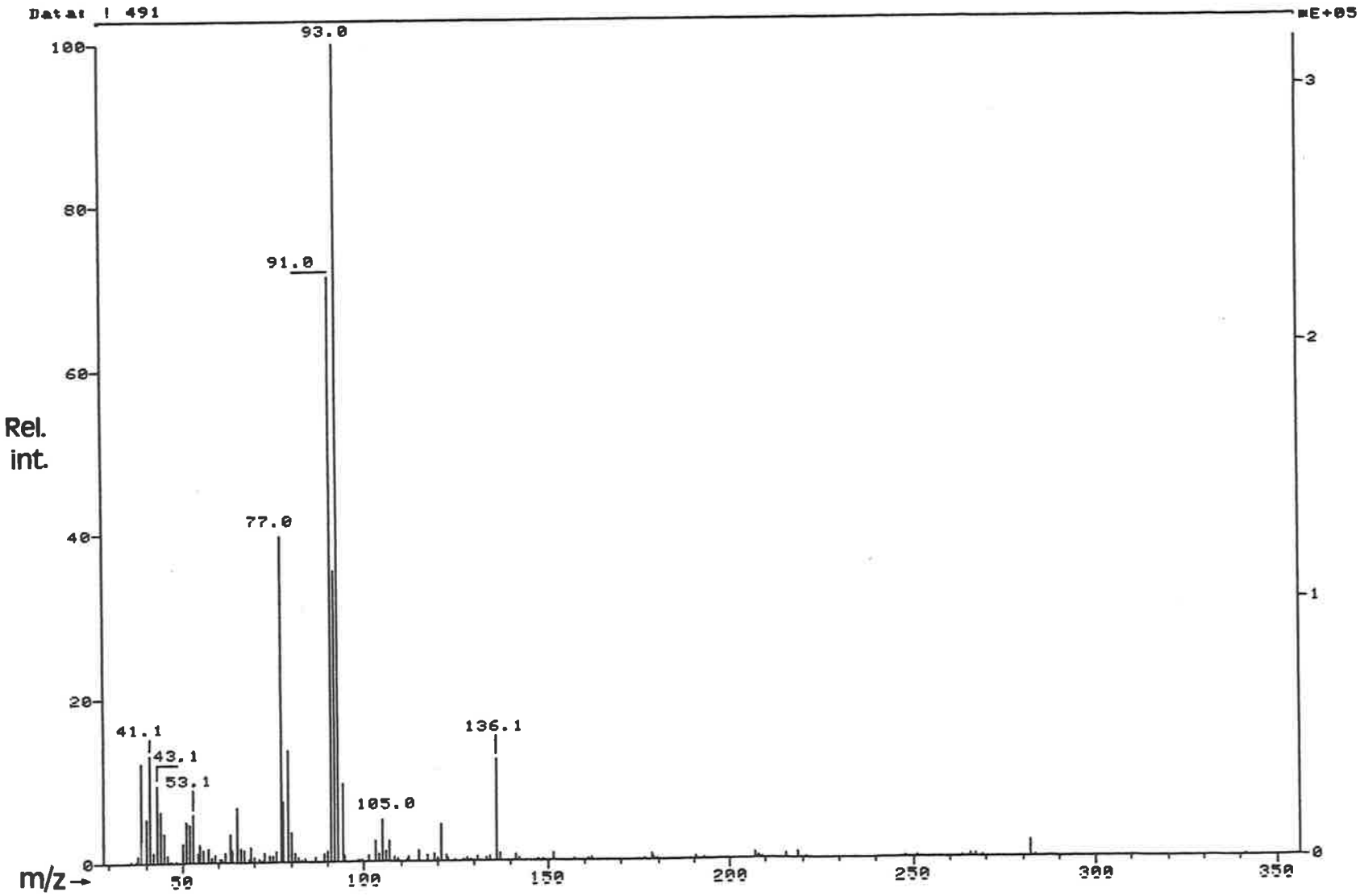
Appendix 6.11. Mass spectra of 1,8-cineole from the headspace sample of *E. macrocarpa*.



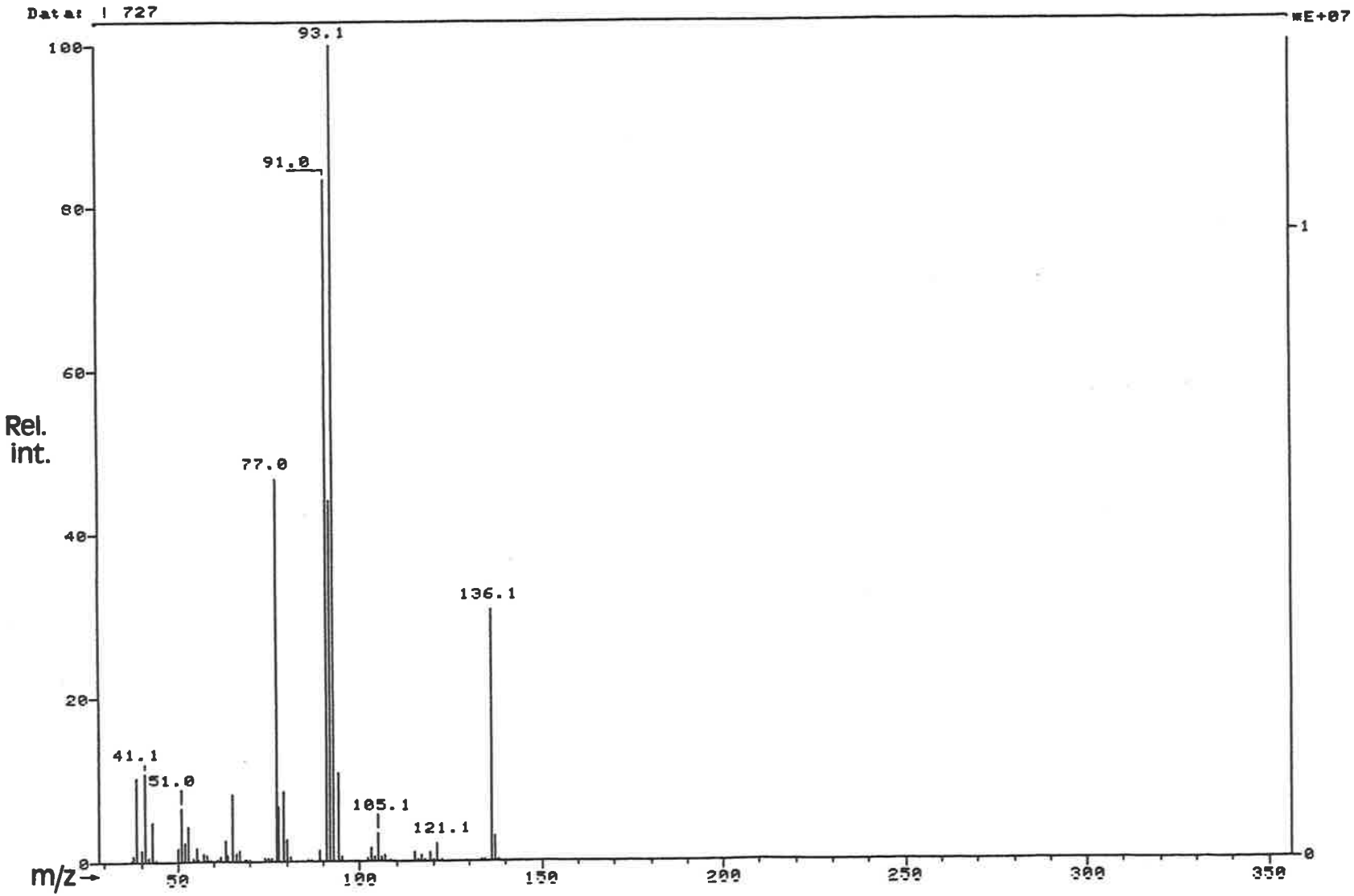
Appendix 6.12. Mass spectra of alloaromadendrene from the headspace sample of *E. macrocarpa*.



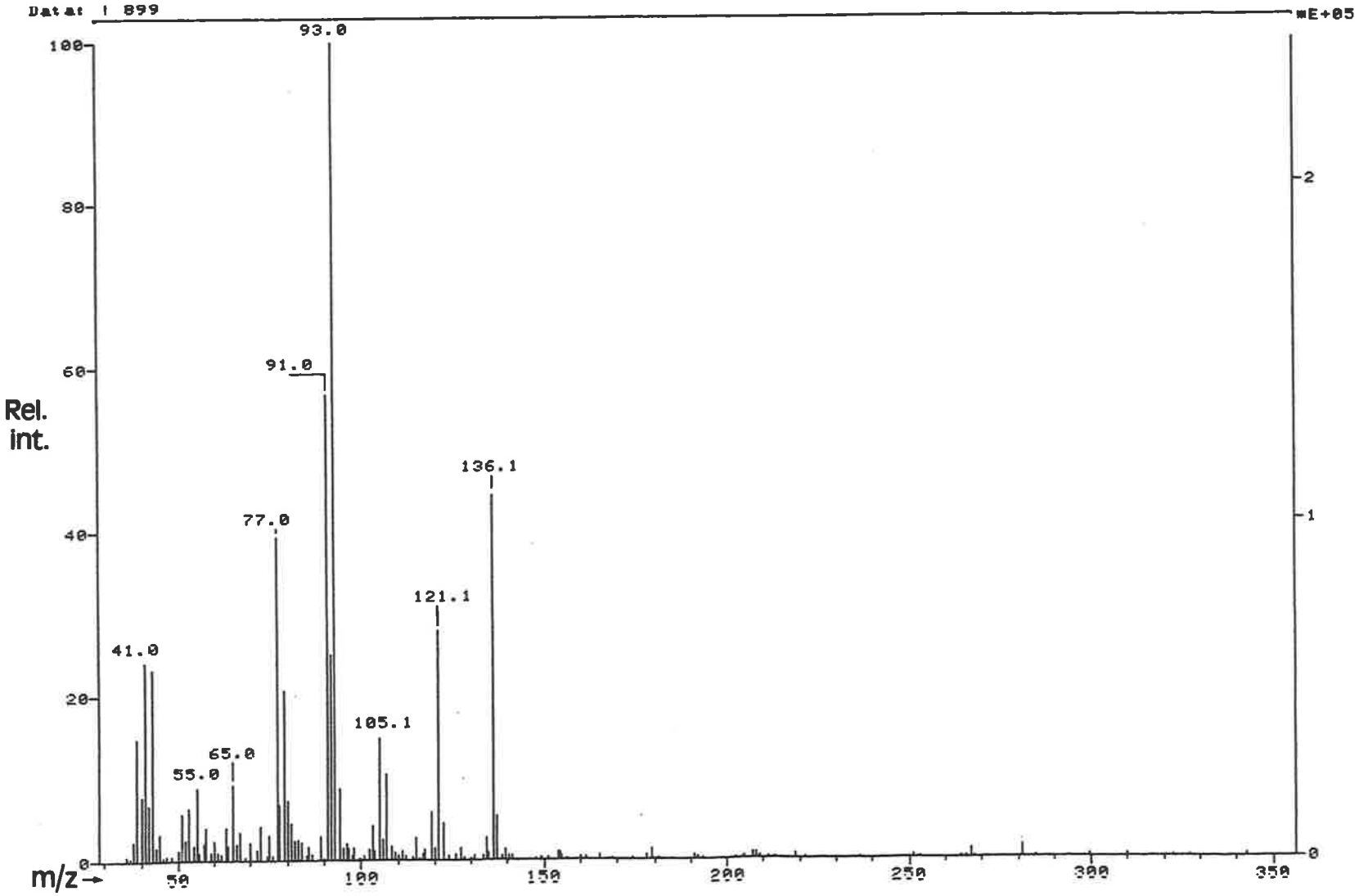
Appendix 6.13. Mass spectra of viridiflorene from the headspace sample of *E. macrocarpa*.



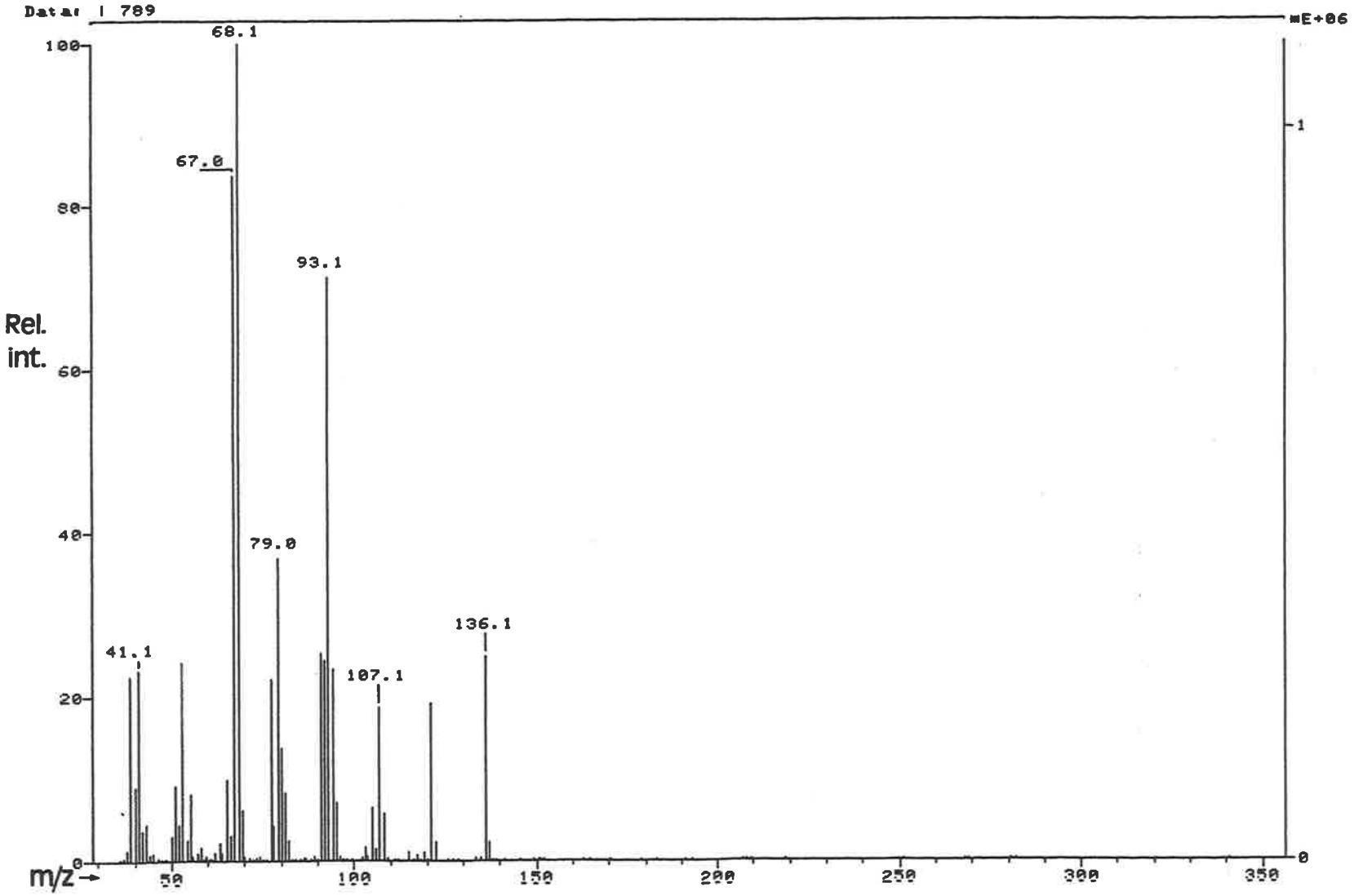
Appendix 6.14. Mass spectra of α-thujene from the headspace sample of *E. macrocarpa*.



Appendix 6.15. Mass spectra of α-phellandrene from the headspace sample of *E. macrocarpa.*



Appendix 6.16. Mass spectra of γ-terpinene from the headspace sample of *E. macrocarpa.*



Appendix 6.17. Mass spectra of limonene from the headspace sample of *E. macrocarpa*.

Appendix 6.18. Diagram of the headspace vial assembly with the SPME fiber holder in place to show relative dimensions.